Universidade de Lisboa

Faculdade de Farmácia

Balance between pro-inflammatory/anti-inflammatory indicators of SOD1^{G93A} microglia in steady state conditions and modification by immunomodulation

Catarina Alexandra Barbosa Ezequiel

Dissertação orientada pela Professora Doutora Dora Maria Tuna de Oliveira Brites e, coorientada pela Doutora Ana Rita Mendonça Vaz Botelho.

Mestrado em Ciências Biofarmacêuticas

2017

Universidade de Lisboa

Faculdade de Farmácia

Balance between pro-inflammatory/anti-inflammatory indicators of SOD1^{G93A} microglia in steady state conditions and modification by immunomodulation

Catarina Alexandra Barbosa Ezequiel

Dissertação orientada pela Professora Doutora Dora Maria Tuna de Oliveira Brites e, coorientada pela Doutora Ana Rita Mendonça Vaz Botelho.

Mestrado em Ciências Biofarmacêuticas

2017

Balance between pro-inflammatory/anti-inflammatory indicators of SOD1^{G93A} microglia in steady state conditions and modification by immunomodulation

The studies presented in this master thesis were performed in the Neuron Glia Biology in Health and Disease Group, at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisbon, under the supervision of Dora Brites, Ph.D. (group leader) and Ana Rita Vaz, Ph.D.

Part of the results discussed in this thesis was presented in the following National Scientific Meetings:

Ezequiel C., Cunha C., Vaz AR., Brites D. Mouse microglia N9 cell line transduced with hSOD1^{G93A} show a heterogeneous inflammatory response to LPS. XV SPN Meeting, May 25-26th, 2017 (*poster presentation*) (Annex VI.1).

Cunha C., Barbosa M., **Ezequiel C**., Vaz A.R., Brites D. Upregulation in hSOD1^{G93A} microglia is depicted in their derived-exosomes. Grupo de Estudos do Envelhecimento Cerebral e Demência, June 30th, 2017 (*oral communication*) (Annex VI.2).

Cunha C., Barbosa M., **Ezequiel C.**, Vaz A.R., Brites D. Microglia Acquire a Pro-inflammatory phenotype in ALS and release miR-155 through exosomes. 9th Postgraduate iMed.ULisboa Students Meeting, July 13-14th, 2017 (*oral communication*) (Annex VI.3).

Ezequiel C., Cunha C., Vaz AR., Brites D. Expression of hSOD1^{G93A} causes microglia activation, but unresponsiveness to LPS, while hSOD1^{WT} has a calming effect and sustains reactivity to LPS. 9th Postgraduate iMed.ULisboa Students Meeting, July 13-14th, 2017 (*poster presentation*) (Annex VI.3).

Ezequiel C., Cunha C., Vaz A.R., Brites D. Transfection of N9 microglia with hSOD1^{G93A} inhibits their response to LPS and triggers M1 polarization, which is reversed by GUDCA. 3rd Mind-Brain College of the University of Lisbon, October 19th and 20th (*poster presentation*) (Annex VI.4).

Work presented in this master thesis was supported by Santa Casa da Misericórdia de Lisboa [Ela Project 2015-002 (DB)] and in part by Fundação para a Ciência e Tecnologia [project Pest-UID/DTP/04138/2013 iMed.ULisboa project].

Para a minha família

“It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul.”

From William Ernest Henley

Acknowledgments

Terminar mais uma etapa na minha vida académica é para mim um enorme orgulho. No entanto, não o teria conseguido sem a presença das pessoas que me apoiaram ao longo dos dois últimos anos.

Primeiro que tudo, gostaria de agradecer à **Professora Doutora Dora Brites**, orientadora desta dissertação de Mestrado. Obrigada por me ter dado a oportunidade de integrar o seu grupo de investigação e por estar sempre presente e disposta a ajudar nos momentos mais determinantes deste percurso. Este ano foi sem dúvida uma experiência muito enriquecedora para mim, onde pude acompanhar de perto a sua dedicação e exigência no que toca a alcançar a excelência em todos os trabalhos científicos que passam por si. Tenho a certeza de que todo o conhecimento que me transmitiu vai acompanhar-me ao longo de toda a minha vida quer profissional, quer pessoal.

A ti, **Rita**, agradeço-te toda a paciência que tiveste comigo ao longo deste ano. Sei que sou ‘’o teu trabalho’’, mas é muito bom saber que tenho alguém ali tão perto a quem recorrer nas situações mais difíceis. No meio de tantos imprevistos, foste tu que estiveste lá para tentar dar a volta à situação. Obrigada por todo o apoio e pelas tuas palavras quando tudo parecia muito negro. **Muito obrigada.**

Um agradecimento também aos **Professores Rui, Adelaide, Alexandra** e ainda à **Andreia** pela simpatia e disposição em ajudar sempre que necessário.

Querida **Carolina**, para ti o meu maior agradecimento. Ajudaste-me desde o primeiro dia no laboratório. Ensinaste-me todas as técnicas e acompanhaste-me até considerares que eu era capaz de as fazer sozinha, mesmo que continuasses a estar sempre lá se fosse preciso. E foi muitas vezes! Foi a ti que eu recorri sempre quando alguma coisa corria mal, ou mesmo se corresse bem! Fizeste-me confiar em mim e em que eu era capaz de fazer um bom trabalho. Sempre foste um exemplo não só no laboratório pelo teu trabalho e exigência contigo própria, mas também como pessoa. Valorizo muito a tua paciência, as tuas palavras, os teus conselhos e tudo o que aprendi contigo e espero levar-te comigo para o futuro.

Ás minhas queridas meninas **Gisela**, **Marta**, **Ritinha** e **Sara**. Sem vocês teria sido tão mais difícil. À **Gisela** por ter sempre respostas e soluções para tudo, mas especialmente por ser um coração mole e uma pessoa muito doce por baixo de todo o ar de má! À **Martinha** por todos os miminhos e por estar sempre disposta a ajudar e a partilhar experiências! À **Ritinha** por ter sido a minha companheira de ‘’guerra’’ neste ano, especialmente no fim em que quase

verdadeiramente andávamos em guerra por causa da ultra! Foi um prazer ‘’lutar’’ ao teu lado xD!! E finalmente à **Sara** por ser sempre um poço de boas energias e caretas! És alguém muito especial e sem dúvida muito única! Obrigada a todas pela paciência para me ouvirem todos os dias e aturarem todos os meus dramas. Obrigada por me terem ajudado sempre que eu achava que não ia conseguir, por me levarem para longe do trabalho, por todos os chocolates e gordices partilhadas! Obrigada por me animarem todos os dias e fazerem com que valesse a pena sair de casa e vir para o CPM! Gosto tanto de vocês! Saio daqui muito mais rica e muito melhor pessoa porque vos conheci, acreditem! <3

À **Fabiana**. Admiro imenso a maneira como vês o mundo e como tratas as pessoas. Obrigada por todos os cafés e por estares sempre disposta a ouvir-me. Aprendi muito contigo este ano e também gosto muito de ti!

Um agradecimento mais recente à **Cátia** e ao **Gonçalo** que embora tenham aparecido já muito no fim, também estiveram sempre dispostos a ajudar, aconselhar e motivar!

Ao meu **João Pedro** que esteve comigo dia após dia, todos os dias, não só neste ano mas em todos os outros. Obrigada por toda a paciência e dedicação. Obrigada por estares sempre lá por mais cedo ou mais tarde que fosse, por cederes os teus fins de semana ao meu trabalho e por estares sempre disposto a ajudar, nem que ajudar significasse apenas a tua companhia. Obrigada por acreditares em mim mais do que qualquer pessoa, e por nunca deixares de me dizer que eu ia conseguir por mais difícil que pudesse ser. És a melhor pessoa que eu alguma vez conheci e sem ti teria sido tudo bastante mais difícil.

Obrigada também à **Lena**, ao **João** e à **Maria** por toda a companhia e apoio que me deram e por tentarem sempre ajudar e facilitar a minha vida <3

Aos meus avós **Gabriela, Graciano, Isabel e Artur** por serem os melhores avós do mundo como sempre, e estarem sempre prontos a dar-me todo o apoio, mimos e motivação!

Obrigada ao resto da minha querida família: à **Susana**, ao **Paulo Zé**, ao **Carlos**, à **Mariana**, ao **Diogo**, ao **Carlos**, à **Sara**, ao **Rafael**, à tia **Fatinha** e ao tio **Fernando** e ao meu querido **João Ricardo** por toda a boa disposição e todos os belos almoços de família aos domingos! Não vos trocava por nada, são os melhores de sempre!

Aos meus pais, **Aurora e João**, por me terem sempre dado a oportunidade para seguir os meus sonhos e por me terem apoiado em continuar o meu percurso académico. Obrigada por terem abdicado de mim e do meu tempo convosco tantas e tantas vezes e por me continuarem

a ouvir e tentar compreender, ainda que na maioria das vezes não fosse fácil. Sem vocês eu não estaria onde estou hoje e não seria a pessoa em que me tornei. Agradeço-vos o mundo inteiro e espero um dia ser um exemplo para os meus filhos como vocês são para mim.

Um obrigada especial ao meu bebé **João Miguel** que um dia, daqui a não muito tempo, vai saber ler isto! De cada vez que sorriste, me agarraste e puxaste para brincar contigo, encheste o meu dia de alegria. És o meu amor maior. A mana ama-te até mil, como tu dizes!!!!! :D <3

Index

Figure Index

I. Introduction

II. Materials and Methods

III. Results

IV. Discussion

Table Index

II. Materials and Methods

III. Results

Abstract

Amyotrophic Lateral Sclerosis (ALS) is the third most common neurodegenerative disease, mostly sporadic, with limited identified targets, biomarkers and therapeutic options. The most widely used animal model and experimental cellular models to study ALS pathological mechanisms are based on mutations in the anti-oxidant protein SOD1, particularly that of G93A. ALS affects mainly motor neurons, but it is widely accepted that immune unbalance plays a crucial role in the ALS disease, and microglial dysfunction is described to be associated with neuronal injury influencing disease onset and progression. As the immune cells of the central nervous system, microglia produce inflammatory responses towards an insult by secreting pro-inflammatory mediators to the extracellular milieu in the form of soluble factors, or in membrane-bound vesicles called exosomes, an important component in intercellular communication and in disease dissemination.

In this thesis we aimed to better understand the role of microglia in ALS disease using the mutant $SOD1^{G93A}$ microglia, and assessing their reactivity upon the immunostimulation by lipopolysaccharide (LPS), and immunomodulation by glycoursodeoxycholic acid (GUDCA) and vinyl sulfone (VS), having in mind the goal of fighting ALS neurodegeneration. For that, we assessed microglia function/dysfunction and reactivity after human SOD1 overexpression in the N9 cell line, either wild type ($hSOD1^{WT}$) or mutated in G93A ($hSOD1^{G93A}$), alone or treated with LPS, and when exposed to GUDCA and VS, known for their potential anti-inflammatory effects.

Data showed that overexpression of $hSOD1^{WT}$ in N9 cells leads to a decrease in all analyzed pro- and anti-inflammatory markers, whereas $hSOD1^{G93A}$ increases both pro-inflammatory *TNF-α, IL-1β, MHCII* and *HMGB1* gene expression levels, together with anti-inflammatory *Arg1* and *SOCS1* indicators, and reduces *iNOS, Fizz1, IL-10, TLR4*, miR-125b and miR-21. Interestingly we found an elevated cargo of miR-155 and miR-146a in $hSOD1^{G93A}$ microglia-derived exosomes. Upon LPS exposure, all cells switched from ramified into amoeboid morphology. LPS-treated transgenic microglia showed equivalent pro-inflammatory markers, when compared to LPS-treated naïve cells. However, they revealed decreased levels of the anti-inflammatory *Arg1, Fizz1* and *IL-10*, thus reducing the ability to later balance the microglia reactivity to the insult. Surprisingly, cells also evidenced reduced miR-155 expression, what may even compromise an adequate pro-inflammatory response. In contrast with $hSOD1^{WT}$ cells, $SOD1^{G93A}$ microglia displayed decreased gene expression of *S100B* and equal of *TNF-α* mRNA, when compared to naïve cells. Additionally, the ability of ingesting a high number of beads (≥ 11) was found diminished. Treatment with GUDCA or VS decreased the cell body area of reactive microglia, and *SOCS1* and *Arg1* mRNA expression. Nevertheless, both immunomodulators increased *TLR4*, as well as reduced *IL-1β* and *S100B*

gene expression, which may represent benefits for response to selected insults, while protecting from destructive secondary damage, respectively. In addition, though it decreased cellular *MFG-E8* and enhanced miR-125b in exosomes, GUDCA markedly increased the cellular gene expression of the anti-inflammatory *IL-10*. On the other hand, VS was the only one able to reduce the pro-inflammatory MMP-9 activity and to elevate the exosomal cargo in the anti-inflammatory miR-21.

In conclusion, this work demonstrates the advantageous hSOD1^{WT} overexpression in balancing pro- and anti-inflammatory mediators in microglial cells, but overall that upregulation of hSOD1^{G93A} increases their reactivity and may have a detrimental role in reducing their wound repair ability after insult, thus causing homeostatic imbalance between anti-inflammatory and pro-inflammatory gene expression mediators. In addition, the study also highlights that, although with different potential roles, both VS and GUDCA may have benefits over specific hSOD1^{G93A} polarized microglia subtypes.

Keywords: hSOD1^{G93A}-microglia activation; glycoursodeoxycholic acid; vinyl sulfone; miRNAs; lipopolysaccharide.

Resumo

A Esclerose Lateral Amiotrófica (ELA) é a terceira doença neurodegenerativa mais comum, sendo maioritariamente esporádica, e limitada em termos de alvos, biomarcadores e opções terapêuticas. Os modelos animais e celulares mais usados no estudo dos mecanismos envolvidos na patogénese da ELA consideram mutações na enzima antioxidante SOD1, particularmente, a mutação G93A. A ELA afeta maioritariamente neurónios motores. No entanto, é considerado que existe uma desregulação inflamatória nesta doença que contribui para a sua progressão. A disfunção de células microgliais é associada ao dano neuronal, o que consequentemente leva ao início e progressão da doença. No Sistema Nervoso Central (CNS), as células da microglia são responsáveis pela produção da resposta inflamatória em consequência da presença de moléculas estranhas no ambiente extracelular. Esta resposta baseia-se na secreção de mediadores pro-inflamatórios para o meio extracelular sob a forma de fatores solúveis ou incorporados em vesículas membranares denominadas de exossomas, um importante meio de comunicação intercelular na disseminação da patologia.

Na presente tese, pretendeu-se compreender melhor o papel da microglia na ELA, utilizando células da microglia sobreexpressando $SOD1^{G93A}$, e avaliando a sua reatividade após estimulação com lipopolissacárido (LPS), e após tratamento com os imunomoduladores ácido glicoursodesoxicólico (GUDCA) e vinil sulfona (VS), com o objetivo de combater a neurodegeneração na ELA. Para isso, avaliámos a função/disfunção e reatividade microglial após a sobreexpressão da enzima SOD1 na linha celular N9, na conformação WT ($hSOD1^{WT}$) ou mutada em G93A ($hSOD1^{G93A}$) da enzima, em células sem tratamento ou tratadas com LPS. Adicionalmente, avaliámos o potencial anti-inflamatório dos compostos GUDCA e VS nas células sobreexpressando $hSOD1^{G93A}$.

Os nossos resultados demonstraram que a sobreexpressão de $hSOD1^{WT}$ em células N9 leva a uma diminuição de todos os parâmetros pro- e anti-inflamatórios analisados, enquanto que da sobreexpressão de $hSOD1^{G93A}$ leva a um aumento da expressão génica dos marcadores pro-inflamatórios *TNF-α, IL-1β, MHCII* e *HMGB1* em conjunto com os marcadores anti-inflamatórios *Arg1* e *SOCS1*, reduzindo *iNOS, Fizz1, IL-10, TLR4*, miR-125b e miR-21. Curiosamente, exossomas derivados de microglia sobreexpressando $hSOD1^{G93A}$ revelaram transportar maiores quantidades de miR-155 e miR-146a. Após exposição ao LPS, todas as células modificaram a sua morfologia ramificada para uma forma ameboide. Células N9 $hSOD1^{G93A}$ tratadas com LPS demonstraram marcadores pro-inflamatórios com níveis equivalentes ao das células controlo. No entanto, revelaram também uma diminuição dos marcadores pró-inflamatórios *Arg1, Fizz1* e *IL-10*, reduzindo assim a capacidade da microglia de resposta ao insulto. Surpreendentemente, estas células demonstraram ainda uma diminuição de miR-155, o que pode sugerir uma resposta pró-inflamatória adequada. Ao contrário de células

sobreexpressando hSOD1^{WT}, microglia SOD1^{G93A} apresentou uma diminuição nos níveis de expressão génica de *S100B* e igual expressão de *TNF-α* quando comparadas ao controlo. Adicionalmente, estas células evidenciaram uma diminuição da capacidade de ingestão de um elevado número de beads [≥11]. O tratamento com GUDCA ou VS demonstrou diminuir a área do corpo celular das células reativas da microglia, em conjunto com uma diminuição da expressão génica de *SOCS1* e *Arg1*. Contudo, ambos os imunomoduladores aumentaram a expressão de *TLR4*, diminuindo a expressão de *IL-1β* e *S100B*, o que pode sugerir o efeito benéfico destes compostos na resposta a insultos, protegendo contra efeitos secundários destrutivos, respetivamente. Adicionalmente, apesar da diminuição da expressão de *MFG-E8* e aumento da expressão de miR-125b em exossomas, o composto GUDCA evidenciou um aumento significativo da expressão do marcador anti-inflamatório *IL-10*. Por outro lado, apenas o tratamento com VS foi bem-sucedido na diminuição da atividade da MMP-9 e aumento do transporte do anti-inflamatório miR-21 em exossomas.

Em conclusão, este trabalho demonstra o benefício da sobreexpressão de hSOD1^{WT} no equilíbrio de marcadores pro- e anti-inflamatórios nas células da microglia, enquanto a sobreexpressão de hSOD1^{G93A} aumenta a reatividade microglial, podendo ter um papel prejudicial na redução da sua capacidade de resposta a estímulos externos, causando assim um desequilíbrio na expressão génica de marcadores pro- e anti-inflamatórios. Adicionalmente, este estudo foca ainda que, apesar de com diferentes funções, os compostos GUDCA e a VS que podem ser benéficos para as células da microglia hSOD1^{G93A} com diferentes polarizações.

Palavras-chave: ativação da microglia-hSOD1^{G93A}; ácido glicoursodesoxicólico, vinil sulfona, miRNAs, lipopolisacárido.

Abbreviations

ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor
BA	Bile acids
BAC	Bacterial Artificial Chromosome
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CCL12	Chemokine Ligand 12
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CSF-1	Colony Stimulating Factor 1
CX3CR1	Fractalkine Receptor or G-protein Coupled Receptor 13
DAMP	Damage-Associated Molecular Protein
DIV	Days *in vitro*
DNA	Deoxyribonucleic Acid
DRP	Dipeptide Repeat Proteins
EAAT-2	Excitatory Amino Acid Transporter 2
ED	Embryonic Day
ER	Endoplasmatic Reticulum
ERAD	ER-associated Degradation System
EVs	Extracellular Vesicles
fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal Bovine Serum
FDA	Food and Drug administration
Fizz1	Found in Inflammatory Zone
FUS	Fused in Sarcoma
GFP	Green Fluorescent Protein
GLT-1	Glutamate Transporter-1
GUDCA	Glycoursodeoxycholic Acid
H_2O	Water
H_2O_2	Hydrogen Peroxide
HMGB1	High Mobility Group Box 1
$hSOD1^{WT}$	Human SOD1 Wild-Type
$hSOD1^{G93A}$	Human SOD1 mutated in G93A
IFN	Interferon
IL	Interleukin
ILVs	Intraluminal Vesicles
IMS	Intermembrane space

iNOS	Inducible Nitric Oxide Synthase
iPSCs	Induced Pluripotent Stem Cells
L-Glu	L-Glutamine
LMN	Lower Motor Neuron
LPS	Lipopolysaccharide
MFG-E8	Milk-fat Globule EGF factor-8
miR	MicroRNA
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MN	Motor Neuron
MND	Motor Neuron Disease
mRNA	Messenger RNAs
MVB	Multivesicular Body
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor-kappa B
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
O_2^-	Superoxide Anion
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate-buffer Saline
PDL	Poly-D-lysine
Pen-Strep	Penicilin-Streptomicin
PNS	Peripheral Nervous System
PRRs	Pattern Recognition Receptor
qRT-PCR	Quantitative Real-Time PCR
RAGE	Receptor for Advanced Glycation Products
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
S100B	S100 Calcium Binding Protein B
sALS	Sporadic Amyotrophic Lateral Sclerosis
SC	Spinal Cord
SOCS1	Suppressor of Cytokine Signaling 1
SOD1	Superoxide Dismutase 1
TARDBP	Transactive Response DNA-binding Protein
TDP-43	TAR DNA-binding Protein 43
TGF-β	Transforming Growth Factor β
TLR	Toll-like Receptor
TNF- α	Tumor Necrosis Factor α
TREM-2	Triggering Receptor Expressed on Myeloid Cells 2

TUDCA	Tauroursodeoxycholic Acid
UBQLN2	Ubiquilin-2
UDCA	Ursodeoxycholic Acid
UMN	Upper Motor Neurons
VAPB	VAMP Associated Protein B and C
VCP	Valosin-containing Protein
VS	Vinyl Sulfone
WT	Wild-Type

I. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal motor neuron disease (MND) that affects both the upper motor neurons (UMN) in the motor cortex and lower motor neurons (LMN) in brainstem and spinal cord (SC) (Chiò et al., 2013; Robberecht and Philips, 2013) (**Figure I.1**). ALS is the third most common neurodegenerative disease (Renton et al., 2013) and may have bulbar involvement or a spinal-onset appearance (Gordon, 2013). The earliest cases of ALS were described in 1848 and 1853, but only in 1869 the disease we recognize today as ALS was formally defined and identified (Al-Chalabi and Hardiman, 2013; Gordon, 2013).

Progression of the disease is characterized by gradual muscle atrophy and weakness, spasticity, increased fatigue and problems swallowing which typically progresses to respiratory failure, ultimately leading to death (Chiò et al., 2013; Robberecht and Philips, 2013).

ALS age of clinical onset is highly variable, but almost always occurs after the fourth decade of life; juvenile ALS is rare (Robberecht and Philips, 2013). Incidence rates for ALS range from 1.2-4.0 per 100 000 persons in Caucasians (Gordon, 2013), although may be lower in some ethnic populations (Gordon, 2013). In Portugal there are 700-800 estimated people affected with the disease. Overall median survival since diagnostic ranges from 2 to 5 years and, only 5% to 10% of patients survive beyond 10-20 years (Chiò et al., 2013; Robberecht and Philips, 2013).

ALS is traditionally classified in two categories: familial ALS (fALS) and sporadic ALS (sALS). fALS is caused by mutations in a heterogeneous set of genes and is predominantly hereditary and almost always autosomal dominant. On the other way, sALS affects patients without precedence of the disease in any relatives and it is thought that sALS possibly have both genetic and environmental causes, although the ultimate cause still remains to be discovered (Gordon, 2013; Robberecht and Philips, 2013). However, despite all the

differences, clinically both ALS categories are very similar and genetic advances have contributed greatly to our knowledge of ALS.

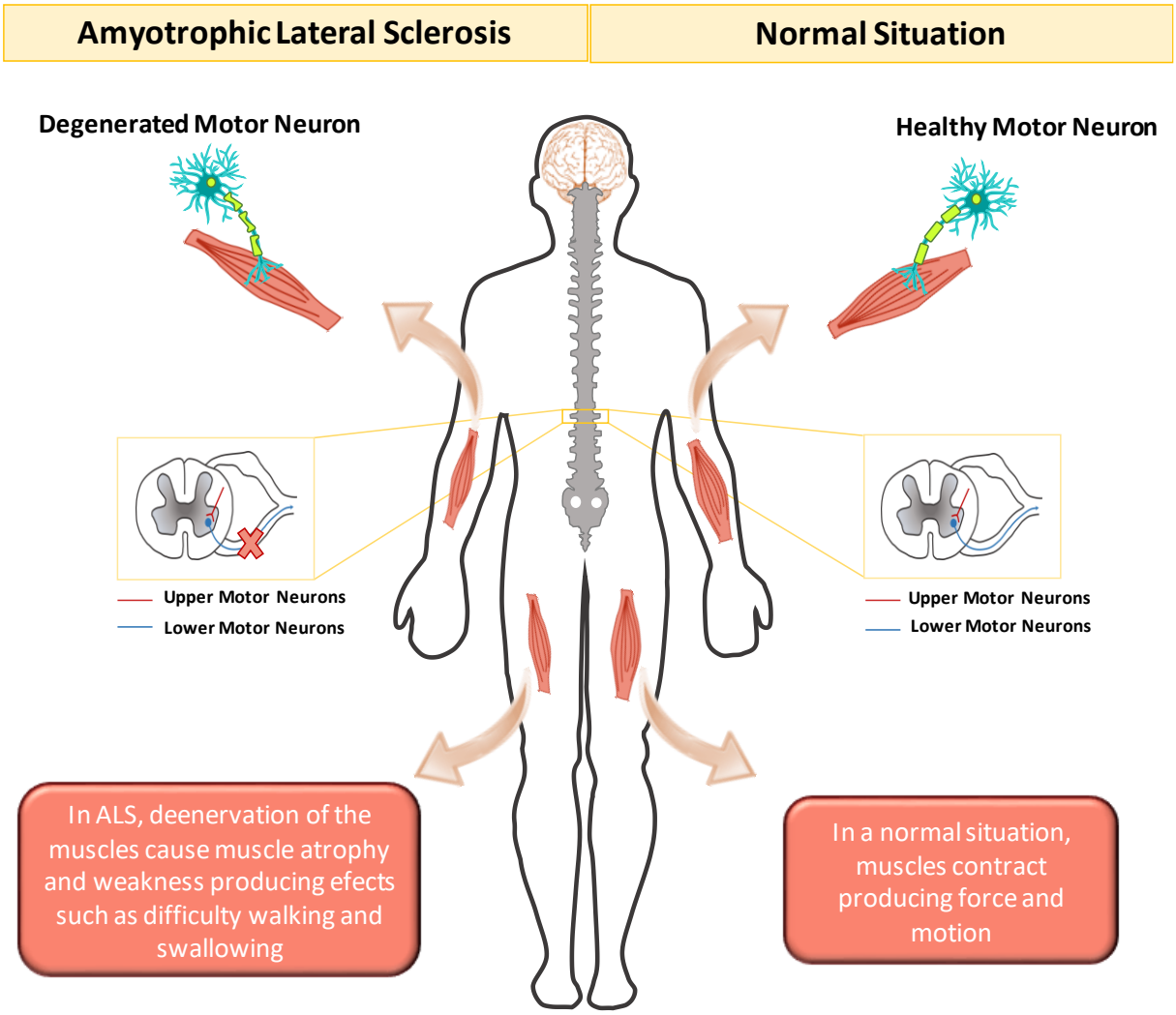


Figure I. 1. **Schematic representation of motor neuron degeneration in amyotrophic lateral sclerosis (ALS).** Amyotrophic lateral sclerosis affects UMNs and LMNs neurons in the motor cortex and in the spinal cord. In a normal situation, muscles are stimulated by UMNs to contract, producing the movement of the body. On the contrary, in ALS disease, degenerated UMNs de-enervate LMNs which are not able to make the connection to the muscle, causing muscle atrophy leading to the loss of the movements of the body. **LMNs,** Lower Motor Neurons**; UMNs,** Upper Motor Neurons**.**

Underlying the development of ALS disease are several multifactorial pathophysiological mechanisms which are a combination of genetics and environmental risk factors (Kiernan et al., 2011). It is presently considered that neurodegeneration in ALS may result from an alteration in a series of complex pathways inside the cells such as glutamate excitotoxicity, generation of free radicals, as well as cytoplasmic, axonal and neurite protein aggregates, together with mitochondrial dysfunction, and disruption of axonal transport processes (Kiernan et al., 2011; Vucic et al., 2014).

Neurodegenerative diseases have as a common hallmark, aggregates of mutated proteins. It is considered that in ALS, these protein aggregates interfere with normal protein homeostasis and induce cellular stress. By disturbing cellular functions such as intracellular transport, cytoskeletal architecture and mitochondrial function, cellular stress leads to axonal retraction and ultimately, cell death (Robberecht and Philips, 2013). Among the 33 genes reported to originate ALS disease, there is evidence that supports a pathogenic role for Cu/Zn superoxide dismutase 1 (SOD1) in 20% of the fALS (http://alsod.iop.kcl.ac.uk/Overview/gene.aspx?gene_id=SOD1).

Although is it not entirely clear how these mutations influence the death of this particular group of neurons, current understanding links genetic mutations to a toxic gain of function of this enzyme, which leads to protein misfolding and aggregation of the peptide intracellularly (Pasinelli and Brown, 2006; Han-Xiang et al., 2008; Vucic and Kiernan, 2009; Bunton-Stasyshyn et al., 2015) and result in the accumulation of free radicals that eventually lead to cell damage and death. However, protein aggregation occurs not only in mutated but also in wild-type (WT) proteins. This process is linked to non-genetic de-regulations, such as metal depletion, quaternary structure disruption and oxidation which leads WT protein to acquire a toxic conformation, similarly to mutated SOD1 proteins in ALS (Rotunno and Bosco, 2013).

I.1. Motor neuron pathomechanisms

The major hallmark of ALS is the presence of abnormal accumulation of protein inclusions or aggregates in degenerating motor neurons. Inclusions are present in different central nervous system (CNS) regions such as frontal and temporal cortices, hippocampus and cerebellum. As above mentioned, neurodegeneration in ALS is currently associated with several mechanisms such as glutamate excitotoxicity, generation of free radicals, cytoplasmic, axonal and neurite protein aggregates, mitochondrial dysfunction, and disruption of axonal transport processes (Kiernan et al., 2011; Vucic et al., 2014), all considered to play a role in motor neuron (MN) dysfunction (**Figure I.2**).

Glutamate is the major excitatory neurotransmitter released from presynaptic nerve terminals, diffusing across the synaptic cleft to further activate specific post-synaptic receptors such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Termination of the nervous signal is only achieved upon removal of all glutamate molecules from the cleft by glutamate uptake transporters such as glutamate excitatory amino acid transporter-2 (EAAT2) localized both in neurons and in astrocytes (Ilieva et al., 2009; Vucic and Kiernan, 2009; Colonna and Butovsky, 2017).

Upon excessively release of this neurotransmitter, neurotoxicity, axonal swelling and depolarization are some of the effects produced on neurons. This excitotoxicity is observed both in the SOD1 mutant mouse model, the most studied *in vivo* mice model mutation, and in

familial and sporadic ALS patients. In addition, overstimulation by glutamate can induce a cascade of toxic events in the postsynaptic neurons with a consecutive activation of glutamate receptors which give origin to an increase in calcium influx that is too high for mitochondria and endoplasmic reticulum (ER) storage capacity (Ilieva et al., 2009; Tan et al., 2014), resulting in the production of free radicals that may further damage intracellular organelles and therefore lead to cell death (Bondy and Lee, 1993).

The ER is a cellular compartment in which post-translational protein processing occurs. Upon a stress stimulus, the ER activates two adaptive pathways: (1) the unfolded protein response, which aims to refold misfolded proteins and (2) the ER-associated degradation (ERAD) that exports misfolded proteins to the proteasome, the cell's machinery for eliminating abnormally folded proteins from the cytoplasm (Ilieva et al., 2009; Bunton-Stasyshyn et al., 2015).

Mutant SOD1 (mutSOD1) has been implicated in the direct activation of ER stress (Ilieva et al., 2009; Bunton-Stasyshyn et al., 2015), was found to be accumulated in ER membranes and showed to inhibit ERAD degradation (Ilieva et al., 2009).

Mitochondria are the major source of intracellular reactive oxygen species (ROS). ROS species, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are products of normal oxygen metabolism in cells but when excessively present can harm the structure and function of the cell (Vehviläinen et al., 2014). Degeneration of mitochondria as well as increased production of ROS has been reported both in ALS patients and in the transgenic SOD1 mouse model (Mattiazzi et al., 2002; Lin and Beal, 2006).

In normal cases, the presence of SOD1 in the intermembrane space (IMS) of the mitochondria is thought to exert a protective effect in the handling of O_2^- as it has in the cytosol, converting this anion radical to H_2O_2 and furtherly to water (H_2O) (Vehviläinen et al., 2014). However, the expression of mutSOD1 in neuronal cell lines or in cultures primary motor neurons has been shown to depolarize, impair calcium homeostasis and reduce mitochondria ATP production (Pasinelli and Brown, 2006). It is described that mutant forms of SOD1 protein selectively accumulates onto the cytoplasmic face of the outer membrane of the mitochondria where it forms aggregates and disrupts the proteins translocation machinery (Liu et al., 2004; Vucic and Kiernan, 2009). Accumulation of the mutated protein may interfere with mitochondrial protein import, mitochondrial fission/fusion, ionic balance or regulation of apoptosis (Ilieva et al., 2009; Tan et al., 2014). Although it is not fully understood how the protein affects mitochondrial function, this accumulation, incorrect dismutation of O_2^- anion and accumulation of H_2O_2 may contribute to mitochondrial damage (Vehviläinen et al., 2014).

Autophagy and apoptosis are two basic physiologic processes that contribute to the maintenance of cellular homeostasis. Autophagy is an intercellular catabolic process involved in the turnover of several cell components, important to maintain cellular homeostasis. This

process is essential for neuron survival due to its high specialization and because the cell does not undergo cell division as much as other cells in the body. Hence, they are susceptible to accumulation of misfolded protein aggregates and need a constants protein control to maintain cell viability (Ramesh and Pandey, 2017). Defects or alterations in autophagy pathway or in autophagy related-genes have been described in several pathologies including neurodegenerative diseases (Ghavami et al., 2014) such as ALS, since impairments in this pathway and its machinery can be contributors to the pathological formation of toxic protein aggregates (Ramesh and Pandey, 2017). In particular, beclin-1 is a key regulation of autophagy initiation described to be downregulated in the SC of SOD1-ALS patients where mutSOD1 impedes the vesicle nucleation step of autophagy, through abnormal interaction with beclin-1, thus impeding the cell to clear mutant protein aggregates (Lee et al., 2015; Ramesh and Pandey, 2017).

Accumulation of SOD1 aggregated protein is also described as a contributor to cell death by apoptosis (Ghavami et al., 2014). Apoptosis is a process of programmed cell death which has been described as deregulated in several pathologies, including neurodegenerative diseases (Agostini et al., 2011). The major molecular components of the apoptosis program in neurons include proteins of the Bcl-2 family of oncoproteins and caspases which respond to cell death signals such as DNA damage, oxidative stress or limited trophic support. In ALS, changes in the levels of members of the Bcl-2 family result in a predisposition towards apoptosis. MutSOD1 is highly associated with Bcl-2 in the mitochondria, which results in a conformational change of this protein that weakens mitochondria, resulting in cytochrome c release and activation of mitochondria apoptosis machinery such as capase-9 and -3 (Sathasivam and Shaw, 2005), which mediate cell death in motor neurons in the mutSOD1^{G93A} transgenic mouse model (Zhang et al., 2013).

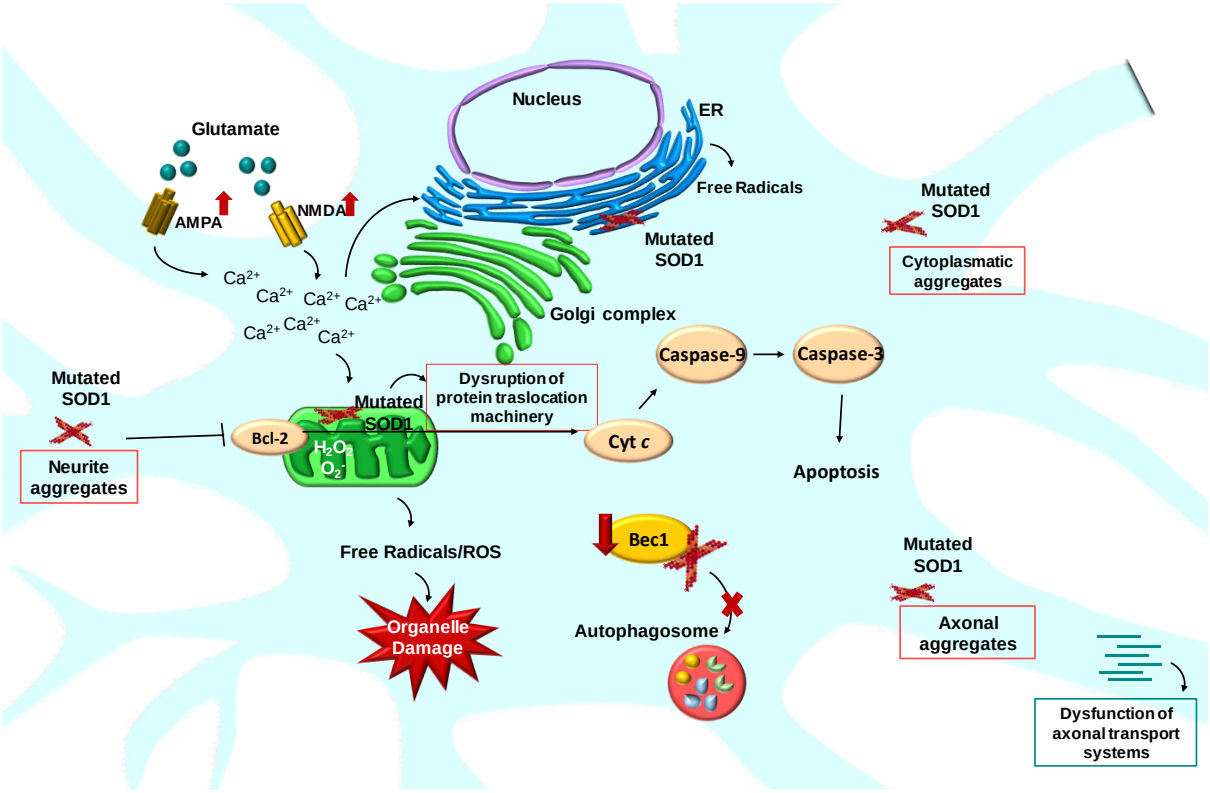


Figure I. 2. Motor neuron pathomechanisms in the amyotrophic lateral sclerosis disease. The neurotransmitter glutamate is released from the pre-synaptic nerve terminals and diffuse across the synaptic cleft to activate NMDA and AMPA receptors in the post-synaptic neuron. When glutamate is released in excess or is insufficiently removed from the synaptic cleft by astrocytes, neurotoxic effects are produced on neurons. Overstimulation by glutamate induces an overactivation of NMDA and AMPA receptors which produce an influx of calcium too high for mitochondria and ER to storage. Also, mutant SOD1 was found to accumulate in ER and mitochondria membranes, resulting in the generation of free radicals and resulting in the disruption of mitochondria machinery. Furthermore, in the mitochondria, mutant SOD1 interferes with the dismutation of O_2^- anion, resulting in the accumulation of H_2O_2, which contributes to mitochondrial damage. In addition, mutant SOD1 aggregated in the cytoplasm interferes with autophagy and apoptosis processes by precipitating with beclin-1 and impeding the formation of autophagosome vesicles and by interfering with the viability of Bcl-2 in the mitochondria which leads to cytochrome *c* release and caspase activation, culminating in apoptosis, respectively. **AMPA,** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; **Bcl-2,** B-cell lymphoma 2; **Bec1**, Beclin1; **Ca^{2+},** calcium; **Cyt *c***, Cytochrome *c*; **ER,** endoplasmatic Reticulum; **H_2O_2,** Hydrogen Peroxide; **NMDA,** N-methyl-D-aspartate; **O_2^-,** Oxygen Anion; **ROS,** Reactive Oxygen Species; **SOD1**, Superoxide Dismutase 1.

I.1.1. ALS-related proteins

It is considered that in ALS protein aggregates interfere with normal protein homeostasis and induce cellular stress. By disturbing cellular functions such as intracellular transport, cytoskeletal architecture and mitochondrial function, cellular stress leads to axonal retraction and ultimately, cell death (Robberecht and Philips, 2013).

In ALS, MNs contain intracellular protein inclusions, which consist of aggregated proteins, thought to be caused by mutations, protein damage such as oxidation, or protein seeding (Robberecht and Philips, 2013).

Besides the pathogenic role attributed to Cu/Zn SOD1, other genes have been reported to contribute to ALS disease origin such as transactive response DNA-binding protein of 43 kDa (TDP-43), *fused in sarcoma* (*FUS*) and *C9orf72* genes (Gordon, 2013). Furthermore, there are some other mutations currently implicated in fALS include the *Angiogenin*, *ataxin-2*, *Optineurin*, *Ubiquilin-2 (UBQLN2), Valosin containing protein (VCP)* and *VAMP-associated protein type B (VAPB)* genes (Gordon, 2013).

Despite the progression and ultimate stage of the disease being almost equal despite which mutation is detected in the patient, mutations in these different genes cause MN death through different pathways: SOD1 mutations lead mostly to oxidative stress; TDP-43, *FUS* and *C9orf72* induce disturbances in RNA machinery; *VAPB* affects endosomal vesicle trafficking; and *UBQLN2* contributes to ubiquitination (Gordon, 2013).

I.1.1.1. SOD1 protein and animal models

SOD1 is composed of 153 amino acids in which more than 150 different mutations have been reported to be pathogenic, representing mutations in this enzyme for about 20% of familial ALS (Gordon, 2013; Robberecht and Philips, 2013). This protein is a member of the human SOD protein family, which includes proteins that function as anti-oxidizing enzymes that catalyze the dismutation of O_2^- to H_2O_2, normally protecting the cell from ROS accumulation. SOD1 is highly abundant, comprising approximately 1% of total proteins in the cell, being located mainly in the cytosol and in a less amount in the IMS (Rotunno and Bosco, 2013).

SOD1 mutations are found in 20% of familial ALS cases and about 1%-5% of sporadic ALS cases globally (Gordon, 2013; Allen et al., 2014; Nardo et al., 2016). The discovery that SOD1 mutations causes ALS early led to the development of the SOD1 transgenic mouse expressing high levels of human SOD1 containing a substitution of the amino acid glycine to the amino acid alanine at position 93 of the sequence (Gurney et al., 1994; Renton et al., 2013). In the mutSOD1 mouse model, several cellular functions, such as mitochondrial energy production, axonal transport and others, have been found to subsequently fail, resulting in axonal retraction and denervation followed by cell death (Robberecht and Philips, 2013).

MutSOD1 accumulates as oligomers and later as aggregates, which lead to stress responses. Afterwards, either because of further protein accumulation or because of an additional stressor, glial cells, such as astrocytes and microglia are activated and start an inflammatory cascade (Robberecht and Philips, 2013).

Although SOD1 appears to trigger disease in motor neurons, astrocytes and microglia are the cells that promote disease progression being hypothesized that SOD1 is linked to ALS inducing oxidative damage to the mitochondria, ER stress, axonal transport dysfunction, excitotoxicity from excess glutamate at synapse, defects in protein degradation machinery and

overproduction of neurotoxic molecules through neuroinflammation (Komine and Yamanaka, 2015).

It has been previously shown that aggregation of misfolded SOD1 protein, influences nearby WT protein to change conformation and also become misfolded, being part of the explanation of how a disease that begins in one area is transmitted widely in the brain (Mackenzie et al., 2007; Gordon, 2013).

Due to the similarities in many genetic features and physiology, anatomy and metabolism between humans and mice, the latter have been the preferred mammalian model for genetic research. In the context of ALS, mouse models based on gene abnormalities associated with ALS pathology have been used to characterize the disease pathophysiology as well as in the design and test of potential targeted therapeutics (Nardo et al., 2016).

The transgenic mice overexpressing the human $SOD1^{G93A}$ mutation was the first model to be developed (Gurney et al., 1994) due to the similarity of the clinical signs between the model and the pathology in humans (Vinsant et al., 2013). Since then, several other transgenic models have been created in mice, rats (Nagai et al., 2001; Howland et al., 2002), zebrafish (Ramesh et al., 2010), *Drosophila melanogaster* and *Caenorhabditis elegans* (Watson et al., 2008).

Nowadays there are 12 different *SOD1* human ALS mutations expressed in the mouse as well as artificially induced *SOD1* mutations that prevent copper binding or truncate the protein (Wang et al., 2003; Han-Xiang et al., 2008; Turner and Talbot, 2008).

I.1.1.2. *TARDBP, FUS* and *C9ORF72* mutations

TDP-43 is a ubiquitously expressed nuclear protein encoded by the *TARDBP* gene. This protein was identified as the major disease accumulated protein (Kabashi et al., 2008; Rutherford et al., 2008) in neuronal intracellular inclusions in proteinopathy diseases such as ALS where TDP-43 is relocated from the nucleus to the cytoplasm and sequestered into inclusion mainly composed of hyperphosphorylated and C-terminally truncated TDP-43 fragments (Rutherford et al., 2008). Mutations in this gene account for about 4-5% of fALS cases (Gordon, 2013; Renton et al., 2013). Nearly 50 mutations have been identified in this gene, mostly involving the C-terminal glycine rich region of the protein, that may influence protein-protein interaction (Kabashi et al., 2008; Gordon, 2013). Mutant forms of protein TDP-43 were described to fragment more rapidly than WT and to caused neural apoptosis and development delay (Sreedharan et al., 2008).

Mutations in the *FUS* gene account for about 5% of fALS and less than 1% sALS (Gordon, 2013; Renton et al., 2013; King et al., 2015). Similarly, to *TARDBP*, more than 50 mutations have been identified, most affecting the last 17 amino acids of the protein, the commonest being Arg521Cys. *FUS* mutations cause ALS with age-of-onset younger than 40

years in one-third of cases, usual onset in the arm, and survival of less than two years (Gordon, 2013; King et al., 2015).

The expansion of the sequence GGGCCC hexanucleotide repeat upstream the *C9orf72* coding region is the most common cause of ALS in Caucasians, although the pathophysiological mechanisms involved are unknown (Mori et al., 2013; Edbauer and Haass, 2016). This mutation is a hexanucleotide repeat in the gene accounting for up to 40% of fALS and 7% of sALS (Gordon, 2013; Floeter et al., 2017). The repeat GGGCCC is translated into dipeptide repeat proteins (DRP). DRPs are known to inhibit the proteasome and sequester other proteins leading to brain atrophy and cognitive impairment. Also, DPRs impair nucleocytoplasmic transport and promote TDP-43 aggregation and mislocalization (Balendra et al., 2016; Edbauer and Haass, 2016).

Besides the SOD1 animal models, others have been created since the discovery of *TARDBP* gene mutations and TDP-43 protein (for review see (Wegorzewska and Baloh, 2011)). More recently, a *C9orf72* bacterial artificial chromosome *(*BAC) mouse model was successfully created being the first to develop the molecular, behavioral, and neurodegenerative features of the disease (Liu et al., 2016).

A new emerging technique in the research field comprises the use of induced pluripotent stem cells (iPSC) generated from fibroblasts of ALS patients. iPSCs from patients with *TARDBP, C9orf72* (Devlin et al., 2015)*, SOD1* (Chen et al., 2014) genes have already been obtained and characterized.

I.2. ALS as a non-cell autonomous disease: studying microglia reactivity and function

Neurodegenerative diseases are characterized by the selective death of a certain group of neuronal cells which are the center of most research studies. However, the activation of glial cells, their role in the death of neurons and spreading of the disease have been gaining attention.

Despite neurons are the most affected cells in neurodegenerative diseases, non-cell autonomous processes also contribute to neuron degeneration, since previous studies in a mice model with overexpression of mutSOD1 in most or all neurons was not sufficient to cause neurodegeneration (Clement, 2003; Yamanaka et al., 2008). Although, these cells acquire an ALS phenotype when surrounded by glial cells carrying the mutation in SOD1 (Clement, 2003; Yamanaka et al., 2008).

Expression of mutSOD1 in motor neurons is determinant for the initial timing of the disease onset and early progression, however there is supporting evidence stating glial cells such as microglia, astrocytes and oligodendrocytes as being actively involved in the later

progression of ALS and motor neuron degeneration (Ilieva et al., 2009; Haidet-Phillips et al., 2011; Lee et al., 2016).

I.2.1. Role of astrocytes in ALS progression

Astrocytes are the most abundant non-neuronal cells in the CNS (Nagai et al., 2007) that have as major functions the clearance of glutamate neurotransmitter upon the nervous impulse and providing metabolic support to neuron cells (Philips and Rothstein, 2014). Upon stress stimuli proliferating and acquiring a reactive phenotype characterized by the development of long and thick processes with an increase expression of glial fibrillary acidic protein (GFAP) (Lee et al., 2016).

One of the pathological characteristics of ALS disease is the generation and migration of new astrocyte cells within and around damaged regions of SC (Lee et al., 2016).

It is probable that genetic alterations induced by mutSOD1 along with other sources of stress, play a role in the transformation of astrocytes into a neurotoxic reactive phenotype (Lee et al., 2016). In this context, non-cell autonomous death of motor neurons in ALS may be de result of the astrocytic support loss and/or the secretion of neurotoxic cytokines, which has been proven in several studies to contributes to disease progression in ALS (Nagai et al., 2007; Ferraiuolo et al., 2011; Meyer et al., 2014; Johann et al., 2015). Also, in ALS, reactive astrocytes present an insufficient release of neurotrophic factors, important in motor neuron health which may contribute to neuronal death (Lasiene and Yamanaka, 2011).

Furthermore, being responsible for the clearance of the glutamate neurotransmitter from the synaptic cleft, mostly by uptake of glutamate by glutamate transporters EAAT2 and GLT-1, being responsible for the maintenance of a low extracellular glutamate concentration, since glutamate overabundance leads to neuronal excitotoxicity (Lasiene and Yamanaka, 2011). When these cells become reactive, expression of *EAAT2* gene is decreased and therefore, glutamate will not be as efficiently removed from the synaptic cleft, contributing to excitotoxicity in MNs. Lastly, astrocyte cell activation leads to the increased production of *iNOS/NOS2* expression which leads to increased production of nitric oxide (NO) release also damages MNs (Lee et al., 2016).

I.2.2. Role of oligodendrocytes in ALS progression

Myelination of axons provided by glial cells was the last major step in the evolution of cells. Oligodendrocytes have the ability to wrap long segments of axons with a multilayered sheath of extended cell membrane in the CNS, while Schwann cells have the same function in the peripheral nervous system (PNS) (Nave, 2010). However, oligodendrocytes myelinate several axons simultaneously, while Schwann cells restrict their myelination to one single axonal segment (Nave, 2010). Like other glial cells such as astrocytes, these cells were

recently shown to provide metabolic support to neurons (Nave, 2010). Therefore, and given their neurotrophic function, oligodendrocytes are most likely contributors neurodegenerative diseases characterized by axonal loss and atrophy, such as ALS pathology (Philips et al., 2013). However, until recently, oligodendrocytes had not been considered as potentially involved in ALS, even though some studies available suggested the existence of abnormalities in oligodendrocytes both in human ALS and in rodent models (Niebroj-Dobosz et al., 2007; Yamanaka et al., 2008; Mackenzie et al., 2011; Ferraiuolo et al., 2016). Actually, extensive degeneration of grey matter oligodendrocytes was found in the SC of $SOD1^{G93A}$ mice prior to disease onset (Kang et al., 2013).

I.2.3. Microglial Cells

Microglia account for approximately 10% of cells in the CNS and originate from a pool of primitive macrophages from the yolk sac, appearing in the mouse at embryonic day (ED) 8.5, constituting an entirely different lineage than the other hematopoietic cells (Ginhoux et al., 2013; Colonna and Butovsky, 2017; Wolf et al., 2017). They invade the brain early in the development, transforming into cells with a highly ramified phenotype (Wolf et al., 2017). During development, microglia plays a role in shaping neural circuits by modulating the strength of synaptic transmission and sculpting neuronal synapses (Colonna and Butovsky, 2017). Further in life, microglia perform not only immune functions but other fundamental roles like the control of neuronal proliferation and differentiation as well as in the formation of synaptic connections (Ginhoux et al., 2013).

In the healthy CNS, microglia constantly survey their environment with their motile processes to make contact with synapses, where they contribute for the modification and elimination of synapse elements, astrocytes and blood vessels (Ginhoux et al., 2013; Heneka et al., 2014). Upon an insult, microglia cells migrate to the lesion site and activate their signaling pathways producing pro-inflammatory mediators (Lasiene and Yamanaka, 2011).

In ALS pathology, microglia are an activated state, contributing to MN death through the secretion of neurotoxic factors. Also, in later stages of the disease, impaired microglia contributes to disease progression (Brites and Vaz, 2014).

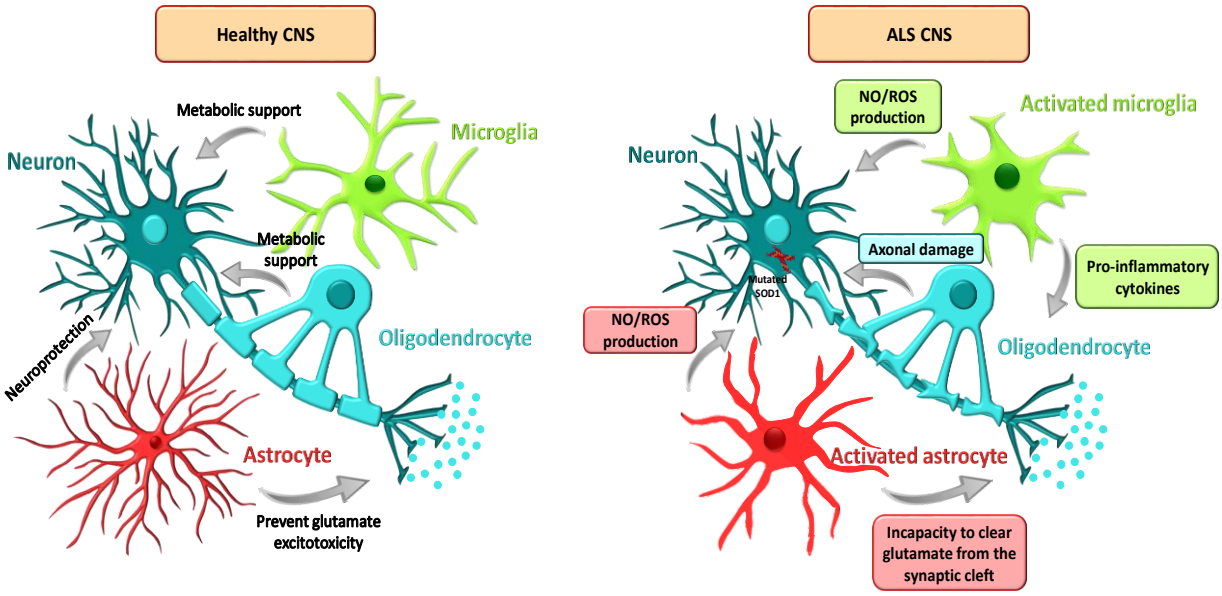


Figure I. 3. Schematic representation of glial cells in amyotrophic lateral sclerosis. Upon an inflammatory stimulus, such as the presence of mutated protein aggregates, glial cells support to neurons is disrupted, conveying in the activation of these cells which produce and release inflammatory cytokines that will be further players in the uncontrolled inflammation generated in the brain. **ALS,** Amyotrophic Lateral Sclerosis; **CNS,** Central Nervous System; **NO**, Nitric Oxide; **SOD1,** Superoxide Dismutase 1; **ROS**, Reactive Oxygen Species.

I.2.3.1. Neuroinflammation

Uncontrolled inflammation constitutes a major component of CNS diseases related to acute or chronic neurodegenerative processes (Park et al., 2015; Cardoso et al., 2016). Although perivascular, choroid plexus and meningeal macrophages can also be found in the brain, brain immunity is mainly sustained by microglia cells, the resident immune cells in the CNS parenchyma (Heneka et al., 2014; Waisman et al., 2015; Cardoso et al., 2016). These cells are equally distributed throughout the brain and spinal cord with increased densities near neuronal nuclei, including *substantia nigra* in the midbrain (Ginhoux and Prinz, 2015).

In the healthy CNS, microglia role is essentially of surveillance, having two photon microscopy studies showed these cells present an extremely ramified morphology with long branches and small soma, always moving (Ginhoux et al., 2013; Cardoso et al., 2016). Also, through this technique it was also possible to see microglia's phenotype rapidly change to a round, branchless morphology in response to injury (Cardoso et al., 2016). Under normal conditions, macrophages from the PNS are not likely to infiltrate into the CNS, unless the blood-brain barrier (BBB) is damaged (Komine and Yamanaka, 2015), although, upon brain injury or fluctuations in CNS homeostasis, microglia cells are capable of recruiting macrophages from the PNS, orchestrating an immunological response (Wolf et al., 2017). At the same time, microglia are responsible for phagocytosis and elimination of pathogens, dead

cells and protein aggregates, acquiring diverse and complex phenotypes and migrating to the lesion site, participating in the cytotoxic response by proliferating and producing inflammatory mediators, participating in immune regulation and injury resolution (Caldeira et al., 2014; Komine and Yamanaka, 2015; Cardoso et al., 2016; Colonna and Butovsky, 2017).

Neuroinflammation concept definition is nothing more than this activation of glial cells and infiltrated macrophages from the PNS, which leads to the production of proinflammatory cytokines and chemokines, characteristic of neurodegenerative diseases like ALS (Ilieva et al., 2009; Komine and Yamanaka, 2015; Lee et al., 2016). However, this process is a vicious cycle, since the production of proinflammatory cytokines and toxic molecules leads to acceleration of neuronal dysfunction and the latter to the production of more of these molecules (Komine and Yamanaka, 2015).

I.2.3.2. Alarmins and microglia response

The majority of neurodegenerative diseases have as a hallmark the deposition and accumulation of aggregated misfolded proteins, which cause an activation of microglia cells that as the immune resident macrophages of the CNS have the function of maintaining homeostasis.

To be able to recognize foreign molecules, microglia cells express pattern recognition receptors (PRRs) that are able to sense pathogen associated molecular patterns (PAMPs) and others that can detect damage-associated molecular patterns (DAMPs) (Colonna and Butovsky, 2017; Wolf et al., 2017). PRRs include toll-like receptors (TLRs), such as TLR4 and TLR1/2 and their co-receptors and NOD-like receptors (NLRs) like the NLRP3 inflammasome (Colonna and Butovsky, 2017). Thus, several molecules associated with neurodegeneration including SOD1 act as a DAMP, activating PRRs, which excessively stimulated, causes prolonged neuroinflammation and ultimately cell death, contributing to neurodegeneration and disease progression. Neurodegenerative conditions activating microglia also induce the release of ROS and NO, through the activation of NADPH oxidase, myeloperoxidase, inducible nitric oxide synthase (iNOS) and release of matrix metalloproteinases (MMPs) (Colonna and Butovsky, 2017; Wolf et al., 2017). In addition, microglia also express chemokine receptors, such as CX3CR1 as well as integrins, such as CD11b and CD11c, that control microglia migration in the CNS and potentiate their ability to bind foreign molecules for elimination (Colonna and Butovsky, 2017).

DAMP molecules are described as a group of endogenous danger signals that play a critical and proinflammatory role in innate immunity. These molecules are also known as ‘’alarmins’’ since they are released upon stress or damage to the cells. They are similar to immune system molecules given their adaptive immune response, activating antigen-presentation in cells and rapid release upon tissue injury (Lee et al., 2014; Feldman et al.,

2015). By binding to specific receptors like TLR2, TLR4 and receptor for advanced glycation end products (RAGE), DAMP molecules induce early innate and adaptive immune inflammation without infection, the so called ‘’sterile inflammation’’ (Lee et al., 2014; Feldman et al., 2015).

DAMPs have been gaining attention from the scientific community in the latest years by their main role in the inflammation process. Originally, DAMP molecules were associated with cell death, however, more recently, these molecules were shown to be secreted by damage cells as an ‘’alarm sign’’ that something is not right in the brain environment. In normal physiological conditions, DAMPs are not detectable to the immune system. However, upon their release after cell death or stress, they play a main role in inflammation, activating almost exclusively macrophages and microglia through TLRs (Feldman et al., 2015; Pandolfi et al., 2016).

On the DAMP category are included S100 proteins, heat-shock proteins and high mobility group box 1 (HMGB1) , which although naturally expressed in the cytosol or nucleus can be secreted to the extracellular media sending signals to the surrounding cells for the presence of damaged tissue (Lotze and Tracey, 2005).

In early stages of ALS pathology, microglia exert a protective effect, increasing the production of brain derived neurotrophic factor (BDNF). However, at a later stage of the disease, these cells start to participate in disease progression and motor neuron death due to the secretion of neurotoxic molecules like HMGB1, which promotes transcription of several proinflammatory genes (**Figure I.4**). HMGB1 is a 25-30 kDa protein constituted by two homologous DNA-binding domains: the A box and the B box (Lee et al., 2014). Functionally, A and B box domains are DNA binding domains although HMGB1 box A also acts as a specific antagonist to HMGB1, showing an anti-inflammatory effect (Lee et al., 2014). On the other hand, B box domain is not only related to DNA binding as it is also involved in the cytokine activity of the protein, stimulating the release of pro-inflammatory cytokines in macrophages, like tumor necrosis factor (TNF)-α (Ellerman et al., 2007; Lee et al., 2014).

HMGB1 is a highly conserved, non-histone mobile chromatin protein. In eukaryotic cells, it is described to mainly remain in the cell nucleus, transiently binding to the DNA and functioning as a chaperone, facilitating DNA replication, recombination, DNA repair, stabilization of nucleosome formation, integration of transposons, and transcription (Lotze and Tracey, 2005; Ellerman et al., 2007; Lee et al., 2014; Pandolfi et al., 2016). While in the cytoplasm, it is involved in the regulation of autophagy and in maintaining balance between autophagy and apoptosis (Lee et al., 2014; Yang et al., 2015). This protein has been implicated in several aspects of the innate immune system as a DAMP molecule, playing a role as a mediator of autophagy and apoptosis as well as a late inflammation mediator. Upon release

by apoptotic or necrotic cells, HMGB1 is able to activate macrophages and produce an inflammatory response (Lotze and Tracey, 2005; Ellerman et al., 2007; Lee et al., 2014).

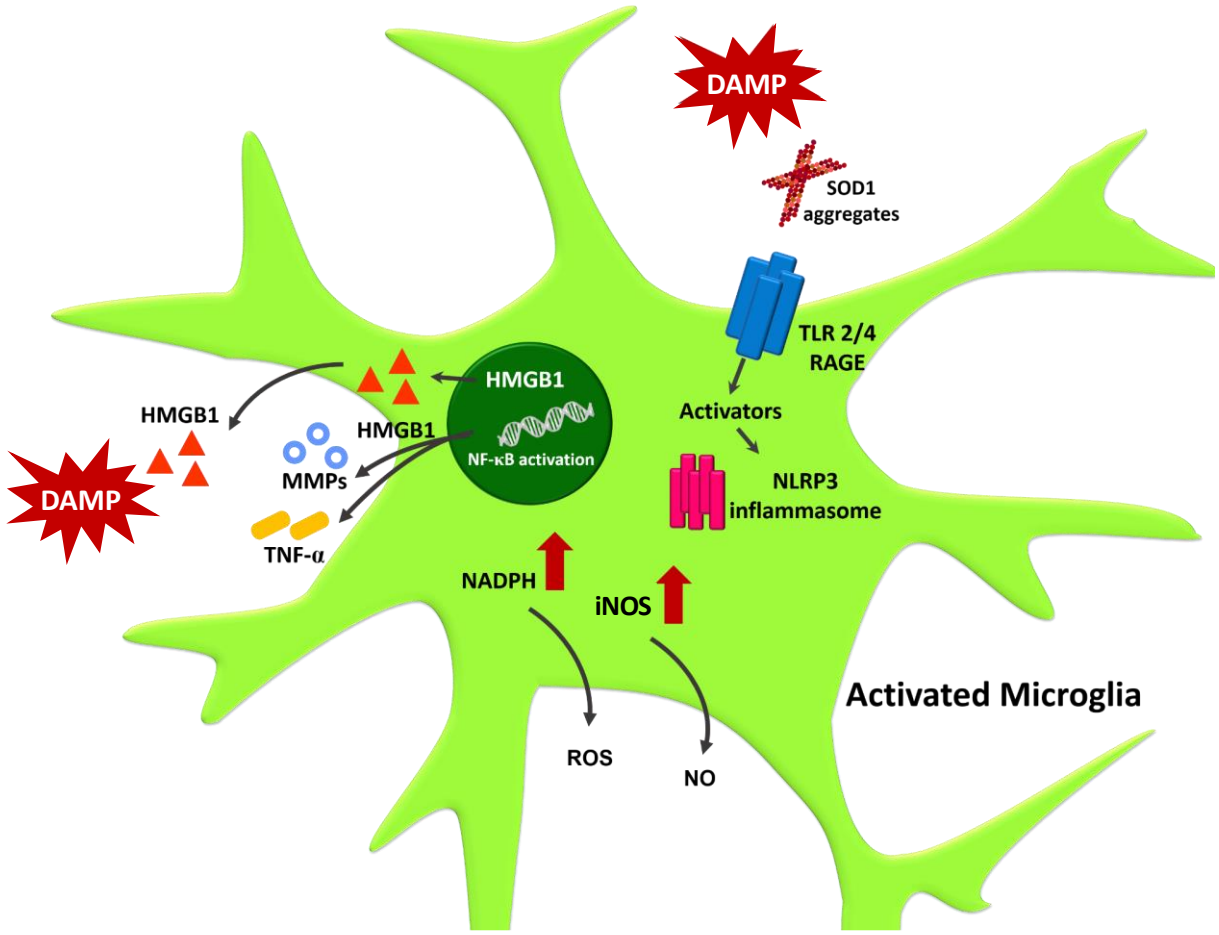


Figure I. 4. Microglia response to DAMP molecules in the CNS. As the immune cells of the central nervous system (CNS), microglia are always into a vigilant state, reacting to abnormal stimulus. When released to the extracellular media by neurons or other CNS cells, SOD1 aggregates act like a damage insult associated molecular pattern (DAMP), also called alarmins, that signalize danger signs. DAMP molecules activate pattern recognition receptors (PRRs) such as toll-like receptors (TLRs)-2 and -4, as well as receptor for advanced glycation end products (RAGE) which will further culminate in the activation of inflammatory cascades that will lead to NLRP3 inflammasome, and NF-κB activation. The latter increases the expression of other alarmin molecules like high molecular group box protein – 1 (HMGB1), matrix metalloproteinases (MMPs), inducible nitric oxide synthase (iNOS) and NADPH oxidase. As a result, HMGB1, MMPs, nitric oxide (NO) and reactive oxygen species (ROS) will be secreted into the extracellular media, producing more inflammation in the surrounding cells. **CNS,** Central Nervous System; **DAMP,** Damage Associated Molecular Pattern; **HMGB1,** High-Mobility Group Box Protein 1; **NF-κB,** Nuclear Factor Kappa B; **NO,** Nitric Oxide; **MMP,** Metalloproteinase; **PRR,** Pattern Recognition Receptor; **RAGE,** Receptor for Advanced Glycation End Products; **ROS**, Reactive Oxygen Species; **SOD1,** Superoxide Dismutase 1; **TLR,** Toll-like Receptor; **TNF,** Tumor Necrosis Factor;

It also contains two binding sites TLRs and RAGE, both crucial in the activation of macrophage cytokine release (Lee et al., 2014; Tsung et al., 2014; Yang et al., 2015; Pandolfi et al., 2016).

HMGB1 can be secreted for the extracellular media after injurious stimuli, transmitting danger signals to neighboring cells that initiate an inflammatory response. Once outside the cell, HMGB1 acts as a proinflammatory cytokine, functioning as a mediator of cellular communication and coordination of cellular activity, through specific receptors to promote the activation of the NF-κB signaling pathway, with a subsequent production of cytokines and chemokines (Lee et al., 2014). Also, its binding ability to RAGE receptor, mediate signals for neuronal outgrowth, polarization of T cells and upregulation of other cell-surface receptors such as TLR2, TLR4 and RAGE, triggering activation of Ras, PI3K and Rho pathways all converging to NF-κB (Lee et al., 2014).

I.2.3.3. Reactivity

Microglia activation occurs upon any kind of insult to the brain and activated cells acquire an amoeboid phenotype. Two types of activation are currently considered, the classical M1 pro-inflammatory and the M2 repair or anti-inflammatory state although the latest knowledge suggests that there is more heterogeneity in activation states than it was initially thought (**Figure I.5**) (Orihuela et al., 2016; Tang and Le, 2016; Wolf et al., 2017).

The classic M1 phenotype is neurotoxic and involved in the release of pro-inflammatory cytokines and chemokines and specialized in pathogen elimination. In this state, TLR and IFN-γ signaling pathways are commonly activated and pro-inflammatory molecules like TNF-α, HMGB1, S100B, iNOS, IL-6, IL-1β, IL-12, CCL2, MMP12, MHCII, costimulatory molecules, RAGE, TLR4 and TREM-2 receptors, Fc receptors and integrins are produced (Komine and Yamanaka, 2015; Colonna and Butovsky, 2017). Furthermore, chemotaxis, microglia ability to migrate towards injured regions, is a property that also seems to be more related to M1 phenotype, being the release of chemotactic molecules like ATP indicated to participate in the recruitment of these cells to lesion sites. If existent, infectious pathogens are phagocytosed by microglia through TLRs or complement receptors that also promote the release of pro-inflammatory cytokines, while apoptotic cells or debris are internalized through phosphatidylserine receptors.

On the other hand, M2 anti-inflammatory phenotype, usually induces MFG-E8, Arginase 1 (Arg1), SOCS1, Fizz1, IL-4, IL-13, IL-10, TGF-β production, contributing to neuroprotection through the release of anti-inflammatory cytokines and growth factors and (Colonna and Butovsky, 2017).

Both these transitional phenotypes were shown to exert beneficial or destructive effects depending on the stimuli, the duration and the surrounding environment, being the balance between both phenotypes considered to be a desirable therapeutic goal (Caldeira et al., 2014).

M1 and M2 phenotypes are also associated with a different expression profile of microRNAs (miRNAs). Thus, miRNAs expressed in a certain moment help to identify which is

the activated phenotype more prevalent in different neurological diseases. This suggests that microglia's phenotype modulation may allow a proper shift of these phenotypes and lead to a possible new way of treatment. Also, different kinetics of miRNA expression and degradation is proved to produce different immune outcomes and changes microglia phenotypes (Cardoso et al., 2016).

I.2.3.4. Microglia phenotypic diversity and microRNAs profile in ALS pathology

Although most of the investigation in ALS disease has been centered in specific proteins, also regulatory mechanisms mediated by small non-coding RNAs have been considered and explored in this complex disease being now considered key to understand pathology and disease progression (Abe and Bonini, 2013).

MiRNAs are conserved, endogenous, non-coding RNAs. They are about 20-24 nucleotides long and post-transcriptionally regulate protein expression or levels of target messenger RNA (mRNA) transcripts (Gascon and Gao, 2012; Abe and Bonini, 2013; Volonte et al., 2015). There are more than 1000 different known human miRNA sequences, being 20-30% of all human protein-coding genes controlled by miRNAs (Volonte et al., 2015).

The link between miRNAs and neurodegeneration was discovered while studying the effect of global disruption of miRNA biogenesis on neuronal development and they have been implicated in the modulation of inflammatory responses and in immune system activation (Paez-Colasante et al., 2015; Karkeni et al., 2016; Thome et al., 2016).

Some specific miRNAs are emerging as important contributors to ALS pathogenesis, being pointed as key regulators of inflammation and as mediators of macrophages/microglia polarization, such as miRNAs-155, -146a and -125b (Parisi et al., 2013; Cunha et al., 2016).

Upon an inflammatory stimulus with lipopolysaccharide (LPS) *in vitro*, inflammatory miRNAs-155 and -146a have been related to microglia polarization towards an M1 pro-inflammatory phenotype (Cunha et al., 2016).

MiRNA-155 is usually upregulated in blood and in SC (Maciotta et al., 2013; Paez-Colasante et al., 2015; Cunha et al., 2017; Pegoraro and Angelini, 2017) and is involved in the targeting of TGF-β1, a set of peptides in control of cell differentiation and proliferation. Additionally, it has shown to increase proinflammatory cytokine secretion (Cunha et al., 2016). Also, a recent study demonstrated that genetic ablation or administration of antisense oligonucleotide of miR-155, elevated in humans and SOD1 mice model, extended survival time of $SOD1^{G93A}$, partly thought restoring microglial functions and controlling neuroinflammation (Komine and Yamanaka, 2015). MiR-146a is reported to be dysregulated in spinal cord, promoting tissue inflammation (Butovsky et al., 2012; Maciotta et al., 2013; Paez-Colasante et al., 2015; Cunha et al., 2017). Although, the role of miR-146a is still quite unclear, since it is

also overexpressed in senescent/dystrophic microglia, which is not able to respond to stress stimuli anymore (Jiang et al., 2012; Cunha et al., 2016).

MiR-125b involves cell growth, movement and division. It is responsible for macrophage activation (Chaudhuri et al., 2011) and when overexpressed in these cells, enhances surface activation markers and become potent stimulators of immune responses. In ALS, this miRNA is reported to be downregulated (Paez-Colasante et al., 2015) due to the release of TNF-α (Parisi et al., 2013).

While miR-155 and miR-146a are considered proinflammatory miRNAs, miR-21 is in control of the extension of the immunological response, repressing M1 phenotype (Iliopoulos et al., 2010; Paez-Colasante et al., 2015; Cardoso et al., 2016; Cunha et al., 2016). The function of miR-21 is to target the expression of *TGF-β1*, causing an overall reduction of *TGF-β1* levels and is upregulated in patients with ALS (Parisi et al., 2013).

I.2.3.5. Microglia capacity of response and aging

Aging is a major risk for the development of many neurodegenerative diseases and brain aging has been intensively studied recently having several processes like mitochondrial dysfunction, oxidative stress and autophagy have been identified as contributors to the loss of synapses in neurons (Bishop et al., 2010; von Bernhardi et al., 2015).

Since glial cells are highly important for all brain functions, it is very likely that aging related changes in glial cells, particularly microglia, are important to the development and progression of neurodegenerative diseases (Biber et al., 2014).

In the healthy aging brain, microglia acquire a hypersensitive phenotype that results in an exaggerated immune responsiveness called microglial ‘’priming’’ (Godbout and Johnson, 2009; Biber et al., 2014; Perry and Holmes, 2014). Priming reflects a shift of microglia towards a pro-inflammatory state known as classically activated M1-state. With age, microglia undergoes several age-related changes that contribute to the generation of a chronic mild inflammatory environment, including the production of pro-inflammatory cytokines and ROS species (von Bernhardi et al., 2015).

Actually, not only aging appears to be a risk factor for neurodegenerative diseases, but the presence of neurodegenerative diseases potentiates the appearance of aging and senescence related markers (Baron et al., 2014; von Bernhardi et al., 2015). After prolonged inflammation in the CNS, and similarly to what happens in the aging brain, microglia acquire a senescent impaired phenotype, contributing to disease progression. Senescent microglia are characterized by a specific phenotype with large soma and thicker processes gathered with the decreased expression of M1 pro-inflammatory markers such as MHC-II, IL-1β, IL-6, TNF-α and activation of NF-κB resulting from the mitochondrial damage caused by oxidative stress,

while M2 markers, involved in neuroprotection are upregulated, suggesting a more protective phenotype (Sierra et al., 2007; Colonna and Butovsky, 2017; Wolf et al., 2017).

Dystrophic/Senescent microglia show different morphological characteristics: they are characterized by a cytoplasmic spheroid formation containing phagocytic-intake material and short, tortuous, swollen processes (Streit, 2006; Sierra et al., 2007; Colonna and Butovsky, 2017). Also, reduced phagocytic capability is described in these cells, diminishing their capacity of clearance of both pathogens and apoptotic cells (Caldeira et al., 2014; Cardoso et al., 2016; Pinto et al., 2017).

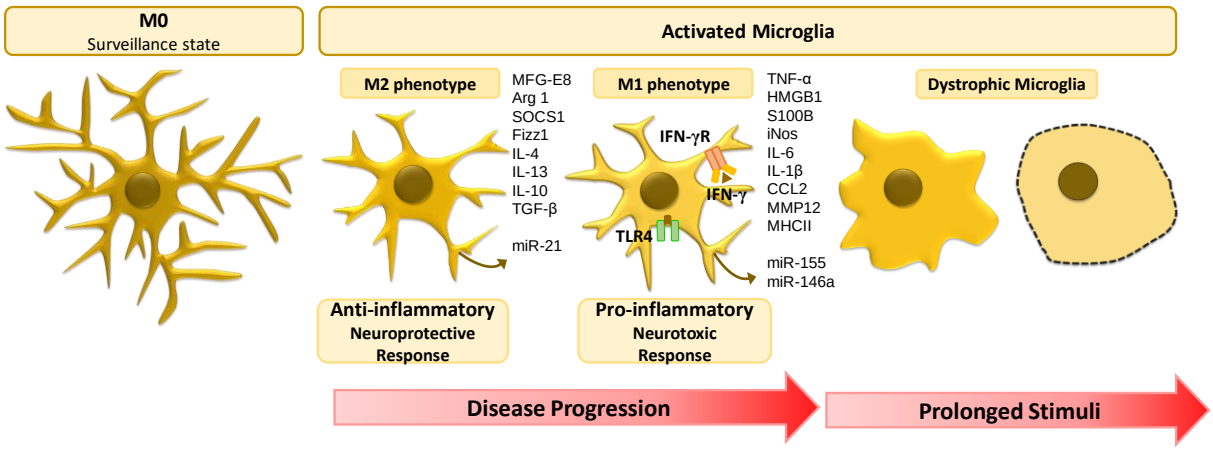


Figure I. 5. Schematic representation of microglia polarization states. Microglia activated cells acquire an amoeboid phenotype. Two types of activation are considered to be prevalent: The M1 pro-inflammatory activation state, involved in a pro-inflammatory and neurotoxic response, and the M2 anti-inflammatory state, considered to be involved in an anti-inflammatory, repair and neuroprotective response. In the first, TLR and IFN-γ signaling pathways are activated and pro-inflammatory molecules are released. In the second one anti-inflammatory cytokines and chemokines as well as growth factors are released. **Arg1**, Arginase 1; **CCL**, Chemokine Ligand; **Fizz1**, Found in Inflammatory Zone 1; **HMGB1**, High Mobility Group Box 1; **IL**, Interleukin; **MHCII**, Major Hitocompatibility Complex class II; **miR**, microRNA; **MMP**, Metalloproteinase; **S100B**, S100 Calcium Binding Protein B; **SOCS1**, Suppressor of Cytokine Signalling 1; **TGF**, Tumor Growth Factor; **TNF**, Tumor Necrosis Factor.

I.3. Intercellular communication in the CNS

The human body is an amazing complex system where everything is tightly connected. Intercellular communication plays an essential role in the development and maintenance of homeostasis and can occur locally, involving direct contact between cells, or through distance, secreting molecules like hormones that send signals through the circulatory system to different parts of the body (Ha et al., 2016). In particular, CNS is characterized by a reciprocal communication among its cellular populations such as neurons, astrocytes and microglia (Basso and Bonetto, 2016).

ALS disease is often originated in a specific part of the body followed by contiguous spread of the disease which is compatible with a propagating process. Recent work on familial

ALS SOD1 mutations, suggested a cell-to-cell transmission of mutSOD1 aggregates (Chia et al., 2010; Munch et al., 2011) which was verified by Munch and colleagues (2011) in Neuro2a cells.

It is considered that misfolded/mutated proteins can be spread from one cell to another in two different ways: 1) by extracellular release of soluble factors that are then collected by other cells or 2) by delivery within membrane-bound structures (Garden and La Spada, 2012).

This last process has recently been gaining attention. Communication through the release of this membrane-bound structures, referred to as extracellular vesicles (EVs) have been shown to be released by all cell types in the CNS and further uptake by neighboring cells (Basso and Bonetto, 2016; Zappulli et al., 2016). EVs are known to participate in multiple processes including maintenance of myelination, synaptic plasticity, antigen presentation and trophic support of neurons (Pitt et al., 2016; Thompson et al., 2016).

I.3.1. Extracellular Vesicles

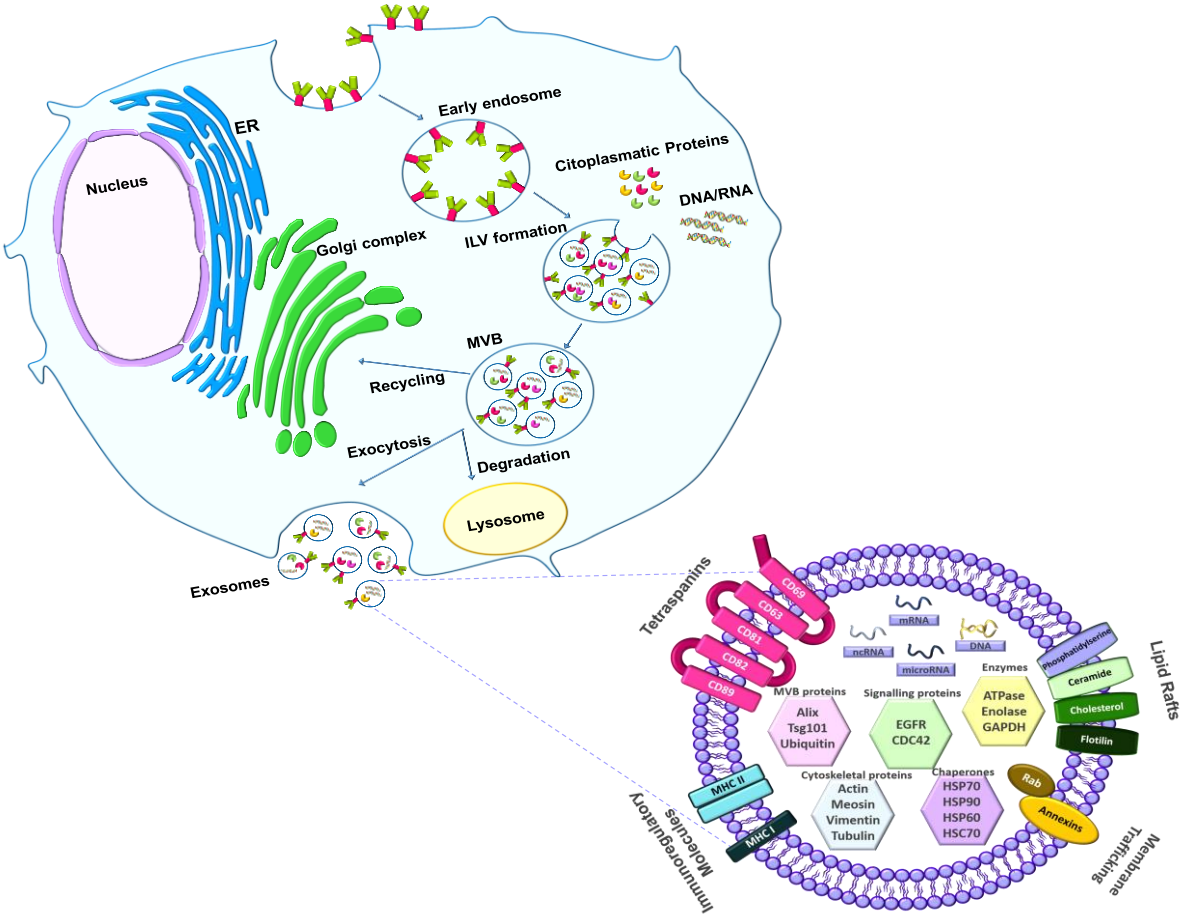
EVs are secreted by every cell of the body and allow the communication among cells both neighboring and long distance (Tkach and Théry, 2016).

These vesicles are divided into three different types accordingly to their subcellular origin: apoptotic bodies, microvesicles and exosomes, each which present different characteristics, like their different compositions (Ha et al., 2016; Tkach and Théry, 2016).

In particular exosomes range in size from 30 to 150 nm in diameter and are originated from the invagination of the endosomal limiting membrane to form the multivesicular body (MVB), containing intraluminal vesicles (ILVs) (**Figure I.6**) (Schneider and Simons, 2013; Ibrahim and Marbán, 2016). They are secreted by all cell types and can be found in most body fluids, including blood, saliva and urine (Ha et al., 2016; Thompson et al., 2016). Exosomes are composed by a lipid bilayer membrane containing proteins and lipids derived from the parent cell (Ha et al., 2016; Tkach and Théry, 2016). The protein constitution includes transport proteins, heat shock proteins, proteins associated with the MVB biogenesis and tetraspanins. Cholesterol, sphingolipids, phosphoglycerides, ceramides, and saturated fatty acid chains are among the presented lipids (Ha et al., 2016). Exosomes are specifically enriched in several proteins such as the integrins and tetraspanins CD63, CD89, CD81, CD9 and CD82; the MVE proteins alix and tsg101; the endosomal and endosome maturation-related proteins flotillin and annexin; and the heat shock proteins hsp70 and hsp90, all of which serve as marker proteins (Simons and Raposo, 2009; Schneider and Simons, 2013).

Once in the extracellular space exosomes can passively traffic through the bloodstream or other bodily fluids or bind to specific cells mediating cell-to-cell communication, through different mechanisms: (A) They can activate intracellular signaling by ligand-receptor interaction without internalization; (B) Their membrane proteins can be cleaved, releasing

soluble ligands that bind to target receptors on the cell surface; (C) They can be taken up by membrane fusion where exosomes release their content into the recipient cell cytoplasm, thereby modifying the physiological state of the recipient cell ; (D) They can be internalized by endocytic mechanisms by recipient cells (Urbanelli et al., 2013; Robbins and Morelli, 2014; Tkach and Théry, 2016). Regarding cells in the CNS, exosomes have been shown to be release *in vitro* by oligodendrocytes, microglia, astrocytes and neurons (Potolicchio et al., 2005; Fauré et al., 2006; Krämer-Albers et al., 2007; Taylor et al., 2007). Vs derived from glial cells, particularly microglia and astrocytes have an active role in neuroinflammation, by spreading proinflammatory signals. Although, they can also be carriers of factors involved in



repair after stress, disease and injury (Budnik et al., 2016).

Figure I. 6. Scheme of the formation and constitution of exosomes. Exosomes originate from the endosomal pathway. The early endosome results from the membrane invagination. This structure invaginates one more time to form ILVs that can incorporate proteins and/or nucleic acids present in the cell's cytoplasm. After the vesicles are formed, the structure that carries them is named the MVB. The MVB can then follow the recycling pathway towards the Golgi complex, follow the recycling pathway towards the lysosome or fuse with the extracellular membrane, releasing the exosomes to the extracellular media. Exosomes are specifically enriched in several proteins that serve as markers to identify them such as tetraspanins, MVB proteins, endosome-maturation related proteins and heat-shock proteins. **dsRNA,** Double Strand RNA**; DNA,** Deoxyribonucleic Acid; **ER,** Endoplasmatic Reticulum; **ILV,**

Intraluminal Vesicle; **MHC,** Major Histocompatibility Complex; **MVB,** Multivesicular Body**; RNA,** Ribonucleic Acid; **ssRNA,** Single Strand RNA.

I.3.2. Disease spreading

One of the mechanisms by which a protein can dominate a pathology is by propagating protein misfolding, like what happens in prion diseases (**Figure I.7**) (Grad et al., 2014). Recently, this mechanism has been increasingly implicated in neurodegenerative diseases as a cell to cell transmission of misfolded protein aggregates (Lee et al., 2014). Prion diseases are fatal neurodegenerative diseases of mammals, in which the infectious agent is a misfolded protein referred to as PrP^{SC}. These proteins are able to self-propagate by binding to monomers of PrP^{C}, the normal prion protein (Kabir and Safar, 2014). Recent studies in cell lines and mouse models suggest a prion-like mechanism is ALS disease.

I.3.2.1. MiRNAs and misfolded proteins

EVs are composed of a lipid bilayer containing transmembrane proteins. These vesicles are capable of enclosing several kinds of molecules, although, how this cargo becomes selected and whether it has a function in the recipient cell is still unclear and a matter of study (Budnik et al., 2016). Published studies reported molecules including cytosolic proteins and nucleic acids like DNA, RNA, mRNA and miRNAs as being carried inside these vesicles (Tkach and Théry, 2016). Several types of nucleic acids have already been detected inside EVs: DNA, including double-stranded and single-stranded DNA, retrotransposons and mitochondrial DNA; as well as RNA, including messenger RNA (mRNA), hairpin and non-hairpin RNA, transfer RNA, noncoding RNA, miRNA, ribosomal RNA fragments, small nucleolar RNA, small nuclear RNA, and small cytoplasmic RNA (Budnik et al., 2016; Thompson et al., 2016).

Also, more than 40 000 proteins – nearly one-quarter of the known human proteome - have been detected within EVs accounting nearly 400 proteins for about 75% of the overall EV-associated protein mass (Thompson et al., 2016). Some of these proteins are specific from the cell of origin, while others are specific across all exosomes (Bellingham et al., 2012).

The export of misfolded SOD1 and uptake into other cells has been show in vitro (Urushitani et al., 2008). SOD1 and TDP-43 proteins can propagate within neuronal cells and transmit to neighboring cells whether secreted to the extracellular environment via exocytosis, transported inside vesicles like exosomes, or released upon apoptosis (Gomes et al., 2007; Munch et al., 2011; Nonaka et al., 2013; Lee et al., 2014; Sábado et al., 2014; Grad et al., 2015). These exosomes, can subsequently be taken up by other cells where misfolded cargo of SOD1 will provide a template for induction of protein misfolding in other cells (Figure I.6) (Grad et al., 2015). MutSOD1 aggregates have been shown to be able to enter neuronal cells, where they cause aggregation of the normal cytoplasmic mutSOD1 (Munch et al., 2011). Also,

mutSOD1 induces misfolding of hSOD1^{WT}, which propagates across cultured cells, even if mutSOD1 is absent in these cells (Grad et al., 2011, 2014).

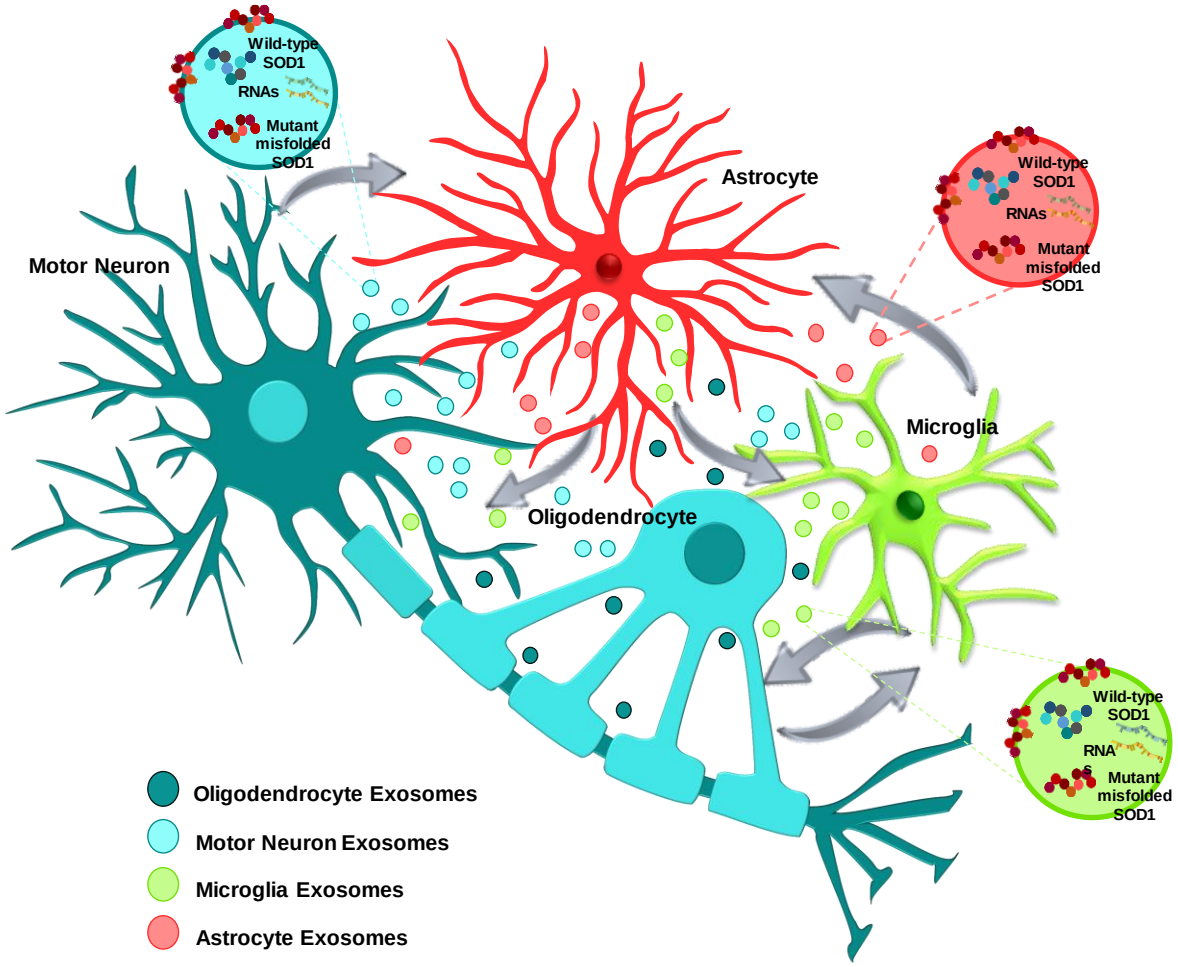


Figure I. 7. Schematic representation of the intercellular communication in the CNS. Extracellular vesicles and their content have recently been gaining attention. In particular, exosomes released by all cell types, have been pointed as carriers of proteins such as aggregates of SOD1 observed in experimental models of amyotrophic lateral sclerosis, as well as several types of genetic material, playing a key role in inflammation and disease spreading. **CNS,** Central Nervous System; **RNA**, Ribonucleic Acid**; SOD1,** Superoxide Dismutase 1.

I.4. Diagnostic and therapeutic strategies

Currently, diagnostic criteria in ALS assume that clinical presentations are subdivided into bulbar-onset or spinal-onset disease, and by the degree of upper motor neuron and lower motor neuron involvement. With the recent novel technologies of neuroimaging, advanced genetics and bioinformatics, the existence of many subtypes of ALS, comprising a wide clinical and pathological spectrum, is becoming increasingly obvious. There is an awakening of the urgent need for reliable classification, which should include not only motor involvement, but also the degree and type of cognitive and behavioral impairment that is present in each patient (Al-Chalabi and Hardiman, 2013).

Currently, there is still no effective cure for ALS. Only the benzothiazole riluzole, licensed in 1996, had been proved to slow the rate of progression and prolong survival by three months (Gordon, 2013; Renton et al., 2013), until recently when a new compound, Edaravone (Radicava™) was licensed by the Food and Drug Administration (FDA) (https://alsnewstoday.com/edaravone-radicava-for-als/). This compound is a radical scavenger which was found to have a neuroprotective effect, slowing disease progression by removing free radicals in the CNS (Abe et al., 2017).

Several clinical trials have been unable to identify another neuroprotective agent. In progress, are clinical trials that test mediations aiming to interfere with a known cellular event and slow the disease course time. Researchers now aim to slow disease progression by targeting known pathophysiological pathways or genetic defects being most approaches directed at muscle proteins, energetic balance, cell replacement or abnormal gene products resultant from mutations (Gordon, 2013).

I.4.1. Modulating neuroinflammation

Although glial cells are important in the homeostasis of the CNS, their driven inflammation in neurodegenerative diseases contribute to disease progression. It is well known that the resident microglia when activated in response to insults release pro-inflammatory mediators. If the stress stimulus is constant, like what happens if mutated aggregated proteins are present, microglia activation becomes chronic, leading to further exacerbation of their response.

I.4.1.1. Inflamma-MiRNAs and HMGB1 modulation

Inhibition of anti-inflammatory activity of microglia has been considered in order to improve clinical outcome in neurodegenerative diseases (Mosley and Gendelman, 2010). However, a complete inhibition of inflammatory microglial states may be harmful to the CNS and phenotypic microglia changes towards M1/M2 phenotypes are still being discussed.

M1 and M2 phenotypes are also associated with a different expression profile of miRNAs) Thus, miRNAs expressed in a certain moment help to identify which is the activated phenotype more prevalent in different neurological diseases. This suggests that microglia's phenotype modulation may allow a proper shift of these phenotypes and lead to a possible new way of treatment (Cardoso et al., 2016)

In addition, it may be possible to develop strategies to specifically target DAMPs mediated inflammatory responses (Lu et al., 2014). In particular, HMGB1 has been described as a mediator of lethal infection and injury, which led investigators to discover endogenous or exogenous agents which inhibited HMGB1 release, protecting against infection and injury (Lu et al., 2014).

Treatment with HMGB1 inhibitors is beneficial and reduces inflammation in several preclinical animal studies (Yang and Tracey, 2010). Like in other neuroinflammation associated diseases like Alzheimer's disease, HMGB1 was proposed to play a critical pathogenic role in the progression of the pathology, being proposed as a molecular target to delay onset and progression of the diseases (Fujita et al., 2016). There are several described HMGB1 inhibitors. An efficient strategy to perform this inhibition in through the use of HMGB1-antagonists like antagonist A box, which is one of the DNA-binding motifs of HMGB1 (Yang et al., 2004), considered a potential anti-inflammatory agent (Yang et al., 2004; Kokkola et al., 2005). Also, small molecules from natural sources or from chemical synthesis have been used as inhibitors (Mollica et al., 2007; Wang et al., 2010; Musumeci et al., 2014)

I.4.1.2. Anti-inflammatory potential of glycoursodeoxycolic acid (GUDCA) and vinyl sulfone (VS)

Bile acids (BAs) are acidic steroids synthesized from cholesterol (**Figure I.8A**) in the liver. First, primary BAs such as cholic acid and chenodeoxycholic acid are synthesized and then secreted into the intestine where they give origin to secondary BAs upon bacterial dihydroxylation (Cortez and Sim, 2014; Brites, 2015).

Ursodeoxycholic acid (UDCA) is a secondary BA, usually used in the treatment of cholestasis in humans. For centuries isolated from the bile of the black bear, today this species is synthesized by pharmaceutical companies. Once taken orally, UDCA is absorbed in the intestine where it enters the liver through the portal vein and its conjugated with taurine to originate tauroursodeoxycholic acid (TUDCA) but mainly with glycine (79,8%), originating GUDCA (Rudolph et al., 2002; Brites, 2015).

Although the primary function of bile acids is to solubilize dietary fats and vitamins in the intestinal lumen to improve absorption, UDCA, TUDCA and GUDCA have been described to have neuroprotective effects in models of neurodegeneration (Keene et al., 2002; Ramalho et al., 2008; Vaz et al., 2015). In this context, these compounds have been gaining attention as potential therapeutics for neurodegenerative diseases and treatment of non-liver diseases, presenting a low toxicity profile and ability to cross the BBB (Brites, 2015). Cytoprotective effects of UDCA and TUDCA have been attributed to the reduction of ROS formation, prevention of mitochondrial dysfunction and inhibition of apoptosis. Also, these two compounds are described to function as chaperones, reducing accumulation of toxic protein aggregates in disease models (Rodrigues et al., 1998; Cortez and Sim, 2014). Regarding GUDCA, it can prevent mitochondrial swelling, high extracellular levels of glutamate, inflammation, cytochrome *c* release, and neuronal loss, all found in ALS disease. Previous studies from our group with a model of MN-like demonstrates the preventive effects of this compound, pointing GUDCA as a promising therapeutic strategy to slow disease onset and progression.

(Fernandes et al., 2007; Brito et al., 2008; Silva et al., 2010; Vaz et al., 2010, 2015; Brites, 2015).

In the context of anti-inflammatory compounds, the Medicinal Chemistry group of the Research Institute for Medicines has synthesized several vinyl sulfone compounds which have shown inhibitory cysteine protease activity. Cysteine proteases include a particular group of molecules called cathepsins, from the endosomal/lysosomal proteolytic system previously, which were previously shown to be dysregulated in neurodegenerative diseases. Particularly one of the synthesized compounds, dipeptidyl vinyl sulfone (VS) (**Figure I.8B**) was able to prevent Aβ-induced microglia-inflammatory responses using N9 microglia cell line (Falcão et al., 2017). Considering these results, it is possible that VS can also have beneficial effects in other neurodegenerative diseases where inflammation plays a key role, like ALS.

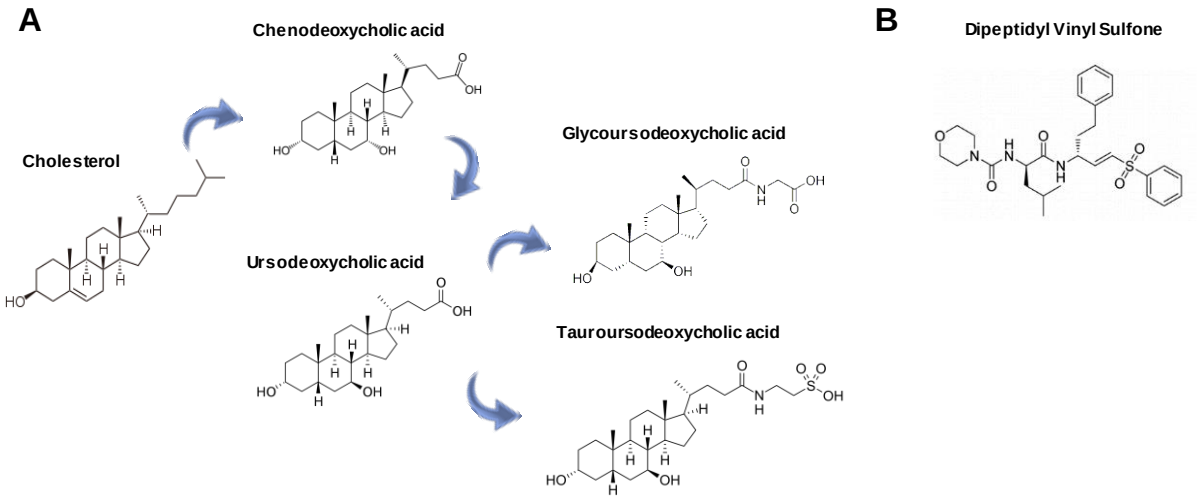


Figure I. 8. (A) Schematic representation of ursodeoxycholic acid conjugation in the liver and (B) Chemical structure of VS. In the scheme are represented the chemical structures of cholesterol which is transformed in the liver into the primary bile acid chenodeoxycholic acid, from which ursodeoxycholic acid is an epimer. Secondary bile acid ursodeoxycholic acid is produced through bacterial dihydroxylation in the small bowel. After entering the portal vein, this product re-enters the liver where it is conjugated with taurine to originate tauroursodeoxycholic acid or with glycine to originate glycoursodeoxycholic acid.

I.5. Aims

It is estimated that among all patients with ALS, the most common neurodegenerative disease affecting the motor neuron system, 5 to 10% of cases are due to an inherited genetic mutation, often in SOD1 gene. The $SOD1^{G93A}$ mutation is the most studied one, mainly because the transgenic mouse model reproduces ALS symptoms, is widespread available and allowed the identification of numerous deficits and impairments, in particular neuroinflammation. $MutSOD1^{G93A}$ microglia isolated from the transgenic mouse model showed to have an inflammatory phenotype, but how the N9 murine microglia cell line transduced with lentiviruses to express $SOD1^{G93A}$ differently display phenotypic characteristics from those overexpressing the WT SOD1 has been scarcely investigated. Therefore, the global aim of this thesis is to assess whether the expression of $hSOD1^{G93A}$ is responsible for changes in microglia activation profile, leading to deregulated response towards LPS immunostimulation and, if so, whether antioxidant and immunomodulatory compounds can recover microglia steady state phenotype. With that in mind, we intend to better elucidate the key pathogenic role of microglia in the onset and progression of ALS disease and to find out targets to develop therapeutic strategies.

Specifc aims of the present study are:

1. Characterization of pro-/anti-inflammatory markers in the mutant $SOD1^{G93A}$ microglia. N9 microglial cells will be transduced with $hSOD1^{G93A}$ to assess induced changes in their reactive status, and data will be compared with $hSOD1^{WT}$ and N9 naïve cells;

2. Assessment of mutant $SOD1^{G93A}$ microglia activation susceptibility upon immunostimulation. Cultures of N9 naïve cells, as well as microglia overexpressing $hSOD^{WT}$ and $hSOD1^{G93A}$, will be treated with LPS for 48 h and the induced activation profile will be evaluated in each cell type and compared;

3. Determination of the benefits produced by immunomodulators on mutant $SOD1^{G93A}$ microglia reactive phenotype towards the steady state phenotype. Microglia overexpressing $hSOD1^{G93A}$ will be incubated with glycoursodeoxycholic acid (GUDCA) or vinyl sulfone (VS) for 48 h and changes in pro-/anti-inflammatory characteristics will be assessed.

II. Materials and Methods

II.1. Materials

II.1.1. Supplements and chemicals

Fetal bovine serum (FBS), Penicillin/Streptomycin and L-glutamine were purchased from Biochrom AG (Berlin, Germany); RPMI-1640 medium, trypsin-EDTA solution (1X), trypsin-EDTA solution (10X), Hoechst 33258 dye, bovine serum albumin (BSA), Coomassie Brilliant Blue R-250 and phenazine methosulfate (PMS) were from Sigma-Aldrich (St. Louis, MO, USA); Glycoursodeoxycholic acid (GUDCA) (minimum 96% pure) and LPS, *E.coli* O111:B4, 437627 were obtained from Calbiochem (Darmstadt, Germany); Triton X-100 and pHrodo™ E. *coli* bioparticles were acquired from Invitrogen Corporation™ (Carlsbad, CA, USA), and Nitrocellulose membrane was obtained from Amersham Biosciences (Piscataway, NJ, USA). MTS reagent powder was purchased from Promega (Madison, Wisconsin, USA). TRIzol® reagent and primers were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). TripleXtractror, GRS cDNA synthesis kit and Xpert Fast Sybr Blue were purchased from GRiSP (Porto, Portugal). miRCURY™ RNA Isolation Kit and miRCURY LNA™ Universal RT microRNA PCR (Universal cDNA Synthesis Kit II and ExiLENT SYBR® Green master mix) as well as the PCR primer mixes for miR-21, miR-125b, miR-146a, miR-155 and SNORD110 were purchased from Exiqon (Vedbaek, Denmark). All the other chemicals were purchased either from Sigma-Aldrich or Merck.

II.1.2. Equipment

Fluorescence microscope (model AxioScope.A1, Zeiss) with integrated camera (AxioCamHRm). Microplate reader (PR 2100 Microplate Reader, BioRad) was used for nitrites measurement and for MTS assay; ChemiDocTM was used for Western Blot and metalloproteinases revelation; gel photos were obtained from Bio-Rad Laboratories (Hercules, CA, USA). To ensure a stable environment to optimal cell growth (37°C and 5% CO_2), cell cultures were maintained in HERAcell 150 incubators from Thermo Scientific (Waltham, MA, USA) and the work performed in sterile conditions in a HoltenLamin Air HVR 2460 (Allerod,

Denmark). For flow cytometry studies, we used the Guava easyCyte 5HT Base System Flow Cytometer (Merck-Millipore, Darmstadt, Germany) and the BDFACSAria (BD Biosciences) cDNA synthesis was performed in a Tpersonal thermocycler from Biometra ® (Göttingen, Germany). For determination of mRNA and miRNA expression, by quantitative real-time PCR (qRT-PCR) we used a QuantStudio Flex 7 from Applied Biosystems (Foster City, CA, USA). Eppendorf 580R (Eppendorf, Hamburg, Germany) and a Sigma 3K30 centrifuges were used for different experimental procedures. For exosome isolation, we used a Beckman Optima™ L-100 XP ultracentrifuge, with a type 90 Ti rotor (fixed angle) and centrifuge tubes of polycarbonate, from Beckman Coulter, Inc. (Fullerton, CA, USA).

II.2. Methods

II.2.1. N9 Cell line

N9 cell line was obtained by immortalization of microglia cells from CD1 mouse cortex and present features similar to microglia in primary cultures such as phagocytosis and inflammation-related features (Righi et al., 1989; Bruce-Keller et al., 2000; Fleisher-Berkovich et al., 2010).

N9 cell line was a gift from Teresa Pais, Institute of Molecular medicine (IMM), Lisboa, Portugal. Cells were cultures in RPMI media supplemented with FBS (10%), L-glutamine (1%) and Penicilin/Streptomycin (1%), grown to confluency and splitted every 2 to 3 days. For each experience, cells were plated at a concentration of $1x10^5$ cells/mL. No coating was required. Cells were maintained as usual in our lab (Cunha et al., 2016).

II.2.1.1. N9 cell transduction: Lentiviral Production and generation of N9 cells expressing hSOD1^{WT}-GFP and hSOD1^{G93A}-GFP protein

Lentiviral particles were produced by co-transfections of HEK293T cells with the packaging plasmids pGal-pol and pRev, the envelope plasmid pVSV-G and the lentiviral expression vectors plvAcGFP-hSOD1wt/plvAcGFP-hSOD1G93A (Addgene, Cambridge, USA) at a ratio of 3:2:1:4, using X-tremeGene HP, according to the manufacturer's instructions (Roche, Mannhein, Germany) (Pereira et al., 2014; Simões et al., 2015).

Twenty-four hours after transfection, cell media was changed. Supernatants containing lentiviral particles were collected after 48 and 72 h, filtered using a 0.22 μm sterile filter and stored at - 80 ˚C for further use.

To stably overexpress hSOD1^{WT}-GFP and hSOD1^{G93A}-GFP in N9 cell line, cells were seeded in 6-well plated at a density of 3×10^5 cells/well. Twenty-four hours after plating, cells were transduced by adding lentiviral particles containing supernatants. Media was changed 4-

5 times to eliminate all the lentiviral particles (**Figure II.1**) Stable cell lines were purified by cell sorting (BDFACSAria, BD Diosciences) of GFP-expressing cells and the percentage of GFP-positive cells was regularly analyzed in the GUAVA flow cytometer. The percentage of GFP-expressing cells was >60% in all experiments (Pereira et al., 2014; Simões et al., 2015).

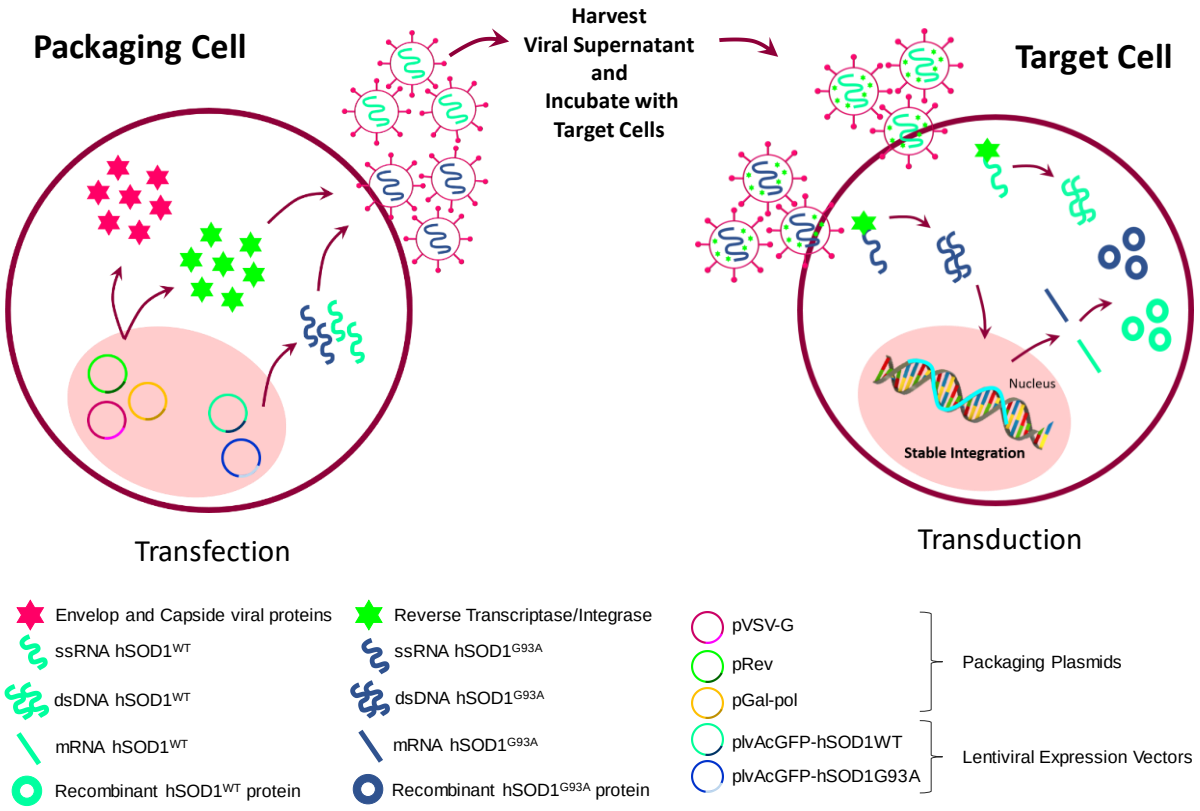


Figure II. 1. Schematic representation of cell transfection for lentiviral production and further generation of N9 cells expressing hSOD1^{WT}-GFP and hSOD1^{G93A}-GFP protein. HEK293T cells were incubated with the packaging plasmids pGal-pol and pRev, the envelop plasmid pVSV-G and the lentiviral expression vectors plvAcGFP-hSOD1wt/plvAcGFP-hSOD1G93A in order to produce lentivirus carrying ssRNA containing the sequence for hSOD1^{WT}/hSOD1^{G93A}. Cell supernatants were collected at 48 h and 72 h and furtherly incubated with N9 naïve cells were reverse transcriptase converted the ssRNA to dsRNA which enters the cell nucleus and stably integrates the genome. **dsRNA**, Double-Strand RNA; **mRNA,** messenger RNA; **ssRNA**; Single Strand RNA; **SOD1**, Superoxide Dismutase 1.

II.2.2. Cell treatments

For N9 characterization and response evaluation, cells were treated with either 300 ng/ml of LPS, GUDCA (50 μM) or VS (10 μM) (**Figure II.2**) to analyze N9 and N9 hSOD1^{WT} microglia response to an inflammatory stimulus and N9 hSOD1^{G93A} cell recovery by GUDCA and VS treatment as it will be further discussed. Experimental determinations were performed after 48 h in order to assess the ability of this bile acid to restore the effects produced by hSOD1 transduction.

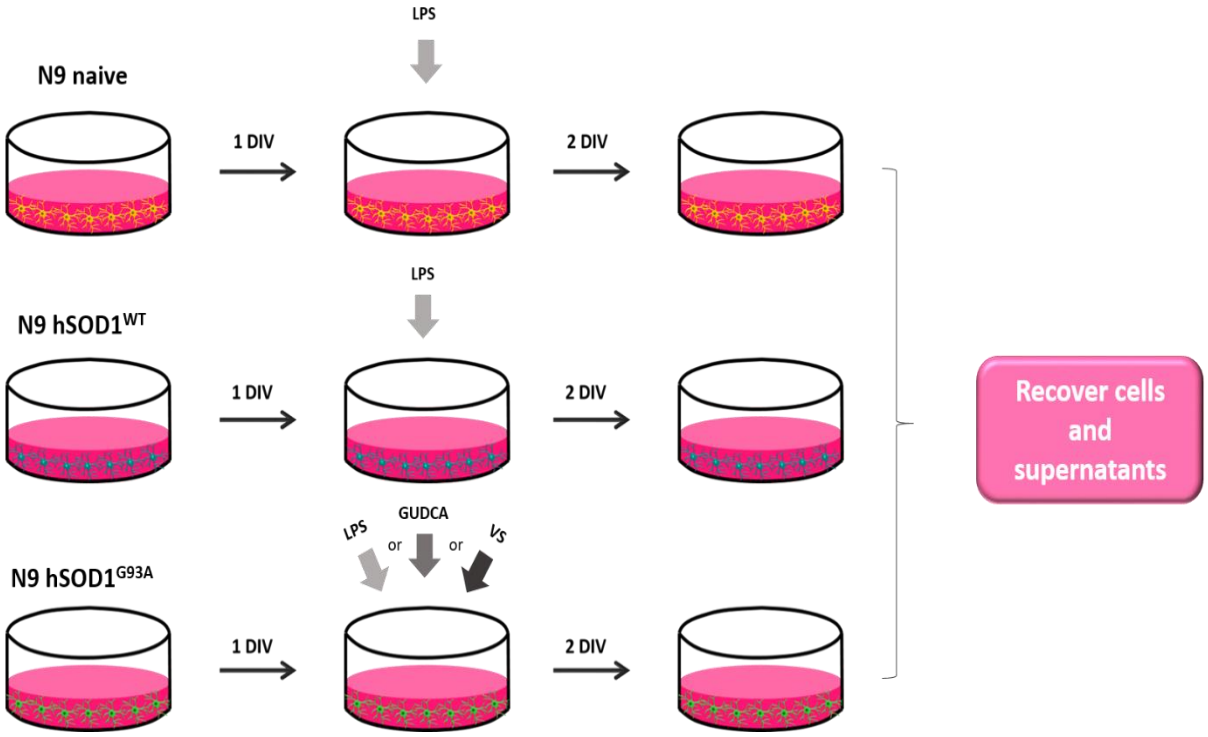


Figure II. 2. Schematic representation of the cell incubation procedure with immunomodulators. N9 naïve, N9 hSOD1^{WT} and N9 hSOD1^{G93A} were plated and left to grow in culture for 1 DIV. Cells were incubated with 300 ng/mL of LPS, 50 µM of GUDCA or 10 µM of VS compound. After 2 DIV, cells and supernatants were collected and kept for further determinations. **DIV,** Days *in vitro;* **GUDCA,** Glycoursodeoxycholic Acid; **LPS,** Lipopolysaccharide; **SOD1,** Superoxide Dismutase 1; **VS,** Vinyl Sulfone.

II.2.3. Differential Ultracentrifugation

For exosome isolation, extracellular media of N9 naïve, N9 hSOD1^{WT} and N9 hSOD1^{G93A} cells with or without the incubated compounds were collected and centrifuged as described by Wang and colleagues (2010), with some minor modifications. Briefly, cell supernatant was centrifuged at 1000 g for 10 min, to pellet cell debris. Then, supernatant was transferred to a different tube and centrifuged at 16 000 g for 1 hour, to pellet microvesicles.

The supernatant was filtered in a 0.22 µm pore filter, transferred to an ultracentrifuge tube and centrifuged at 100 000 g for 2 hours, to pellet exosomes. Afterwards, the pellet of exosomes was resuspended in PBS and centrifuged one last time at 100 000 g for 2 hours in order to wash the pellet (**Figure II.3**). All the procedure was performed at 4 ˚C. (Pinto et al., 2017).

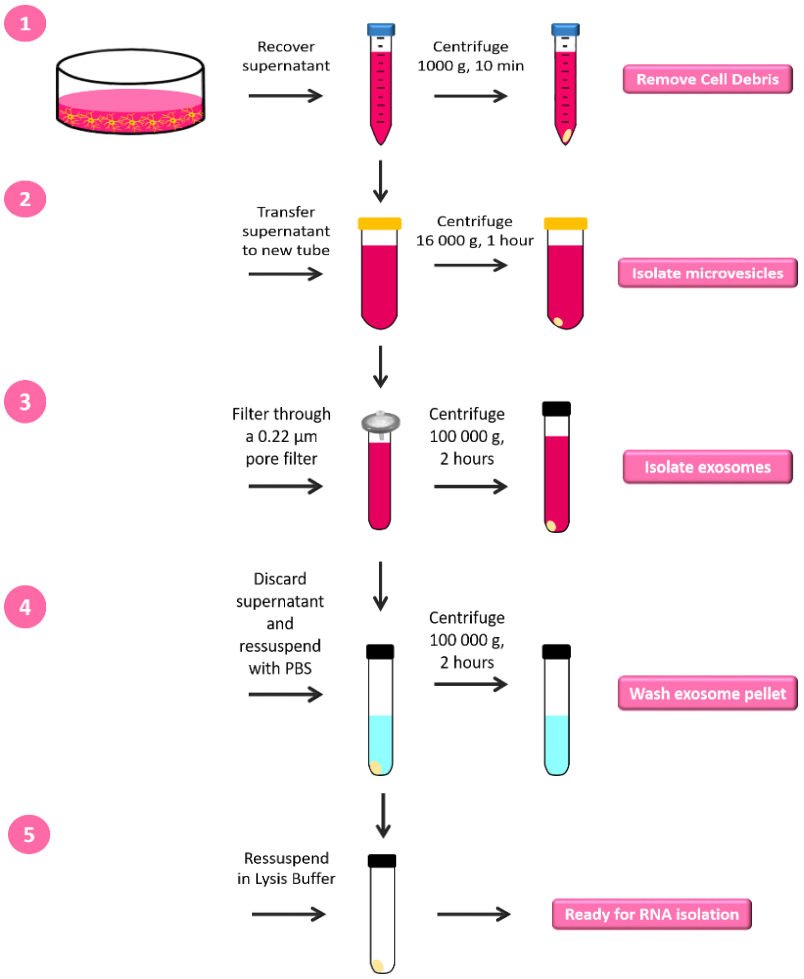


Figure II. 3. Schematic representation of the exosome isolation procedure from culture supernatant. (1) Cell supernatants are recovered and centrifuged 10 min at 1000 g in order to precipitate cell debris; (2) Cell debris pellet is discarded and supernatant is transferred to another tube and centrifuged 1 hour at 16 000 g to pellet microvesicles; (3) Supernatant is filtered through a 0.22 µm pore filter in order to eliminate remaining particles measuring more than 200 µm and centrifuged 2 hours at 100 000 g to pellet exosomes; (4) Supernatant may be discarded or saved for posterior determinations and the pellet containing exosomes is resuspended in PBS and exosome pellet is centrifuged one last time for 2 hours at 100 000 g in order to wash the pellet; (6) Exosome pellet is resuspended in lysis buffer for posterior RNA isolation. **RNA,** Ribonucleic Acid.

II.2.4. Determinations

II.2.4.1. Quantitative RT-PCR

After incubation, cell extracellular media was removed and cells were collected using TRIzol® (Life Technologies, Carlsbad, CA, USA) using a cell scrapper as implemented in the lab (Cunha et al., 2016). Total RNA was then extracted from N9 cells using TRIzol reagent method according to the manufacturer's instructions and posteriorly quantified using NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) (Caldeira et al., 2014).

Determination of *Arg1, Fizz1, HMGB1, IL-1β, IL-10, iNOS, MHCII, RAGE, SOCS1, S100b, TLR4* and *TNF-α* expression as well as miR-21, miR-124, miR-125b, miR-146a and miR-155, was performed by quantitative Real-Time PCR (qRT-PCR). RNA from exosomes was extracted using miRCURY™ RNA Isolation Kit (Exiqon).

For mRNA expression, aliquots of 1000 ng/μl of total RNA were reversed transcribed into cDNA using SensiFAST™ cDNA Synthesis Kit (Bioline) and GRS cDNA Synthesis Mastermix (GRiSP, Porto, Portugal), following the manufacturer's procedure. qRT-PCR was performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems), using a SYBR® Hi-ROX kit (Bioline) and a Xpert Fast Sybr Blue (GRiSP). The sequences of used primers are listed in the **table II.1(A)**. qRT-PCR was performed in 384-well plates, with each sample performed in duplicate, and under optimized conditions: 50˚C for 2 min, 95 ˚C for 2 min, followed by 40/50 amplification cycles at 95 ˚C for 5 seconds and 62 ˚C for 30 seconds. In order to verify the specificity of the amplification, a melting curve analysis was performer, immediately after the amplification protocol. qRT-PCR was performed using β-actin as an endogenous control, to normalize the expression level of *Arg1*, *Fizz1, HMGB1, IL-1β, IL-10, iNOS, MHC class II, RAGE, SOCS1, S100b, TLR4* and *TNF-α*. Fold change was determined by the $2^{-\Delta\Delta CT}$ method.

Expression of miR-21, miR-124, miR-125b, miR-146a and miR-155 was also performed by qRT-PCR. After RNA quantification, cDNA conversion was performed with the Universal cDNA Synthesis Kit (Exiqon), using 5 ng/μl of total RNA according to the following protocol: 60 min at 42˚C followed by heat-inactivation of the reverse transcriptase for 5 min at 95˚C. For miRNA quantification, the ExiLENT SYBR® Green master mix (Exiqon) or the Power SYBR® Green PCR Master Mix (Applied Biosystems) was used in combination with pre-designed primers for miR-21, miR-124, miR-125b, miR-146a, miR-155 and SNORD110 (reference gene) (Exiqon). The sequences used for these miRNAs are listed in **table II.1(B)**. The reaction conditions consisted of polymerase activation/denaturation at 95˚C for 10 min, followed by 50 amplification cycles at 95˚C for 10 seconds and 60˚C for 1 min (ramp-rate 1.6˚/second). Quantification od target miRNAs was made in comparison to the reference gene (SNORD110) and fold change was determined by the $2^{-\Delta\Delta CT}$ method.

Table II.1. (A) - List of primer sequences used for qRT-PCR gene expression.

Gene	Sequence (5'-3')	Sequence (5'-3')
iNOS	5'-ACCCACATCTGGCAGAATGAG-3' (fwr)	5'-AGCCATGACCTTTCGCATTAG-3' (rev)
TLR4	5'-ACCTGGCTGGTTTACACGTC-3' (fwr)	5'-GTGCCAGAGACATTGCAGAA-3' (rev)
IL-10	5'-ATGCTGCCTGCTCTTACTGA-3' (fwr)	5'-GCAGCTCTAGGAGCATGTGG-3' (rev)
TNF-α	5'-TACTGAACTTCGGGGTGATTGGTCC-3' (fwr)	5'-CAGCCTTGTCCCTTGAAGAGAACC-3' (rev)
HMGB1	5'-CTCAGAGAGGTGGAAGACCATGT-3' (fwr)	5'-GGGATGTAGGTTTTCATTTCTCTTTC-3 (rev)
RAGE	5'-CTGGTGGGACTGTGACCTTG-3' (fwr)	5'-TCTGCCTGTCATTCCTAGCTC-3' (rev)
S100B	5'-GAGAGAGGGTGACAAGCACAA-3' (fwr)	5'-GGCCATAAACTCCTGGAAGTC-3' (rev)
MHCII	5'-TGGGCACCATCTTCATCATTC-3' (fwr)	5'-GGTCACCCAGCACACCACTT-3' (rev)
Arg1	5'-CTTGGCTTGCTTCGGAACTC-3' (fwr)	5'-GGAGAAGGCGTTTGCTTAGTTC-3' (rev)
Fizz1	5'-GCCAGGTCCTGGAACCTTTC-3' (fwr)	5'-GGAGCAGGGAGATGCAGATGAG-3' (rev)
Socs1	5'-CACCTTCTTGGTGCGCG-3' (fwr)	5'-AAGCCATCTTCACGCTGAGC-3' (rev)
Il-1β	5'-CAGGCTCCGAGATGAACAAC-3' (fwr)	5'-GGTGGAGAGCTTTCAGCTCATA-3' (rev)
β-actin	5'-GCTCCGGCATGTGCAA-3' (fwr)	5'-AGGATCTTCATGAGGTAGT-3' (rev)

Table II. 1. (B) - List of primer sequences used for qRT-PCR microRNA expression.

microRNA	Sequence (5'-3')
hsa-miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU-3'
mmu-miR-155-5p	5'-CTCAGAGAGGTGGAAGACCATGT-3'
hsa-miR-21-5p	5′- UAGCUUAUCAGACUGAUGUUGA-3′
hsa-miR-125b-5p	5′-UCCCUGAGACCCUAACUUGUGA-3
SNORD110	Reference gene
Spike-in	Reference gene

II.2.4.2. MTS assay

Cellular reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium) is an enzymatic reaction that occurs in functional cells. This can be considered as a marker for cellular viability. Cells were incubated during 1 h at 37°C with 100 µL of the mix MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and PMS (Phenazinemethosulfate) in 900 µL Dulbecco's

Modified Eagle Medium (DMEM-F12) per well. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad Laboratories; Hercules, CA, USA). For each experiment, the mean value of absorbance obtained from control conditions was considered as 100% of cell functionality (Falcão et al., 2017).

II.2.4.3. Microglial phagocytosis and morphology

To evaluate the phagocytic ability of N9 transduced and non-transduced microglia, cells were incubated with pHRodo E. *Coli* particles for 2 hours at 37 ˚C and fixed with paraformaldehyde in PBS. Fluorescence was visualized using an AxioScope.A1 coupled with an axioCam HR camera (Carl Zeiss). UV, green and red fluorescence images of ten random microscopic fields, under 400X magnification, were acquired per sample. The number of beads ingested per cell were counted and distributed by intervals to determine the condition representing with the highest phagocytic capacity. At least 100 cells were counted for each independent condition (Cunha et al., 2016). Morphology measurements were obtained by delineation of at least 100 cells using ImageJ software.

II.2.4.4. Gelatine Zymography

MMP-2 and MMP-9 quantification was performed in the extracellular media of N9 cells incubated with neurons differentiation media, either alone or incubated with LPS, GUDCA or VS, through the gelatine zymography assay. With this method, it is possible to detect the protease activity in the running gel based on the absence of color (white bands), at the particular site of protease action.

The assay was performed as usual in our lab (Silva et al., 2010). Cell supernatants free from cellular debris were used in SDS-PAGE zymography in 0.1% gelatin-10% acrylamide gels, under non-reducing conditions, at 30 mA/gel. After electrophoresis, gels were washed for 1 hour at RT with 2.5% Triton X-100 (in 50 mM Tris pH 7.4; 5 mM $CaCl_2$; 1 µM $ZnCl_2$). For enzyme activity analysis, gels were stained with 0.5% Coomassie Brilliant Blue R-250 for 30 min and de-stained in 30% ethanol/ 10% acetic acid/H_2O to see the bands. Gelatinase activity, detected as a white band on a blue background, was photographed in ChemiDoc™ (Bio-Rad Laboratories) and measured using the Image Lab™ software.

II.2.4.5. Western Blot

Western Blot was performed as usual in our lab (Fernandes et al., 2006). Protein was precipitated from Trizol® after RNA isolation. Following sonication for 45 seconds, the lysate

was centrifuged at 14 000 g for 10 min, 4 ˚C, and the supernatants were collected ad stored. Protein concentration was determined by NanoDrop® at 280 nm. Equal amounts of protein were separated in a gradient of 10% and 15% polyacrylamide electrophoresis gel (SDS-PAGE). After electrophoresis, proteins were transferred to a nitrocellulose membrane. Following, membranes were blocked using a 5% milk solution in TBS-T and incubated with the following primary antibody mouse anti-TLR4 HRP-linked (1:100 in TBS-T with 5% BSA) by overnight incubation with diluted in blocking solution. Membranes were then washed with TBS-T and incubated for one hour at RT with secondary antibody anti-mouse HRP-linked (1:5000, Santa Cruz Biotechnology®) After washing the membraned with TBS-T, chemiluminescence detection was performed by using LumiGLO® reagent and bands were visualized in ChemiDoc (Bio-Rad Laboratories, Hercules, CA, USA). The relative intensities of protein bands were analysed using the Image Lab™ analysis software.

II.2.4.6. Statistical analysis

Results of at least six independent experiments were expressed as a mean ± SEM. Comparisons between different parameters evaluated were made using one-tailed Student's *t*-test for equal or unequal variance, as appropriate. In the characterization of N9 naïve, N9 hSOD1^{WT} and hSOD1^{G93A}, comparisons among the different groups for the assessed parameters were done by one-way ANOVA followed by multiple comparisons using Bonferroni *post-hoc* correction. $p<0.5$ was considered statistically significant. Statistical analysis was made using GraphPad Prism 7 (GraphPad Software Inc, San Diego, CA, USA).

III. Results

III.1. Characterization of pro-/anti-inflammatory markers in the mutant SOD1^{G93A} microglia

The contribution of glial cells to the chronic inflammation process occurring in neurodegenerative diseases, such as ALS, has been gaining attention over the years. In ALS, protein aggregates, such as SOD1, are secreted to the surrounding environment by neurons from where they can be recognized and uptaken by glial cells, being a source of stress that constantly contribute to the inflammatory process. In particular, the activation of pro-inflammatory pathways in microglia, the resident immune cells of the CNS, have been shown to contribute to motor neuron degeneration. In this thesis, we centered our research in microglia cells, using the N9 mouse microglia cell line, and studied the effects of the overexpression of human SOD1, either wild-type (hSOD1^{WT}) or mutated in G93A (hSOD1^{G93A}), in their activation mechanisms, as well as their response to LPS inflammatory insult and the potential protective role of GUDCA and VS as immunomodulators.

III.1.1. Generation of N9 microglial cells overexpressing human SOD1 WT (hSOD1^{WT}) and with G93A mutation (hSOD1^{G93A})

To achieve SOD1 overexpression, N9 naïve cells were transduced with hSOD1^{WT} and hSOD1^{G93A} coupled with a green fluorescent protein (GFP) tail which allowed us to monitor protein overexpression over time, as detailed in methods. After transduction, hSOD1 expression in the different generated cells was confirmed by preparing cell lysates of each cell type for Western Blot analysis. As shown in **Figure III.1**, hSOD1 protein expression was verified at about 40 kDa, which is the weight of human SOD1 coupled with the GFP tail, in the N9 hSOD1^{WT} and hSOD1^{G93A}. As expected, the expression of mouse SOD1 was also detected in all samples, at 14 kDa.

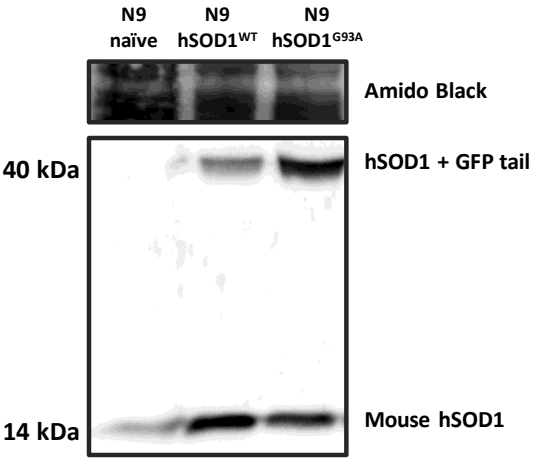


Figure III. 1. SOD1 expression in N9 naïve and transduced N9 microglia with human SOD1 wild-type (hSOD1^{WT}) and mutated in G93A (hSOD1^{G93A}). Transduction and Western Blot analysis were performed as described in the Method section. Cell lysates of all the three cell types were prepared. Samples containing 50 µg of total protein were separated in a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Amido black was used control. **GFP**, Green Fluorescent Protein; **SOD1**, Superoxide Dismutase.

III.1.2. Overexpression of hSOD1 in N9 microglia affects their viability, morphology and phagocytic ability

After confirmation of the success in the transduction process, we assessed viability and morphology of the transduced N9 cells comparing to the naïve controls. Cell viability assessment was essential to prove that cells did not die upon transduction. Morphologic changes were analyzed since it was described that activated N9 microglia change their phenotype to an amoeboid shape once exposed to an inflammatory stimulus (Cunha et al., 2016). Hence, there was a need to confirm that transduction did not work as a source of stress to the cells. Also, phagocytosis was analyzed as an essential characteristic of microglia functionality, critically important in the clearance of cellular debris and pathogenic organisms in the CNS (Nakamura et al., 1999).

First, the obtained results show an increase in cell mitochondria viability (**Figure III.2A**) when naïve cells are transduced with hSOD1^{WT}, probably deriving from the presence of an increased amount of the anti-oxidant SOD1 which may be helping the cell to get rid of ROS species. On the other hand, hSOD1^{G93A} transduction produced no effect in cell viability comparing to naïve cells although a difference over the WT transduction was noticed. Additionally, morphological changes were evident in transduced cells, which appear to be larger than naïve ones (**Figure III.2B**) as shown by increased cell area, perimeter and Feret's diameter. However, the percentage of cells with round/oval, ramified or amoeboid morphology did not change between the naïve and transduced cells. Transduction with hSOD1^{WT} appears to not affect microglia phagocytosis ability (**Figure III.2C**), by hSOD^{G93A} overexpression reduced such function. This was demonstrated by an increased number of hSOD1^{G93A} cells phagocytosing beads in the [1-5] subcategory.

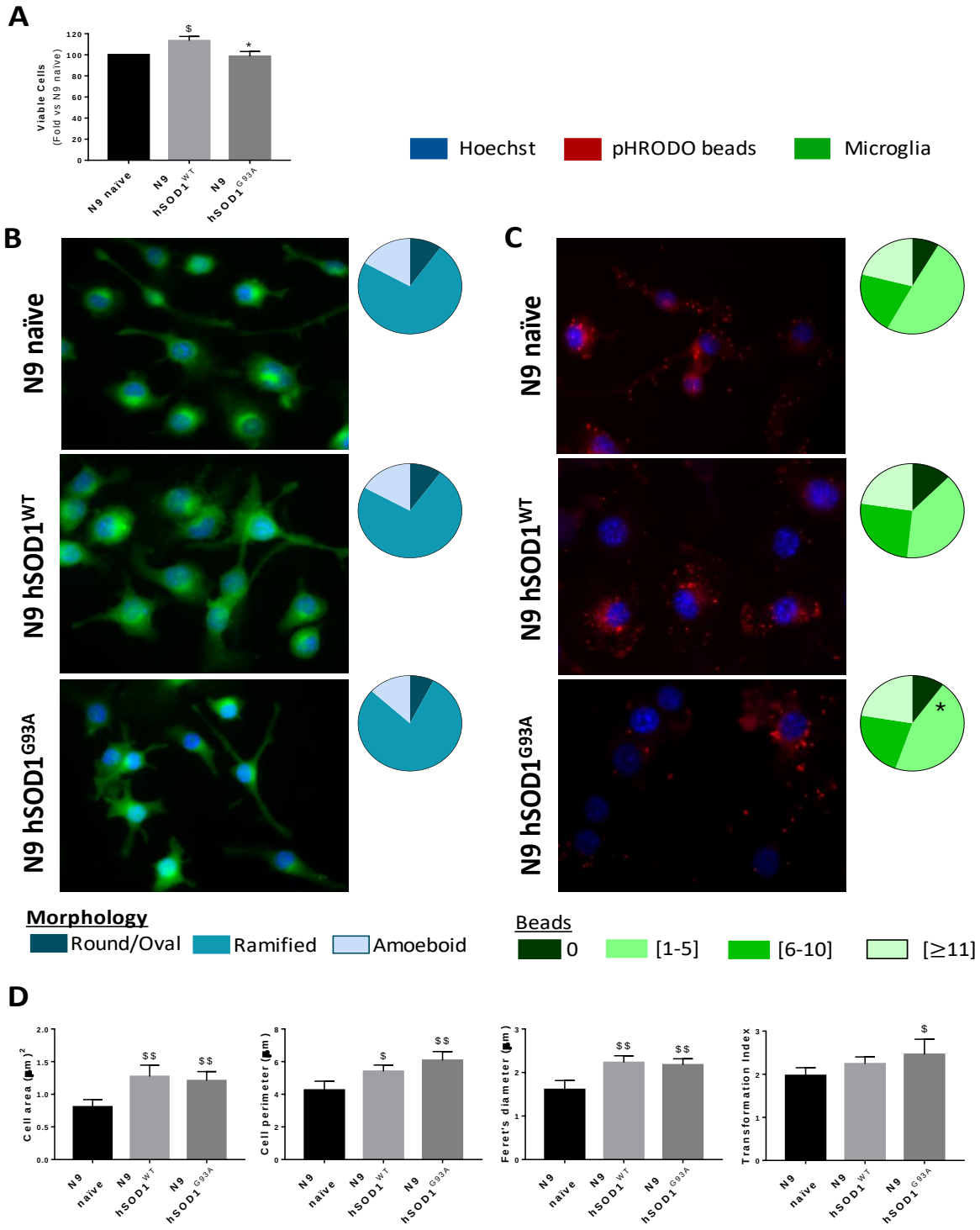


Figure III. 2. Cell viability, morphology and phagocytic ability change in N9 hSOD1^{WT} and N9 hSOD1^{G93A} transduced cells, when compared to N9 naïve cells. Evaluation of cell viability was performed with a mixture of MTS/PMS as described in methods. Morphology was determined by delineation of each cell using 10 random fields in Image J (approximately 100 cells). Phagocytosis was determined by incubation of cells with pHRODO beads as described in the method section and by counting the number of beads in each cell and dividing into intervals to assess capacity differences. (A) Percentage of viable cells considering naïve cells as 100%. (B) Morphometric characterization by immunocytochemistry. (C) Determination of phagocytic ability by the number of engulfed beads. (D) Quantitative assessment of cell area, perimeter, Ferret's diameter and Transformation Index (TI). Results are expressed in graph bars as mean (+SEM) from at least three independent experiments. One-way Anova followed by Bonferroni multiples comparison test was used to compare between the three tested conditions. $^{\$}p<0.05$ and $^{\$\$}p<0.01$ *vs.* N9 naïve cells; *p<0.05 *vs.* N9 hSOD1^{WT} cells. Scale bar represents 100 µm.

III.1.3. Overexpression of hSOD1^{WT} in microglia reduces M1 and M2 activation markers, while that of hSOD1^{G93A} triggers combined expression of pro- and anti-inflammatory indicators

SOD1 is an anti-oxidant enzyme that naturally converts superoxide radicals to molecular oxygen and hydrogen peroxide (H_2O_2) (Barber and Shaw, 2010). As previously mentioned, mutations in this enzyme were found in fALS, where protein aggregates are formed mostly in the cytoplasm of degenerating motor neurons leading to a chronic inflammation environment that ultimately leads to cell death (Wong et al., 1995).

Also, misfolded/aggregated proteins can be released by dying motor neurons into the secretome, either as soluble factors or integrated in the cargo of extracellular vesicles (Gomes et al., 2007), which may then activate the surrounding cells. However, the effect that mutSOD1 expression in microglia may have in their activation process in ALS is not completely clarified.

In **Table III.1**, are presented paired comparisons of the fold changes obtained between naïve and hSOD1^{WT} cells, as well as between hSOD1^{WT} and hSOD1^{G93A}. In the first paired comparison analysis (**Table III.1A**) is evidenced that the overexpression of SOD1 induces alterations in the so called reactive and oxidative markers of the N9 naïve microglial cells. Results for hSOD1^{WT} show a decrease in microglia activation markers, wither for those considered pro-inflammatory and usually associated to the M1 phenotype (*IL-1β, iNOS, TNF-α, MHCII*, the receptors *RAGE* and *TLR4* and the alarmins *HMGB1* and *S100B*) or for the M2 anti-inflammatory markers (*MFG-E8, Arg1, Fizz1, SOCS1* and *IL-10*), as well as for the inflammatory miRNAs (inflamma-miRs) miR-21 and miR-125b. Despite the elevated levels of miR-155 and miR-146a, they were not statistically significant. The same profile was obtained for matrix metalloproteinase (MMP)-2, while MMP-9 only evidenced a slight increase. All decreased significant values are highlighted in light blue in **Table III.1A**.

The second paired comparison comprises the transduced hSOD1^{WT} and hSOD1^{G93A} cells (**Table III.1B**), where differences caused by the mutation are identified. Results show an increase in all the analyzed pro-inflammatory markers (excluding *TLR4* gene receptor) and a decrease in the anti-inflammatory *IL-10*, with no changes in *Arg1* and *Fizz1* M2 markers in hSOD1^{G93A} cells compared with WT ones. Also, the pro-inflammatory miR-155 was increased, together with a decrease in miRs-146a and miR-21 levels, reinforcing the acquired pro-inflammatory state of the mutated cells. Curiously, MMP-9 revealed to suffer a decreased expression, in accordance with previous non-published results in microglia and in opposite to the increased levels observed in motor neurons. All increased significant values are highlighter in yellow.

Overall, transduction with the WT protein exerts calming and protective effects of microglia, while transduction with the mutated protein changes the steady state phenotype towards mixed pro- and anti-inflammatory subclasses.

Table III. 1. hSOD1^{WT} transduction has calming effects on microglia, while hSOD1^{G93A} transduction activates the cells in mixed subtypes.

		(A) Fold change N9 hSOD1^{WT} vs N9 naïve (Mean ± SEM)	P value	(B) Fold change N9 hSOD1^{G93A} vs N9 hSOD1^{WT} (Mean ± SEM)	P value
	Genes				
Pro-inflammatory markers	**IL-1β**	0.30 ± 0.14	0.05	2.06 ± 0.44	0.05
	iNOS	0.19 ± 0.09	0.01	1.55 ± 0.35	0.05
	TNF-α	0.27 ± 0.04	0.01	3.51 ± 1.27	0.05
	MHCII	0.23 ± 0.10	0.01	2.51 ± 0.83	0.05
	RAGE	0.18 ± 0.14	0.01	4.01 ± 1.39	0.01
	TLR4	0.31 ± 0.10	0.01	0.36 ± 0.11	0.01
	HMGB1	0.64 ± 0.11	0.05	4.21± 0.76	0.01
	S100B	0.37 ± 0.08	0.01	2.18 ± 0.62	0.05
Anti-inflammatory markers	**MFG-E8**	0.18 ± 0.09	0.01	2.27 ± 0.61	0.05
	Arg1	0.12 ± 0.07	0.01	1.22 ± 0.26	n.s.
	Fizz1	0.35 ± 0.16	0.01	1.04 ± 0.32	n.s.
	SOCS1	0.15 ± 0.04	0.01	7.30 ± 2.47	0.01
	IL-10	0.25 ± 0.14	0.01	0.56 ± 0.13	0.01
	MicroRNAs				
	miR-125b	0.12 ± 0.07	0.01	1.36± 0.50	n.s.
	miR-21	0.20 ± 0.06	0.01	0.45 ± 0.09	0.01
	miR-155	1.53 ± 0.50	0.32	2.14 ± 0.35	0.01
	miR-146a	3.10 ± 1.03	0.09	0.56 ± 0.15	0.01
	Matrix Metalloproteinases				
	MMP-9	1.57 ± 0.06	0.01	0.71 ± 0.06	0.01
	MMP-2	1.39 ± 0.27	0.22	1.29 ± 0.21	n.s.
	Genes in Exosomes				
	HMGB1	-	-	8.21 ± 2.62	0.05
	SOD1	-	-	3.51 ± 0.45	0.01

Comparisons were made in pairs between N9 naïve and N9 hSOD1^{WT} (A) and N9 hSOD1^{WT} and N9 hSOD1^{G93A} (B). The expression of cytokine mRNA and inflamma-miRs in cells was evaluated by quantitative Real-Time PCR (qRT-PCR) and matrix metalloproteinases (MMPs) activity was determined in the extracellular media by gelatin zymography. Results are mean (± SEM) from at least three independent experiences. Comparisons were made using a paired one-tailed Student's *t*-test. p<0.05 is considered significant. **Arg1**, Arginase 1; **Fizz1**, Found in inflammatory zone 1; **HMGB1**, High mobility group box protein1; **IL-10**, Interleukin-10; **IL-1β**, Interleukin-1β; **iNOS**, inducible nitric oxide synthase; **MFG-E8,** Milk fat globule EGF factor 8; **MHCII,** Major histocompatibility complex class II; **miR,** microRNA; **MMP,** Metalloproteinase; **RAGE**, Receptor for Advanced Glycation End Products; **S100B**, S100 calcium binding protein B; **SOCS1,** Suppressor of cytokine signalling1; **SOD1**, Superoxide Dismutase 1; **TLR4**, Toll like receptor 4; **TNF-α**, Tumor necrosis factor-α.

Although the paired comparisons allowed data on the effects of WT and mutated SOD1 in N9 microglial cells, we decided to make multiple comparison analysis considering the three conditions we have tested, i.e. naïve, hSOD1^{WT} and hSOD1^{G93A} microglial cells to globally understand the effects of the mutated protein transduction in the expression of inflammatory biomarkers (**Figure III.3)**.

Among the inflammatory mediators released by microglia are MMPs and NO. MMP-2 and -9 were found elevated in the spinal cord of hSOD1^{G93A} mice and MMP-9 in NSC-34 neuron-like cell line, whereas NO is known for its contribution to oxidative stress and inflammation in ALS (Fang et al., 2010; Drechsel et al., 2012; Vaz et al., 2015). However, we did not find any significant difference for these molecules between the naïve and the mutated cells (results not shown), although an increased trend was noticed for NO in the hSOD1^{G93A} cells. Further experiments are needed to confirm such results.

When comparing microglia in the three testes conditions, we observed that the reduction of M1 and M2 markers are sustained in WT transduced cells as compared with naïve cell, as our previous data indicated (**Figure III.3).** In the hSOD1^{G93A} transduced cells, although some M2 markers were reduced in a similar way to those obtained for the hSOD1^{WT} cells (e.g. *IL-10* and *Fizz1*), some parameters such as *TNF-α, IL-1β, MHC-II, HMGB1* and *MGF-E8* are not only above the levels obtained in hSOD1^{WT}, but also above naïve cells values, which indicate a stressed microglia that may either respond in a depressed or in an exacerbated way depending on the inflammatory stimulus. Although *MFG-E8* is not actually a pro-inflammatory mediator, it is involved in the phagocytic capacity of cells. *MFG-E8* upregulation may be involved in apoptotic processes caused by the presence of hSOD1^{G93A} as shown for endothelial cells (Brissette et al., 2012). In addition, as a bridge between microglial receptors and phosphatidylserine exposed in apoptotic neurons, this result may also indicate that mutated microglial cells would be able to better recognize apoptotic neurons or debris inducing their engulfment (Hanayama et al., 2002). Interestingly, *Arg1* and *SOCS1* gene expression (and tentatively *S100B* and *RAGE*) levels returned to values of naïve cells. To additionally note the observed decreased of iNOS mRNA (**Figure III.3**), together with that of *TLR4* gene and protein expression (**Figure III.4**). Negative regulation of *TLR4* by miRNA-146a was previously documented (Curtale et al., 2013), reason why we decided to next assess the expression of inflamma-miRs not only in cells, but also in their derived exosomes.

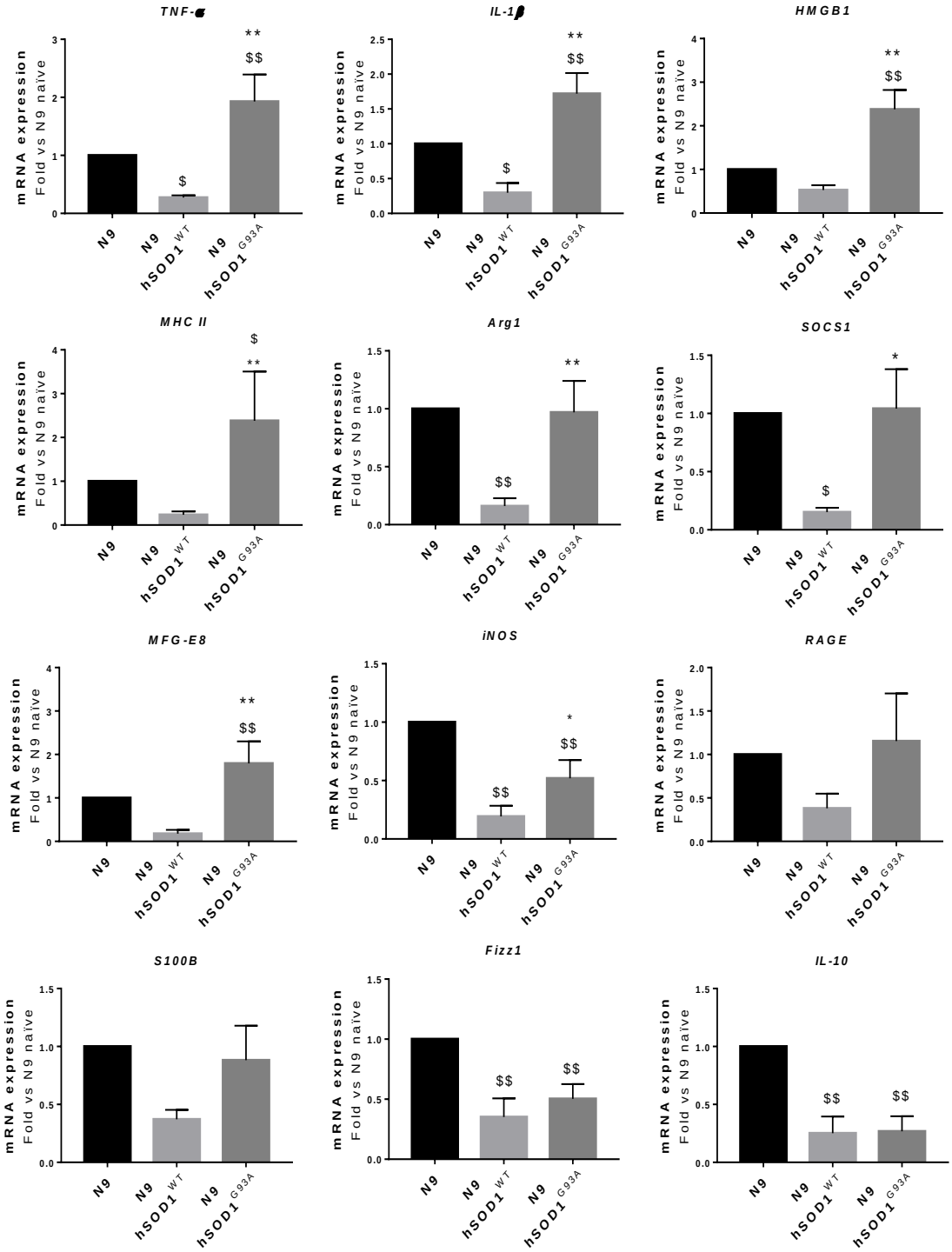


Figure III. 3. Calming effects by the transduction of hSOD1^{WT} in microglia are lost in cells overexpressing hSOD1^{G93A}, which evidence increased gene expression of pro-inflammatory cytokines, alarmin HMGB1, MFG-E8 and MHC class II expression, producing a heterogeneous phenotype. The mRNA expression of pro- and anti-inflammatory mediators was evaluated by quantitative Real-Time PCR (qRT-PCR), as detailed in the Method section. N9 naïve was used as control. Results are mean (± SEM) from at least three independent experiments. One-way ANOVA followed by Bonferroni multiple comparison test was used to compare the three tested conditions. $^{\$}$p<0.05 and $^{\$\$}$p<0.01 *vs.* N9 naïve cells; *p<0.05 and **p<0.01 *vs.* N9 hSOD1^{WT} cells. **Arg1,** Arginase 1**; Fizz1,** Found in inflammatory zone 1**; HMGB1,** High mobility group box protein1**; IL-10,** Interleukin-10**; IL-1β,** Interleukin-1β**; iNOS,** inducible nitric oxide synthase**; MFG-E8,** Milk fat globule EGF factor 8**; MHCII,** Major histocompatibility complex class II**; RAGE,** Receptor for Advanced Glycation End Products**; S100B,** S100 calcium binding protein B**; SOCS1,** Suppressor of cytokine signaling 1**; TLR4,** Toll like receptor 4**; TNF-α,** Tumor necrosis factor-α**.**

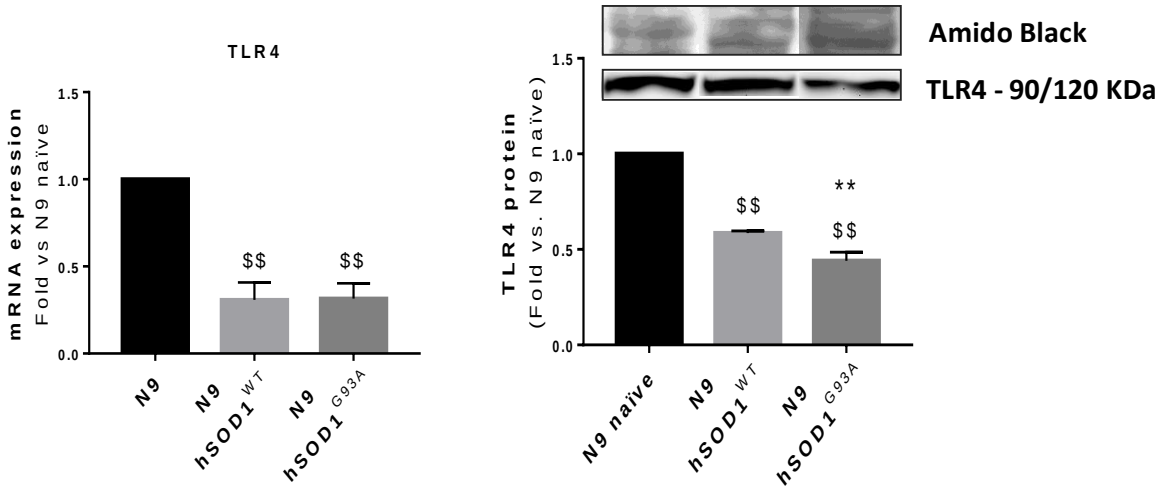


Figure III. 4.TLR4 total protein level is consistent with downregulation of gene expression in both transduced cells. Cell lysates of all the three cell types were prepared. Samples containing 50 µg of total protein were separated in a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Amido black was used as the control of the experiment. The mRNA expression of TLR4 was evaluated by quantitative Real-Time PCR (qRT-PCR), as detailed in the method section. N9 naïve cells were used as control. Results are mean (± SEM) from at least three independent experiments. One-way ANOVA followed by Bonferroni multiples comparison test was used to compare among the three tested conditions. $^{\$\$}$p<0.01 *vs.* N9 naïve cells; **p<0.01 *vs.* N9 hSOD1^{WT} cells. **TLR4**, Toll like receptor 4.

III.1.4. Microglia and their derived exosomes show altered inflammatory-associated miRNA profile after hSOD1^{WT} and hSOD1^{G93A} overexpression

Inflamma-miRs are reported to have a modulator role in microglia activation such as miR-146a and miR-155 (Cardoso et al., 2012; Saba et al., 2012), which were also found as being part of exosome cargo (Alexander et al., 2015). Exosomes are extracellular vesicles that have recently been gaining attention as part of the transportable cell secretome. Once released, these vesicles can be taken up by nearby recipient cells or even travel longer distances (Sarko and McKinney, 2017).

When analyzing cellular expression of inflamma-miRs (**Figure III.5**), decreased expression of miR-125b and miR-21 was observed in both SOD1 transduced cells. Data obtained, indicate that the first may be increased in exosomes from the hSOD1^{WT} microglia, while the latter seems to increase in those from hSOD1^{G93A} microglia. Relatively to the inflammatory miR-155 and the negative regulator miR-146a, only this last one was increased in WT cells, but both were found elevated in hSOD1^{G93A} -derived exosomes.

MiR-125b was shown to be a key mediator of microglia dynamics in ALS and to be upregulated in response to inflammatory stimulus (Parisi et al. 2016) in order to have a role as an anti-inflammatory molecule (Tili et al. 2007). Likewise, miR-21 is also considered to have an anti-inflammatory effect, thus meaning that the decrease in miR-21 and miR-125b may turn microglia more susceptible to activation (Barnett et al. 2016). More interestingly, although the

overexpression of hSOD1^{wt} determined a cellular increase in miR-146a, such finding was not reflected in the released exosomes. The opposite was observed for the cells overexpressing mutated SOD1 that did not reveal cellular alterations in miR-146a, but determined its increase in the derived exosomes. These two miRNAs were previously shown to be released from dendritic cells and N9 microglia within exosomes after treatment with LPS (Alexander et al., 2015; Cunha et al., 2016), and revealed to have opposite effects. MiR-146a inhibits while miR-155 promotes endotoxin-induced inflammation in mice (Alexander et al. 2015).

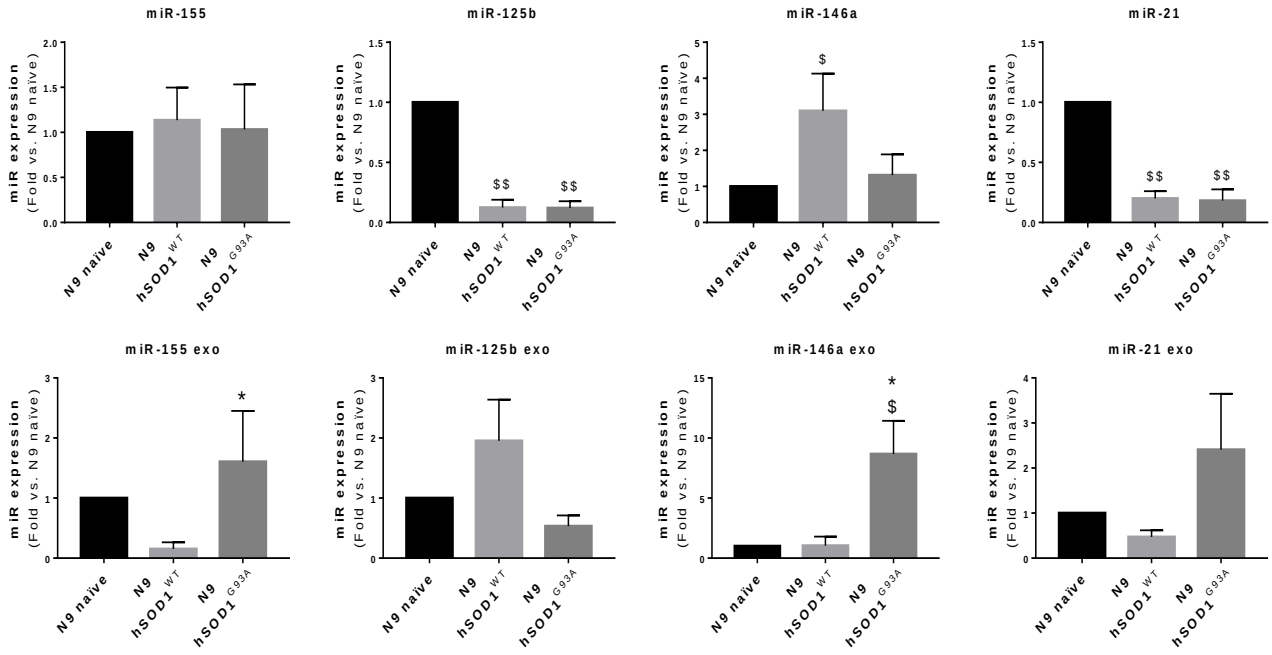


Figure III. 5. MiRNA expression in transduced cells is not reflected in their derived exosomes. Inflamma-miRs expression in both cells and exosomes were analyzed by qRT-PCR. N9 naïve cells were used as control. Results are mean (± SEM) from at least three independent experiences. One-way ANOVA followed by Bonferroni multiple comparison test was used to compare among the three tested conditions. $^{\$}$p<0.05 and $^{\$\$}$p<0.01 *vs.* N9 naïve cells; *p<0.05 *vs.* N9 hSOD1^{WT} cells.

Intriguingly, they were found to be co-induced in the brain by *Toxoplasma* infection (Cannella et al., 2014). Although more data is required to better define the microglia miRNA inflammatory profile and its consequences in ALS disease, our results sustain that exosomes from N9hSOD1^{G93A} microglia may be implicated in the dissemination of miR-155 and miR-146a to other neural cells, namely glial cells. Exosomes may also be involved in the propagation to the alarmin *HMGB1* and the SOD1 protein, whose gene levels were found elevated in N9 hSOD1^{G93A} microglia relatively to the N9 hSOD1^{WT} cells (**Table III.1**). This result is consistent with previous findings where SOD1 was described to be transported into the extracellular media in exosomes, which are further uptaken by other cells (Silverman et al., 2016). HMGB1 that is described as being released upon apoptosis in apoptotic bodies (Bell et al., 2006; Buzas et al., 2014) and in exosomes of different cells (Liu et al., 2006; Sheller-Miller et al., 2017), was never mentioned to be carried by microglia-derived exosomes, what turns our result

particularly interesting. Actually, this finding indicates that mutated microglia also contribute to chronic inflammatory status through the transport of HMGB1 in their released exosomes, besides the propagation of the mutated SOD1.

III.2. Assessment of mutant SOD1^{G93A} microglia activation susceptibility upon immunostimulation

In previous works from our group, it has been demonstrated that intraperitoneal administration of LPS in newborn mice activated microglia (Cardoso et al., 2015) and that LPS-treated N9 cells show a predominant M1 polarized phenotype and release exosomes enriched in miR-155 and miR-146a (Cunha et al., 2016). In addition, it was also demonstrated that SOD1 overexpression plays a significant role in inflammatory signaling in microglia by altering ROS production and reducing neurotoxic inflammatory markers (Dimayuga et al., 2007), even in the presence of LPS. Therefore, we thought that would be interesting to assess differences in SOD1^{G93A} microglia reactivity towards LPS treatment relatively to the WT and naïve cells, to better understand how ALS microglia may behave when facing and inflammatory status condition in patients.

III.2.1. LPS induces an inflammatory response of N9 microglia

Before assessing the effects of LPS stimulation in the transgenic microglia we evaluated the changes caused on viability, morphology and phagocytosis upon treatment and attested the activation properties of LPS on the N9 microglial cells, some of them already determined in a previous work of the group (Cunha et al., 2016) (**Table III.2**). LPS induced the amoeboid shape, with elevation in Feret's diameter, cell area, cell perimeter and Transformation Index, while significantly decreased cell viability. It also increased the number of cells with any bead, although enhancing the expression of the phagocytic-associated protein *MFG-E8*. As expected, LPS induced the majority of the inflammatory mediators (except the reactive S100B protein and the receptor RAGE), together with some anti-inflammatory ones (*Fizz1* and *IL-10*). It also produced an upregulated inflammatory miR-21, miR-155 and miR-146a, but a decreased miR-125b, which where all reduced in cell derived exosomes, excepting miR-146a. Relatively to the released MMP-2 and MMP-9, only the last one was found elevated in the extracellular media of microglia treated with LPS. Globally, we may conclude that LPS determined a predominant M1 polarization and an amoeboid morphology in the LPS-treated N9 microglia.

Table III. 2. Changes induced by lipopolysaccharide (LPS) on the morphology, phagocytosis, viability and inflammatory profile of N9 naïve cells.

		Fold change N9 naïve + LPS vs N9 naïve (Mean ± SEM)	P value
	Viability	0.53 ± 0.44	0.01
	Phagocytosis		
	[0]	2.05 ± 0.34	0.05
	[1-5]	0.98 ± 0.07	n.s.
	[6-10]	0.90 ± 0.15	n.s.
	[≥11]	0.87 ± 0.19	n.s.
	MFG-E8	3.41 ± 0.99	0.05
	Morphology		
	Round/Oval	0.36 ± 0.09	0.05
	Ramified	0.12 ± 0.02	0.01
	Amoeboid	5.00 ± 0.07	0.01
	Cell area	2.91 ± 0.14	0.01
	Cell perimeter	1.53 ± 0.17	0.01
	Feret's Diameter	1.82 ± 0.07	0.01
	Tansformation Index	1.27 ± 0.09	0.05
	Genes		
M1 Markers	*IL-1β*	6.59 ± 4.17	n.s
	iNOS	1.56 ± 0.68	n.s.
	TNF-α	10.56 ± 3.87	0.01
	MHCII	1.46 ± 0.58	n.s.
	RAGE	0.46 ± 0.26	0.05
	TLR4	2.00 ± 0.65	n.s.
	HMGB1	1.69 ± 0.28	0.01
	S100B	0.32 ± 0.09	0.01
M2 Markers	*Arg1*	1.11 ± 0.37	n.s.
	Fizz1	8.49 ± 1.78	0.01
	SOCS1	0.31 ± 0.09	0.01
	IL-10	11.09 ± 7.67	n.s.
	MicroRNAs		
	miR-125b	0.25 ± 0.12	0.01
	miR-21	7.49 ± 1.78	0.01
	miR-155	6.28 ± 1.95	0.01
	miR-146a	5.34 ± 1.1	0.01
	Matrix Metalloproteinases		
	MMP-9	2.23 ± 0.34	0.01
	MMP-2	1.20 ± 0.15	n.s.
	Nitric Oxide	2.74 ± 0.47	n.s.
	MicroRNAs in Exosomes		
	miR-125b	0.48 ± 0.07	0.01
	miR-21	0.36 ± 0.11	0.01
	miR-155	0.33 ± 0.33	0.05
	miR-146a	1.25 ± 0.36	n.s.

Comparison was made between N9 naïve and N9 naïve + LPS. Cells were cultured for 48 h in the presence of 300 ng/mL LPS. The expression of cytokine mRNA and inflamma-miRs in cells was evaluated by quantitative Real-Time PCR (qRT-PCR) and matrix metalloproteinases (MMPs) activity was determined in the extracellular media by gelatin zymography. Results are mean (± SEM) from at least three independent experiences. Comparisons were made using a paired one-tailed Student's *t*-test. $p<0.05$ is considered significant. **Arg1**, Arginase 1; **Fizz1**, Found in inflammatory zone 1; **HMGB1,** High mobility group box protein1; **IL-10,** Interleukin-10; **IL-1β,** Interleukin-1β; **iNOS,** inducible nitric oxide synthase; **MFG-E8**, Milk fat globule EGF factor 8; **MHCII,** Major histocompatibility complex class II; **miR.** microRNA; **MMP,** Metalloproteinase; **RAGE,** Receptor for Advanced Glycation End Products; **S100B**, S100 calcium binding protein B; **SOCS1,** Suppressor of cytokine signalling1; **SOD1,** Superoxide Dismutase 1; **TLR4,** Toll like receptor 4; **TNF-α,** Tumor necrosis factor-α.

III.2.2. LPS induces amoeboid morphology in microglia, independently of hSOD1 expression, and increases hSOD1^{WT} microglia phagocytosis, but diminishes such ability in hSOD1^{G93A} cells

In previous studies it was shown that SOD1 transgenic microglia (mutSOD1 and WT) have a lower capacity to sense tissue alterations in steady state conditions, including a reduced ability to phagocytose and respond to extracellular ATP stimulation (Sargsyan et al., 2011). Thus, we first evaluated whether LPS produced alterations in the phagocytic ability of SOD1 transgenic microglia relatively to the naïve cells, as well as on cell viability and morphology. For that we used the concentration of 300 ng/ml of LPS already showed by us to polarize the naïve cells into the M1 phenotype (Cunha et al., 2016). As depicted in **Figure III.6A**, LPS stimulation not only did not change the viability of the transgenic microglia relatively to the N9 LPS-treated microglia, but also did not modify the acquired amoeboid morphology of the activated microglia (**Figure III.6B**). Actually, ramified cells were only marginal in all cases. Interestingly, based on the results obtained for Ferret's diameter (**Figure III.6D**) hSOD1^{G93A} microglia are the most spheroid ones. In addition, while the experiments with hSOD1^{WT} N9 microglia revealed as compared to naïve cells lower number of cells with no beads and an increase in microglia containing 6-10 beads, upon LPS treatment, those of hSOD1^{G93A} showed a reduced number of cells able to swallow more than 11 beads (**Figure III.6C**). Therefore, LPS stimulus was more injurious to hSOD1^{G93A} microglia in terms of morphological alterations and impaired phagocytosis.

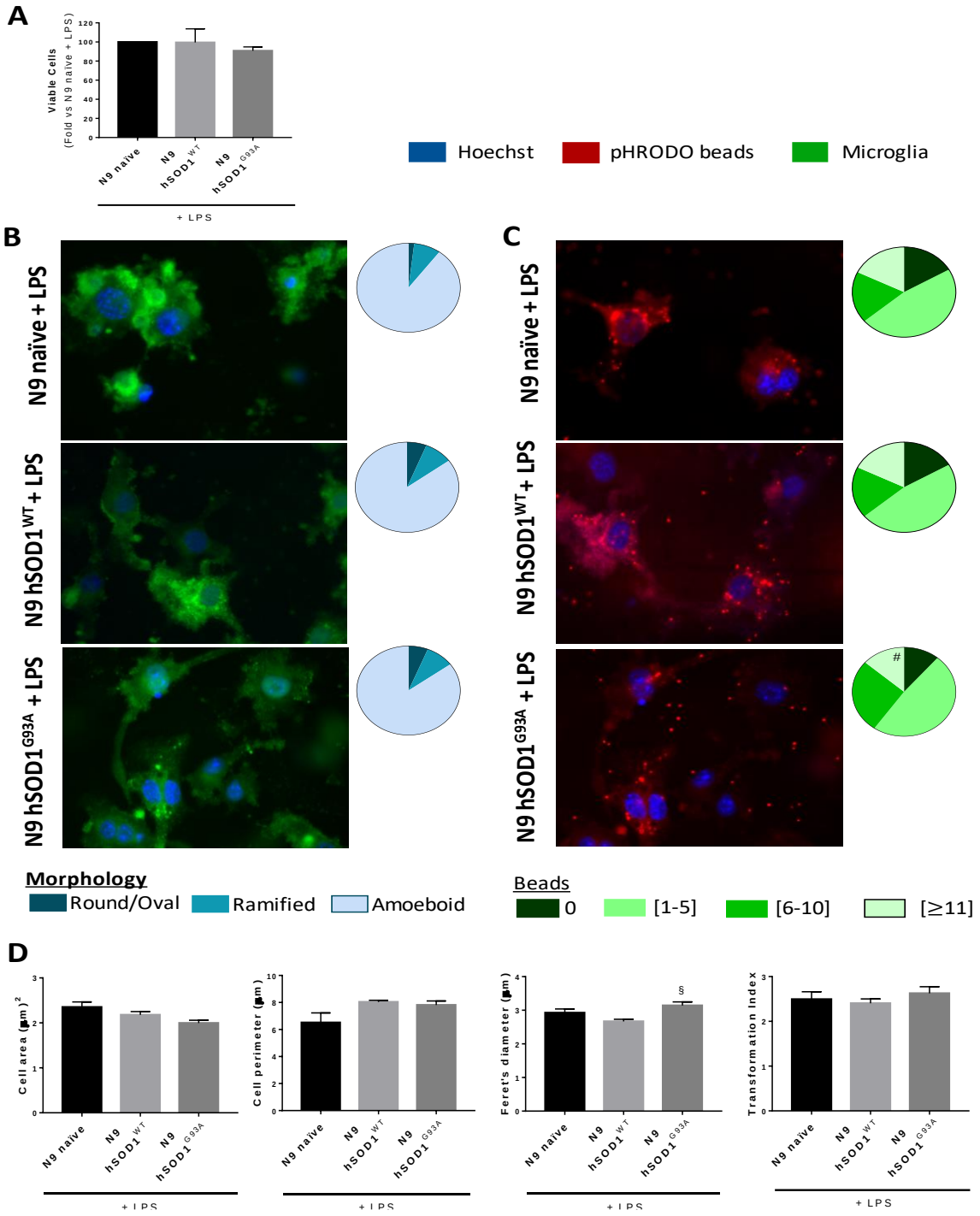


Figure III. 6.Lipopolysaccharide (LPS) triggers microglia amoeboid shape in all conditions, while enhances phagocytosis in hSOD1^{WT} cells and decreases it in hSOD1^{G93A} ones. N9 naïve and transgenic microglia were treated with 300 ng/mL LPS for 48 h. Evaluation of cell viability was performed with a mixture of MTS/PMS as described in methods. Morphology was determined by delineation of each cell using 10 random fields in Image J (approximately 100 cells). Phagocytosis was determined by incubation of cells with pHRODO beads as described in the method section and by counting the number of beads in each cell and dividing into intervals to assess capacity differences. (A) Viable cells considering naïve cells as 100%. (B) Morphometric characterization by immunocytochemistry. (C) Determination of phagocytic ability by the number of engulfed beads. (D) Quantitative assessment of cell area, perimeter, Ferret's diameter and Transformation Index (TI). Results are expressed in graph bars as mean (+SEM) from at least three independent experiments. One-way ANOVA followed by Bonferroni multiples comparison test was used to compare between the three conditions. $^{\#}p<0.05$ *vs.* N9 naïve cells+ LPS; $^{\S}p<0.05$ *vs.* N9 hSOD1^{WT} cells + LPS. Scale bar represents 100 µm.

III.2.3. Transgenic hSOD1^{G93A} microglia stimulated by LPS showed downregulation of anti-inflammatory markers and similar inflammatory response when compared with LPS-treated naïve cells

When stimulated with LPS, N9 naïve cells responded to LPS (**Table III.2**) by increasing pro-inflammatory markers. Thus, we decided to compare whether N9 hSOD1^{WT} and N9 hSOD1^{G93A} would respond differently from LPS-treated naïve cells upon LPS exposure. Pro- and anti-inflammatory molecules were evaluated as previously indicated and depicted in **Figure III.7.** Overall, we observed that *HMGB1, SOCS1* and *MFG-E8* did not present significant differences among the three conditions tested. Both transduced cells showed a low expression of the M2 markers (*Arg1, Fizz1* and **IL-10**), as well as of the reactive marker S100B. Intriguingly, hSOD1^{WT} cells showed a trend to IL-1β increased gene expression and downregulated *TNF-α* mRNA, which we found to be similarly to naïve microglia levels in hSOD1^{G93A} cells. In a similar way*, iNOS, MHCII* and *RAGE* expression levels were close to the values observed in LPS-treated naïve cells and slightly elevated than those in hSOD1^{WT} microglia. Therefore, we may assume that the expression of mutated SOD1 did not increase or decrease the ability of microglia in mounting an inflammatory response towards an immunostimulatory injury. To note that no significant differences were found for MMP-2, MMP-9 or NO (results not shown). Now considering the effects of LPS on its receptor *TLR4*, although a slight decrease was observed for the gene expression, we found an upregulation of the protein in the hSOD1^{WT} cells upon LPS treatment, as indicated in **Figure III.8.** To that it may account the fact that *TLR4* gene expression was shown to be upregulated upon LPS (Ren et al., 2010) after 6 to 8 hours of exposure and we used 48 h incubation.

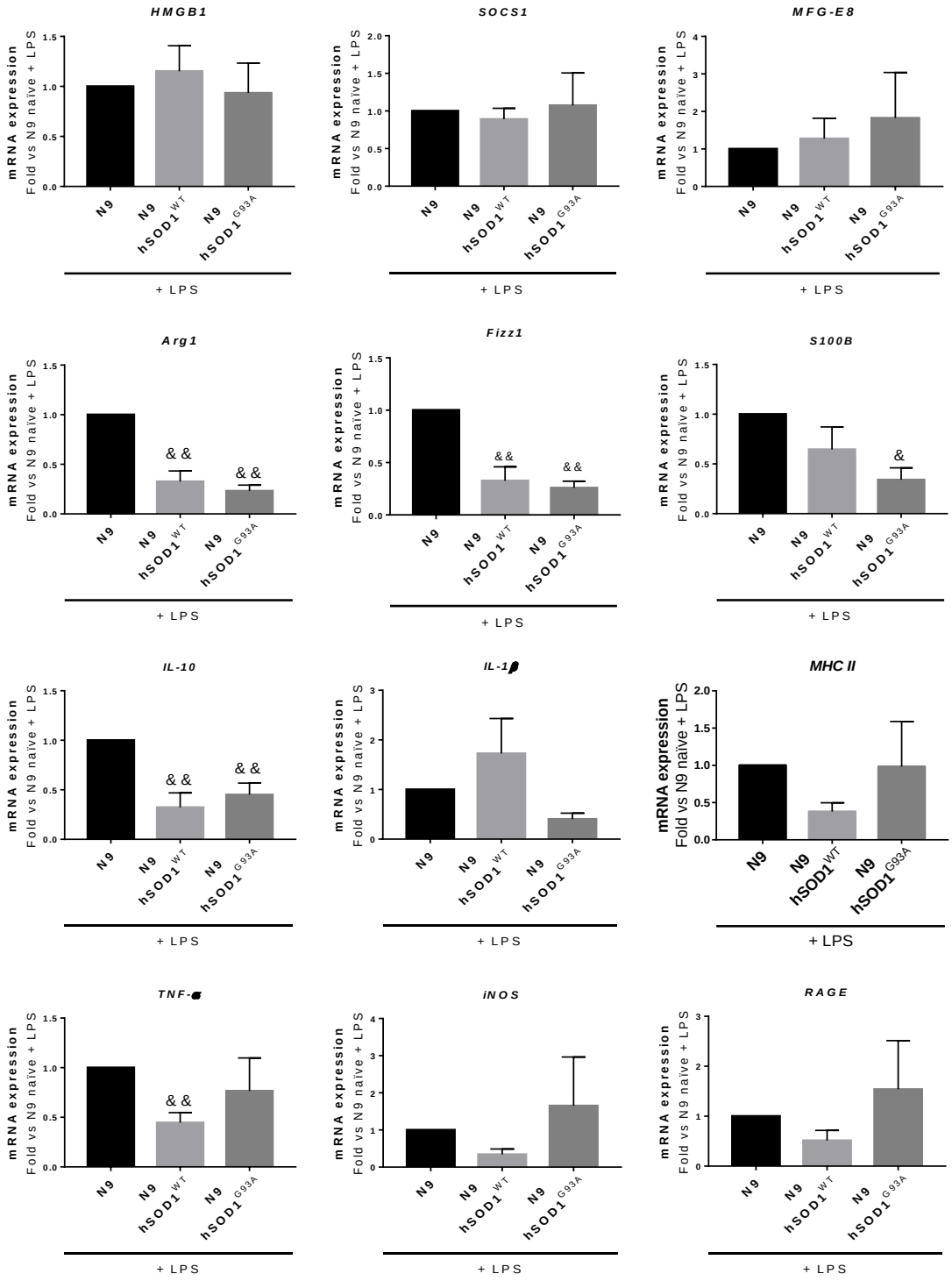


Figure III. 7. Lipopolysaccharide triggers downregulation of anti-inflammatory markers in transgenic microglia, which apparently react similarly to naïve cells in terms of inflammatory-related indicators. The mRNA expression of pro- and anti-inflammatory mediators in cells were evaluated by quantitative Real-Time PCR (qRT-PCR), as detailed in the method section. N9 naïve + LPS was used as control. N9 naïve and transgenic microglia were treated with 300 ng/ml LPS for 48 h. Results are mean (± SEM) from at least three independent experiences. One-way ANOVA followed by Bonferroni multiple comparison test was used to compare among the three tested conditions. $^{\&}p<0.05$ and $^{\&\&}p<0.01$ *vs.* N9 naïve cells + LPS. **Arg1,** Arginase 1**; Fizz1,** Found in inflammatory zone 1**; HMGB1,** High mobility group box protein1**; IL-10,** Interleukin-10**; IL-1β,** Interleukin-1β**; iNOS,** inducible nitric oxide synthase**; MFG-E8,** Milk fat globule EGF factor 8**; MHCII,** Major histocompatibility complex class II**; RAGE,** Receptor for Advanced Glycation End Products**; S100B,** S100 calcium binding protein B**; SOCS1,** Suppressor of cytokine signaling 1**; TNF-α,** Tumor necrosis factor-α.

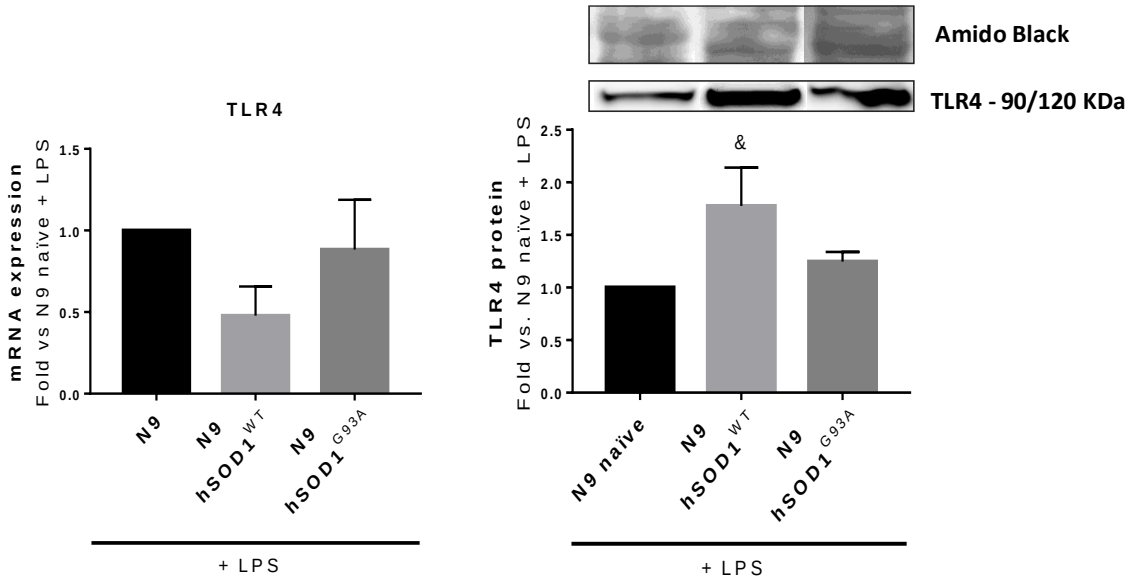


Figure III. 8. Lipopolysaccharide triggers upregulation of TLR4 protein expression in N9 hSOD1^{WT} cells, but not in hSOD1^{G93A} microglia. Cell lysates of all the three cell types were prepared. Samples containing 50 µg of total protein were separated in a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Amido black was used as the control of the experience. The mRNA expression of TLR4 was evaluated by quantitative Real-Time PCR (qRT-PCR), as detailed in the method section. N9 naïve + LPS cell were used as control. N9 naïve and transgenic microglia were treated with 300 ng/ml LPS for 48 h. Results are mean (± SEM) from at least three independent experiences. One-way ANOVA followed by Bonferroni multiple comparison test was used to compare the three tested conditions. $^{\&}p<0.05$ *vs.* N9 naïve cells + LPS. **TLR4,** Toll like receptor 4.

III.2.4. Transgenic microglia stimulated by LPS and their derived exosomes show altered inflammatory-associated miRNA profile

To more deeply evaluate the inflammatory response of the SOD1 transgenic microglia upon LPS immunostimulation, we next evaluated both intracellular and exosomal cargo miRNAs (**Figure III.9**). Surprisingly, we noticed that miR-155 decreased by LPS in both transgenic cells, while no other differences were obtained for the remaining inflammatory-associated miRNAs in both transgenic cells. However, only the exosomes from hSOD1^{WT} microglia showed an elevated cargo of miR-155, despite the same trend exhibited by miR-21, suggesting that LPS increases their dissemination in exosomes in the WT transgenic microglia to decrease the harmful influence of LPS. The variability we found in the expression of these miRNAs indicates that further determinations must be performed to confirm these yet preliminary results.

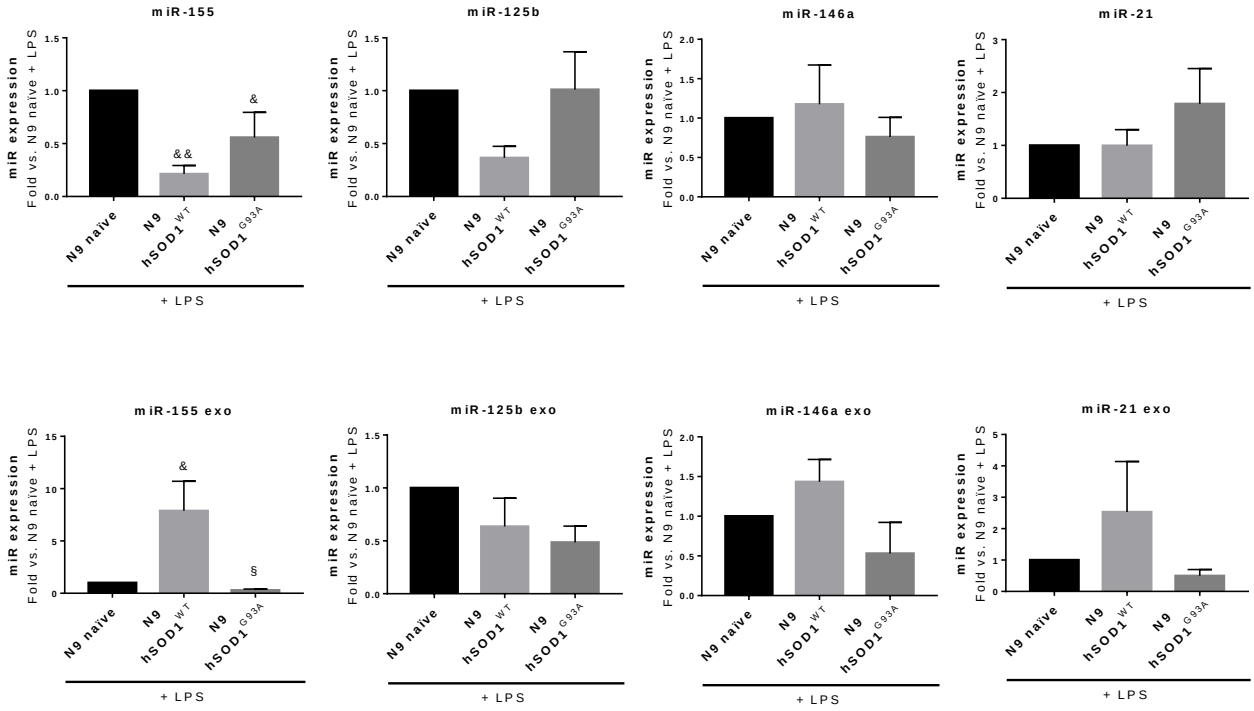


Figure III. 9. MiRNA expression in transduced cells upon LPS treatment reveals reduced miR-155 levels, together with its elevation in exosomes derived from hSOD1WT cells. Inflamma-miRs expression in both cells and exosomes were analyzed by qRT-PCR. N9 naïve + LPS cells were used as control. N9 naïve and transgenic microglia were treated with 300 ng/mL LPS for 48 h. Results are mean (± SEM) from at least three independent experiences. One-way ANOVA followed by Bonferroni multiple comparison test was used to compare the three tested conditions. $^{\&}p<0.05$ and $^{\&\&}p<0.01$ *vs.* N9 naïve cells + LPS; $^{\S}p<0.05$ *vs.* N9 hSOD1^{WT} cells+ LPS.

III.3. Determination of the benefits produced by immunomodulators on mutant SOD1G93A microglia reactive profile towards the steady state phenotype

We have observed that the transduction of mutated SOD1 in microglia induced alterations in cell morphology and in expression of pro-inflammatory and anti-inflammatory markers.

In an attempt to rescue the hSOD1^{G93A} overexpression effects on microglia, we used the bile acid GUDCA, previously shown to have anti-apoptotic, anti-oxidant and anti-inflammatory properties (Fernandes et al., 2007; Brito et al., 2008; Silva et al., 2012; Vaz et al., 2015) and VS, which was lately demonstrated to reduce the inflammatory responses of N9 microglia to Aβ (Falcão et al., 2017). Both compounds were used in order to analyze its capacity to prevent some cellular features implicated in the ALS pathology, namely those associated to neuroinflammation. Indeed, as already commented there are no effective therapeutic strategies for ALS and usage of anti-inflammatory compounds are a subject of controversy in neurodegenerative diseases.

III.3.1. GUDCA and VS do not change viability or phagocytosis, but reduces cell area of N9 hSOD1G93A microglia

First in our approach, we evaluated GUDCA and VS effects on cell viability. Results presented in **Figure III.10A** reveal that both compounds did not significantly cause any differences in cell viability. Our data show modifications in cell morphology mostly upon treatment with GUDCA that switch the cells from ramified to amoeboid morphology ($p<0.01$), probably because GUDCA is thought to act on the cell membrane, stabilizing its structure and dynamic properties (**Figure III.10B**). However, both GUDCA and VS reduced cell area. In addition, despite no alterations in the phagocytic ability, VS seem to enhance the number of cells without any bead by decreasing cells able to ingest [1-5] bead interval subcategory.

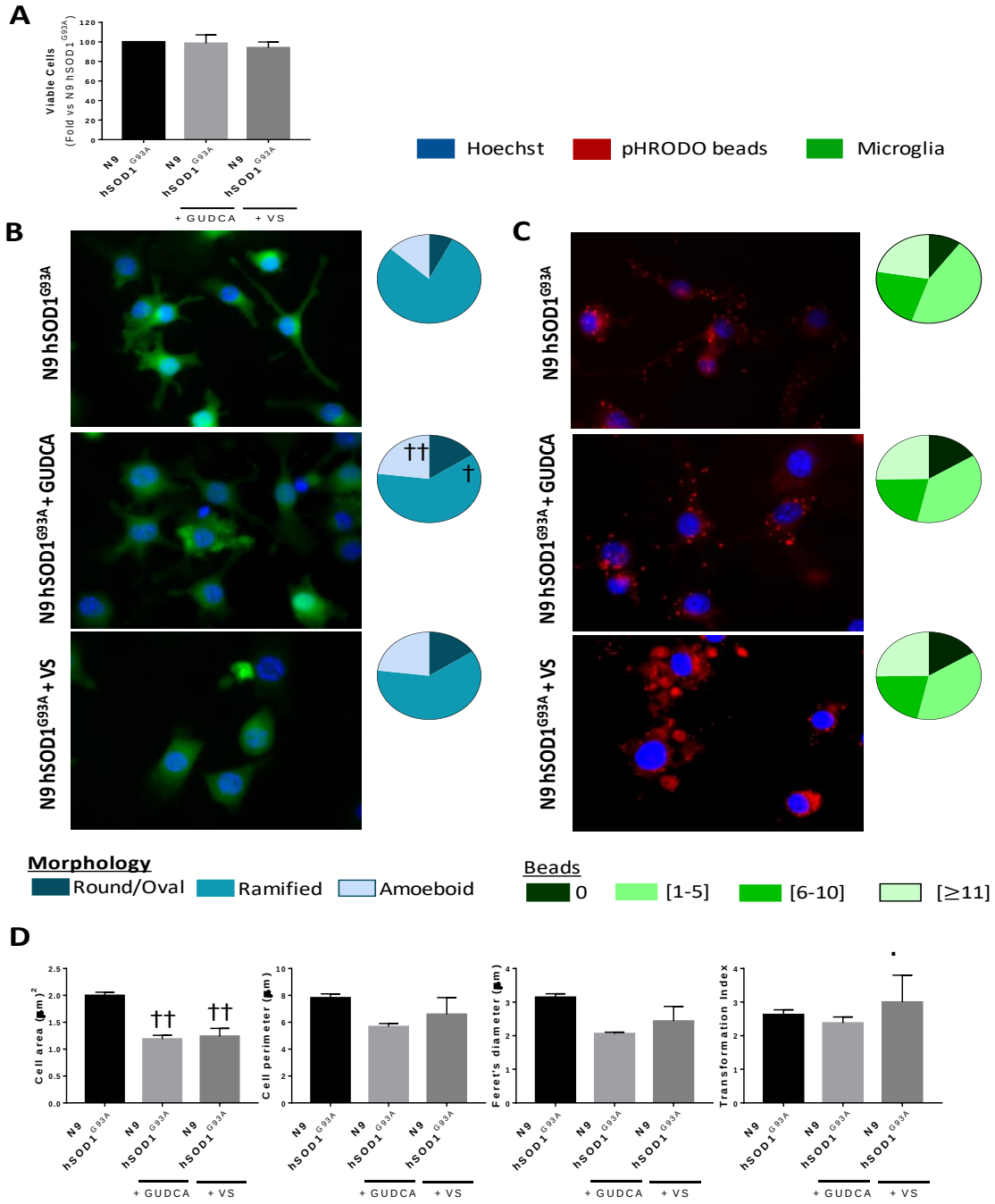


Figure III. 10. Glycoursodeoxycholic acid (GUDCA) and vinyl sulfone (VS) treatment does not affect cell viability or phagocytosis, while modify hSOD1G93A morphological appearance. Transgenic microglia were incubated with 50 µM of GUDCA or 10 µM of VS compound for 48 h. Evaluation of cell viability was performed with a mixture of MTS/PMS as described in methods. Morphology was determined by delineation of each cell of 10 random fields in Image J (approximately 100 cells) and measuring of cell area, perimeter, Ferret's Diameter and Transformation Index (TI). Phagocytosis was determined by incubation of cells with pHRODO beads as described in the method section and was determined by counting the number of beads of each cell and dividing the counting into intervals in order to compare each interval in every cell line. N9 hSOD1^{G93A} were used as control. (A) Percentage of viable cells considering naïve cells as 100%. (B) Morphometric characterization by immunocytochemistry. (C) Determination of phagocytic ability by the number of engulfed beads. (D) Quantitative assessment of cell area, perimeter, Ferret's diameter and Transformation Index (TI). Results are expressed in graph bars as mean (+SEM) (A) Results of viable mitochondria in the cells. (B) Morphologic differences by immunocytochemical assessment. (C) Phagocytosis analysis by immunocytochemical assessment. (D) Quantitative assessment of cell measurements. One-way ANOVA followed by Bonferroni multiple comparison test was used to compare the three tested conditions. $^{††}p<0.01$ and $^{†}p<0.05$ *vs.* N9 hSOD1^{G93A} cells. Scale bar represents 100 µm.

III.3.2. GUDCA and VS differently influence the gene expression of pro-inflammatory and anti-inflammatory mediators in N9 hSOD1^{G93A} microglia

Evaluation of mRNA expression of usual of genes associated to microglia activation and deactivation revealed that both GUDCA and VS exert no effects on *TNF-α, MHCII, Fizz1* and *iNOS*, but decreased anti-inflammatory M2 (*SOCS1, Arg1*) and pro-inflammatory M1 (*S100B* and *IL-1β*) indicators and increased TLR4 receptor, thus influencing the response of the cells towards LPS (**Figure III.11**). Furthermore, only GUDCA revealed ability to decrease *HMGB1* gene expression and increase that of IL-10. However, we also observed that it reduced the expression of MFG-E8 drastically, with possible consequences on the phagocytic ability, although this was not observed in the experiment with fluorescent beads (**Figure III.10B**).

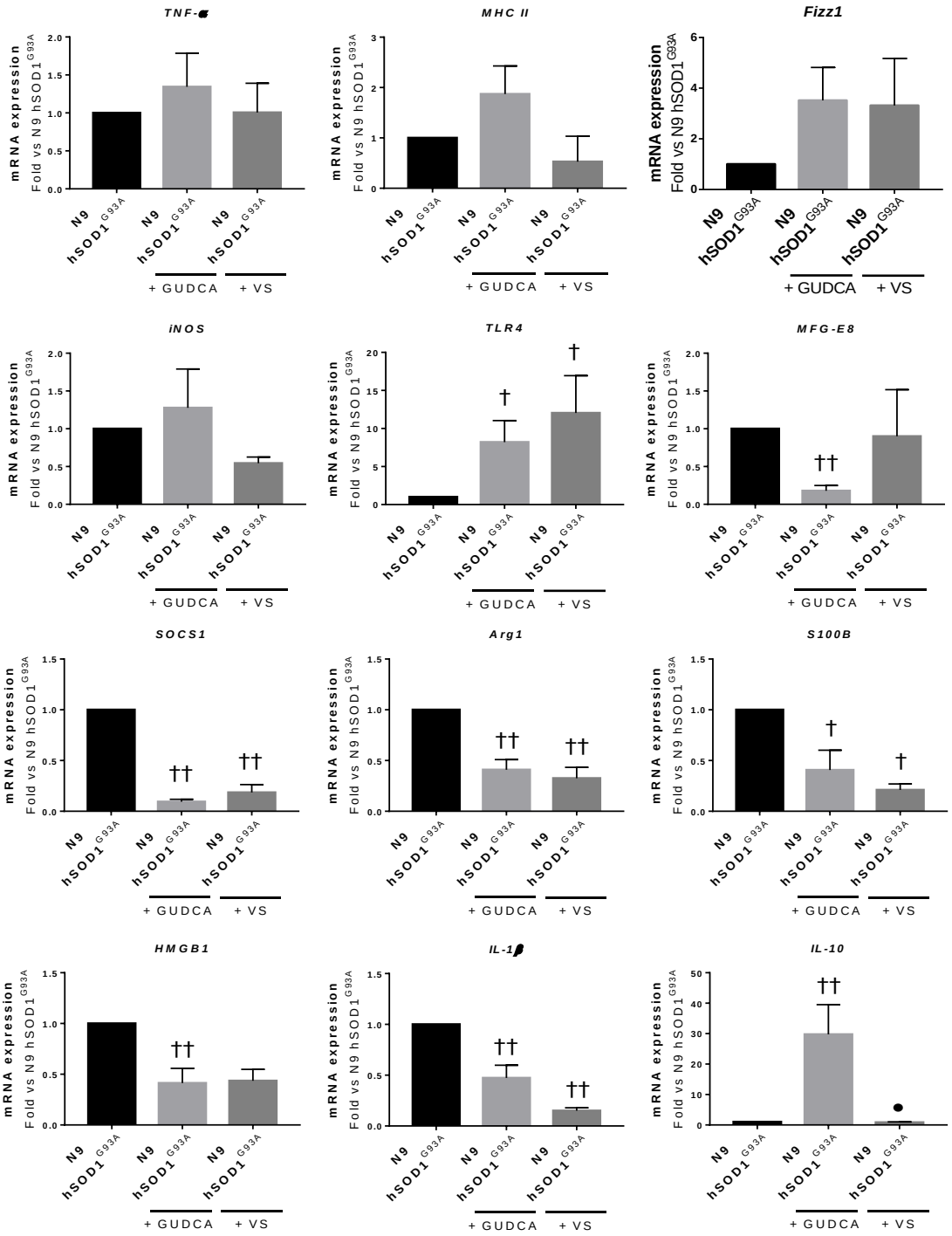


Figure III. 11. Pro-inflammatory and anti-inflammatory indicators are differently regulated by glycoursodeoxycholic (GUDCA) and vinyl sulfone (VS) in hSOD1G93A microglia. Transgenic microglia were incubated with 50 µM of GUDCA or 10 µM of VS compound for 48 h. The mRNA expression of pro- and anti-inflammatory mediators in cells were evaluated by quantitative Real-Time PCR (qRT-PCR), as detailed in the method section. N9 hSOD1^{G93A} was used as control. Results are mean (± SEM) from at least three independent experiences. One-way ANOVA followed by Bonferroni comparison test was used to compare the three tested conditions. $^{\dagger\dagger}p<0.05$ and $^{\dagger}p<0.01$ and *vs.* N9 hSOD1^{G93A}; $^{\bullet}p<0.05$ *vs*. N9 hSOD1^{G93A} + GUDCA. **Arg1**, Arginase 1; **Fizz1**, Found in inflammatory zone 1; **HMGB1**, High mobility group box protein1; **IL-10,** Interleukin-10; **IL-1β,** Interleukin-1β; **iNOS** – inducible nitric oxide synthase; **MFG-E8**, Milk fat globule EGF factor 8; **MHCII**, Major histocompatibility complex class II; **RAGE**, Receptor for Advanced Glycation End Products; **S100B**, S100 calcium binding protein B; **SOCS1,** Suppressor of cytokine signaling 1; **TLR4**, Toll like receptor 4; **TNF-α**, Tumor necrosis factor-α.

III.3.3. GUDCA and VS diversely influence inflammatory-associated miRNA profile in N9 hSOD1^{G93A} microglia and in their derived exosomes

It is well known that the expression of a particular set of miRNAs is directly related with microglia activation and that some of those are transferred into exosomes and exert anti-inflammatory or pro-inflammatory effects on the recipient cells (Brites and Fernandes, 2015; Pinto et al., 2017). While VS was previously indicated to decrease miR-155 and miR-146a in N9 microglial cells incubated with the amyloid-β peptide, no similar studies were so far performed for GUDCA, and none were assessed in ALS experimental models. Therefore, we evaluated the expression of the same inflammatory-miRNAs determined in the conditions we investigated before after the application of both experimental treatments in N9 hSOD1^{G93A} microglia.

From all the pro-inflammatory miRNAs evaluated in the N9 hSOD1^{G93A} microglia after the application of the two immunomodulators, only miR-21 was shown to increase by GUDCA and miR-125b by VS (**Figure III.12**). Curiously, passage into cell derived exosomes was just noticed for miR-21 by VS, what may have benefits due to its anti-inflammatory effects. However, the same seems to occur for miR-155, although not significantly, what may in part counteract the influence of miR-21. Again, the variability we found in the expression of these miRNAs indicates that further determinations must be performed to confirm these yet preliminary results.

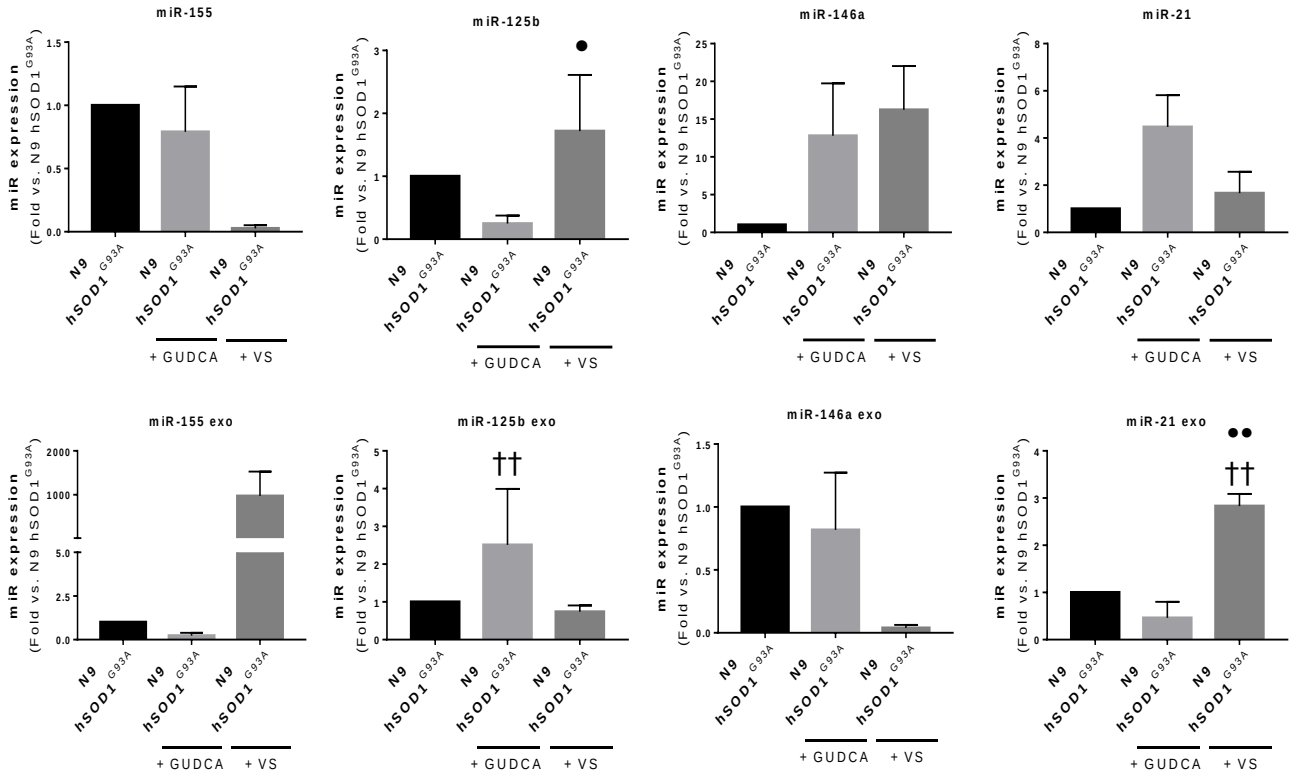


Figure III. 12. Treatment of N9 hSOD1^{G93A} microglia with glycoursodeoxycholic acid (GUDCA) and vinyl sulfone (VS) does not significantly change the cellular expression of inflammatory miRNAs, but determines alterations in their exosomal cargo. Transgenic microglia were incubated with 50 μM of GUDCA or 10 μM of VS compound for 48 h. Inflamma-miRs expression in both cells and exosomes were analyzed by qRT-PCR. N9 hSOD1^{G93A} was used as control. Results are mean (± SEM) from at least three independent experiences. One-way ANOVA followed by Bonferroni comparison test was used to compare the three tested conditions. ††p<0.05 and †p<0.01 *vs.* N9 hSOD1^{G93A} cells; •p<0.05 *vs*. N9 hSOD1^{G93A} cells + GUDCA. **miR,** microRNA.

III.3.4. Pro-inflammatory matrix MMP-9 is downregulated by VS treatment in N9 hSOD1^{G93A} microglia.

Besides exosomes, soluble factor released by activated microglia into the extracellular milieu are also very important in the neuroinflammatory processes. We noticed that treatment, with either GUDCA or VS, did not change the generation of NO and activation of MMP-2 (data not shown). Interestingly, we found that MMP-9 activity was downregulated by VS (p<0.01) and slightly by GUDCA (**Figure III.13**). Since MMP-9 plays a role in disrupting the BBB, neurodegeneration, and allows the propagation of glial cells activation (Sargsyan et al., 2005; Könnecke and Bechmann, 2013), decreased secretion of these soluble factor to the extracellular media could constitute an advantage in the treatment with such potential VS compound. Moreover, reduction of neuronal MMP-9 function by diverse procedures revealed to delay muscle de-enervation, reason why it was proposed as a candidate therapeutic target for ALS (Kaplan et al., 2014).

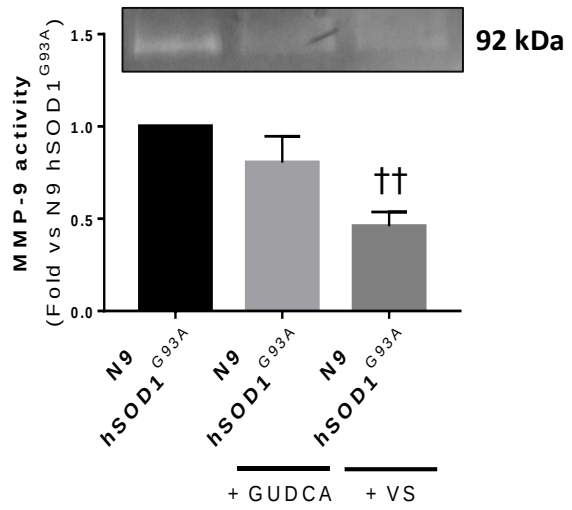


Figure III. 13. Matrix metalloproteinase (MMP)-9 activity is reduced in hSOD1G93A microglia upon VS treatment. Transgenic microglia were incubated with 50 μM of GUDCA or 10 μM of VS compound for 48h. Culture media of the three conditions were loaded into gelatin gels to quantify metalloproteinase activity, as detailed in the method section. N9 hSOD1^{G93A} was used as control. Results are mean (± SEM) from at least three independent experiences. One way-ANOVA followed by Bonferroni multiple comparison test was used to compare the three tested conditions. ††p<0.01 *vs.* N9 hSOD1^{G93A}. **MMP,** Metalloproteinase.

IV. Discussion

ALS pathogenesis results from alterations in several intracellular processes such as autophagy, mitochondria and miRNA profile dysfunction (Chen et al., 2012a; Cozzolino and Carrì, 2012; Parisi et al., 2013). Several mutations are involved in this disease and new *in vitro* models have been recently created in addition to those based in SOD1 mutations, which is still the most used model (Myszczynska and Ferraiuolo, 2016). Although based in a familial form of the disease (fALS), both fALS and sporadic cases (sALS) have the very similar clinical symptoms highlighting the relevance of such models. The role of glial cells such as astrocytes and microglia in neurodegenerative diseases, and particularly in ALS, have been thoroughly investigated and discussed being these cells pointed as key contributors to the neuroinflammation processes and disease progression (Lasiene and Yamanaka, 2011; Philips and Rothstein, 2014). *In vitro* studies have shown that murine astrocytes and microglia expressing mutSOD1 can induce motor neuron death (Xiao et al., 2007; Haidet-Phillips et al., 2011).

Our study was focused on microglial cells. Particularly, we used the N9 microglia cell line, derived from the mouse brain. This line is characterized by sharing many phenotypical characteristics with primary mouse microglia. Hence it is an interesting model to study microglia without using live animals (Stansley et al., 2012).

First, we transduced N9 naïve cell line with hSOD1^{WT} or hSOD1^{G93A}. Afterwards, we looked at cell viability and whether or not it was affected by the overexpression of the foreign proteins. Actually, N9 hSOD1^{WT} cells presented a higher viability comparing to naïve cells, as previously described by Dimayuga and colleagues (2007), probably because of the anti-oxidant role of SOD1, which decreases the oxidative stress in the cell. On the other hand, N9 hSOD1^{G93A} cells did not show any increase or decrease in viability, which may result from the mutation-loss ability to efficiently scavenge ROS. Morphological alterations in cells were previously reported upon transduction with SOD1, which is consistent with our results in either WT or G93A transduced cells (Sargsyan et al., 2009) that revealed to be larger in size. In addition, phagocytic capacity did not change upon transduction, contrarily to what was described by Sargsyan and colleagues (2011), in a study performed in microglial primary

cultures from mice brain (Sargsyan et al., 2011). Our microglial cell line may have increased resistance, and therefore be more able to retain some of the inner capacities of the cells even upon the transduction process. Since morphological properties, such as cell area, perimeter and diameter are not sufficient to infer on microglia activation status, we analyzed gene and miRNA expression in the cells, as well as secreted soluble factors and exosomes in order to determine if the overexpression of a foreign protein could somehow activate the cells or make cells more dysfunctional. Overexpression of human SOD1 in transgenic mice has been shown to be highly protective in models of Parkinson's disease and global ischemia (Nakao et al., 1995; Murakami et al., 1997). In addition, a more recent study reported the decrease of pro-inflammatory cytokine *TNF-α* and NO, which are major players in ALS (Urushitani and Shimohama, 2001; Dimayuga et al., 2007). Indeed, we found that all pro- and anti-inflammatory parameters decreased upon $hSOD1^{WT}$ expression, which could mean that cells are in a more steady state and resistant condition, considering the protection from the oxidative stress in the cell. Therefore, the same was not observed for the transduction with the mutated protein. Overexpression of the $hSOD1^{G93A}$ led to heterogeneous activation and polarization of the cells mainly towards the M1 phenotype. Contrarily to the profile observed for the WT protein, G93A transduced cells increased their expression in pro-inflammatory mediators (*TNF-α, IL-1β, HMGB1* and *MHCII*) and in the phagocytic-related protein MFG-E8, while revealing similar downregulated anti-inflammatory indicators (*Fizz1* and *IL-10*) and *TLR4*. Indeed, increased expression in pro-inflammatory markers in $SOD1^{G93A}$ but not $SOD1^{WT}$ mice had already been described (Hensley et al., 2006; Jeyachandran et al., 2015), and primary microglia, isolated from $SOD1^{G93A}$ mice was reported to be more neurotoxic than LPS-activated WT microglia, also due to the increased production of superoxide, NO and pro-inflammatory cytokines *IL-1β* and *TNF-α* (Tang and Le, 2016).

When analyzing miRNA profile in both cells and released exosomes, our data evidenced that both $hSOD1^{WT}$ and $hSOD1^{G93A}$ have a reduction of miR-21 and miR-125b, but that exosomal increase in the pro-inflammatory miR-155 and miR-146a only occurred in those from mutated cells, as previously observed in N9 microglia polarization by LPS (Cunha et al., 2016). However, increase of miR-146a in cells was only found in the WT ones. As a mediator of microglia dynamics in ALS and reported to be upregulated in the pro-inflammatory response (Chaudhuri et al., 2011; Parisi et al., 2016), the release of miR-125b by WT cells may function through a different mechanism. However, this result can also mean that other pro-inflammatory mediators not analyzed in this work might be upregulated as a response of the cell to the foreign protein (Parisi et al. 2016). Increase of miR-146a in $hSOD1^{WT}$ cells may determine an inhibition of the inflammatory response by targeting NF-κB with consequent downregulation of pro-inflammatory cytokines like *TNF-α* and *IL-1β*, as we observed in these cells. Curiously, gene expression analysis in exosomes revealed that HMGB1 and SOD1 are transported as

part of their cargo. As previously presented in the Results section HMGB1 was never reported as being transported in microglia-derived exosomes, although described for exosomes from other cells (Liu et al., 2006; Sheller-Miller et al., 2017). Our findings in hSOD1^{G93A} overexpressing cells indicate that cells carrying such mutation are more reactive in the steady state condition than the WT microglia, probably influencing the surrounding cells and contributing to an overall inflammatory environment. In addition, our results confirm the transport of mutated/misfolded SOD1 in exosomes. This results are supported by previous studies which not only confirm SOD1 release in exosomes, but state that the enzyme induce exosome formation and release (Basso et al., 2013; Grad et al., 2014; Silverman et al., 2016).

Preconditioned response has been defended by some authors stating that a moderate primary stimulus can stimulate cells to respond more markedly to secondary insults (primed microglia). Thus, a wide range of stimuli have been used, among them LPS (Chen et al., 2012b), which has been described to enhance the pro-inflammatory microglia response towards a given second stimulus (Zhang et al., 2015; Cunha et al., 2016). Our previous work demonstrated that N9 microglia cells become M1 polarized when subjected to LPS exposure (Cunha et al., 2016). Hence, we analyzed the effects, upon stimulation with LPS, in both naïve and transduced cells with 300 ng/mL, a concentration with maximum LPS effect on N9 microglia cells (Cui et al., 2002). We aimed to explore differences in the transduced cell response by an additional stress stimulus, whereas the first one was the overexpression of hSOD1, either WT or mutated in G93A. We first analyzed LPS effects on viability, morphology and phagocytosis. We must notice that 300 ng/mL LPS caused a marked demise of N9 microglial cells that were sustained in the transgenic microglia, what may have determined a selection of a specific set of surviving microglia, thus restricting all our data further obtained to such living cells, but not influencing the comparison among the three tested conditions. However, as expected, LPS exposure caused N9 naïve and both transduced cells morphology to change abruptly from a ramified to an amoeboid phenotype, which was already described by our studies using N9 naïve cells (Cunha et al., 2016). This change in morphology is consistent with cell activation. Also, N9 naïve microglia phagocytic capacity upon LPS exposure was not affected, which can be explained by the fact that higher phagocytic capacity is a main characteristic of M2 polarized cells, while our studies on N9 naïve cells demonstrated a M1 polarization (Cunha et al., 2016; Tang and Le, 2016). On the contrary, overexpression of hSOD1^{WT} appeared to increase phagocytosis in LPS-treated microglia, with a decrease in the amount of cells that phagocytosed zero beads and an increase in the number of cells phagocytosing six to ten beads, suggesting the existence of M2 polarized subclasses. Finally, hSOD1^{G93A} overexpression and treatment with LPS seemed to decrease the number of cells phagocytosing eleven or more beads, which could mean that although the overall cell phagocytic capacity is not affected, it is not fully functional either, at least when comparing with

the two other tested conditions. Regarding the analysis of cytokines and chemokines produced by the cell upon LPS exposure, the majority of studies describe an upregulation of pro-inflammatory cytokines *TNF-α, IL-1β*, as well as the release of NO (Chen et al., 2012b; Dai et al., 2015). However, LPS stimulation in cells transduced with hSOD1^{WT} cells showed protection against the production of these molecules (Chang et al., 2001). Our results showed an overall decrease in all the analyzed pro- and anti-inflammatory cytokines in N9 hSOD1^{WT} over the naïve cells, although there was an increase (not significant) in the pro-inflammatory *IL-1β* suggesting that protection from activation may work for the activation of some pro-inflammatory pathways of the cell, but not for all of them. Nevertheless, our results needs further confirmation to increase their significance, given that *IL-1β* is a key regulator of iNOS/NO induction and *iNOS* gene expression is below the control levels (Kim et al., 2006). Regarding hSOD1^{G93A} overexpression, markers such as *HMGB1* and *IL-1β* were found at the same level as the LPS-treated naïve cells and WT, suggesting that although showing increased inflammatory markers they are not able to mount an overactivated response towards LPS. Despite of that, it deserves to be noted that a slight increase was observed for *TLR4, iNOS, MHCII* and *RAGE* as compared with the LPS-treated transgenic WT cells. On the other hand, anti-inflammatory markers like *Arg1* and *Fizz1* were downregulated in both transgenic microglia, which could mean some pro-inflammatory activation subsequently to LPS in these cells, although miR-155 revealed to be downregulated in each of them.

Upon LPS exposure, miR-155, miR-146a and miR-21 were all reported to be upregulated in several models (Tili et al., 2007; Sheedy, 2015; Cunha et al., 2016; Parisi et al., 2016). While miR-155 was shown to be upregulated upon inflammatory stimulus consequently producing higher levels of *TNF-α*, overexpression of miR-146a was shown to act as a negative feedback loop by downregulating NF-κB activation and further miR-155 upregulation (He et al., 2014). Elevation of miR-146a was also indicated to lead to microglial primed state, and importantly to directly target *IL-1β* downregulation (indicated to be involved in phagocytosis together with *iNOS, MHCII, IL-10* (Saba et al. 2012). In addition, miR-21 is also involved in the cell's immune response, controlling the balance between initial pro-inflammatory responses and later immune-regulatory and anti-inflammatory responses (Sheedy, 2015). MiR-155 and miR-125b are both involved with NF-κB, the first by activating it, and the second by being activated by it, strengthen and prolonging the inflammation state (Ma et al., 2011). Our results show that despite miR-155 reduced levels in transgenic microglia, miR-155 was detected in the exosome cargo of hSOD1^{WT} cells. Both miR-146a and miR-21 expression was not altered in cells, and again miR-21 seems to be preferentially released by hSOD1^{WT} microglia through exosomes, although more experiments are needed to confirm such data. In hSOD1^{G93A} microglia, neither miR-155, nor miR-21, was found to be increasingly transported in exosomes as compared with vesicles from naïve cells. Overall, results point to a less activation state upon LPS exposure in

hSOD1^{WT} cells, by the downregulation of miR-155 and miR-125b in the cells. However, upregulation of miR-155 in the WT cells exosomes also suggests that cell may be signaling inflammation to other cells. On the other hand, miR-155 decrease in hSOD1^{G93A} cells with LPS and its low transport in exosomes can be supporting data of the lower capacity of response of cells carrying the mutation in comparison with hSOD1^{WT} cells.

Having determined microglial reactive profile in non-treated and LPS-treated hSOD1^{G93A} cells, we next focused on the evaluation of the modulatory efficacy of the anti-inflammatory GUDCA and VS compounds to rescue the dysfunctional microglia phenotype towards a more neuroprotective one, based on previous achieving results (Falcão et al., 2007; Vaz et al., 2015). Regarding the two compounds, our approach was to incubate hSOD1^{G93A} transduced cells, with each one of them, for 48 h, as we did for LPS. Despite previous reports, we confirmed the most used concentration of each one for not causing loss of cell viability in our models. In addition, our results did not show alterations in phagocytic capability upon treatment with either compound, although morphology moderately changed by either treatment, with GUDCA significantly increasing the number of amoeboid in detriment of ramified cells, probably by its stabilizing effect on cell membranes (Fernandes and Brites, 2009; Perez and Britz, 2009). The same tendency was verified with VS incubation, although results were not statistically significant. Data indicate a decrease in the pro-inflammatory molecules *S100B* and *IL-1β*, as well as in the anti-inflammatory *SOCS1* and *Arg1* markers and receptor *TLR4*, for both compounds, with a concomitant increase of IL-10 and decrease of HMGB1, together with miR-125b exosomal increase only in GUDCA treated cells. Actually, GUDCA was demonstrated to inhibit the production of *TNF-α* and *IL-1β* in astroglial cells, by preventing the maturation of these cytokines and their consequent release in an experimental model of jaundice (Fernandes et al., 2007). Regarding VS, its anti-inflammatory properties already described included the suppression of miR-155 and miR-146a and of MMP-2 and 9 activities, as well as of *HMGB1* and *IL-1β* in microglia exposed to Aβ peptide, as a model of Alzheimer's disease. These anti-inflammatory properties of GUDCA and VS should be tested in the future in the LPS-treated hSOD1^{G93A} transduced cells to better confirm such effects. Here, we only evaluated their immunomodulatory effects on the reactive, but not LPS-stimulated hSOD1^{G93A} cells, as we intended to assess GUDCA and VS benefits on the ALS microglia phenotype. VS revealed a trend advantage over GUDCA in sustaining *MFG-E8* close to control values and in increasingly inhibiting *IL-1β* and *S100B*, while specifically preventing MHCII and iNOS, and driving exosomal elevation of miR-21.

We hypothesize that miR-155 and miR-21 may increase in exosomes from VS-treated hSOD1^{G93A} cells in the course of their increased clearance, the same occurring for miR-125b this time from GUDCA-treated hSOD1^{WT} cells. In contrast, cellular retention of miR-146a and miR-125b in VS-treated hSOD1^{WT} cells may determine its reduction in exosomal cargo. These

findings are without precedent, as we observed that exosomes released by LPS-stimulated microglia recapitulated cell expression profile (Cunha et al., 2016). It will be interesting to evaluated miRNA-related elimination from cells as depending on changes inducing by compounds in membrane properties. Anyway, VS treatment is probably acting on both pro- and anti-inflammatory pathways, by increasing miR-146a and miR-125b, respectively. In addition, exosome cargo is increased in the expression of the anti-inflammatory miR-21 and the pro-inflammatory miR-155 suggesting the existence of a heterogeneous M1 and M2 microglia population upon VS treatment. Furthermore, MMP-9, although slightly reduced by GUDCA similarly to our previous results in ALS motor neurons (Vaz et al., 2015), was more efficiently downregulated by VS treatment.

We may then conclude that although both compounds showed to differ in their action pathways, either GUDCA or VS appear to have benefic therapeutic effects on modulating microglia reactivity in ALS, although future studies are required to corroborate their use in disease treatment.

Overall, as schematically represented in **Figure IV.1**, our findings gave important information on how transduced hSOD1^{wt}, but above all that hSOD1^{G93A}, caused alterations in the host cell and which signaling mechanisms are upregulated, with or without secondary LPS immune-stimulation. Besides inducing the expression of pro-inflammatory genes, hSOD1^{G93A} also decreased the expression of anti-inflammatory genes, what can potentiate reactivity, but mainly decrease microglia repairing ability after injury and inflammation, thus compromising homeostatic balance. Finally, we propose that treatment with GUDCA/VS may constitute complementary therapies to ALS due to their multi-target effects and the new hope based on the usage of combined therapies for diseases with still not clear pathogenicity.

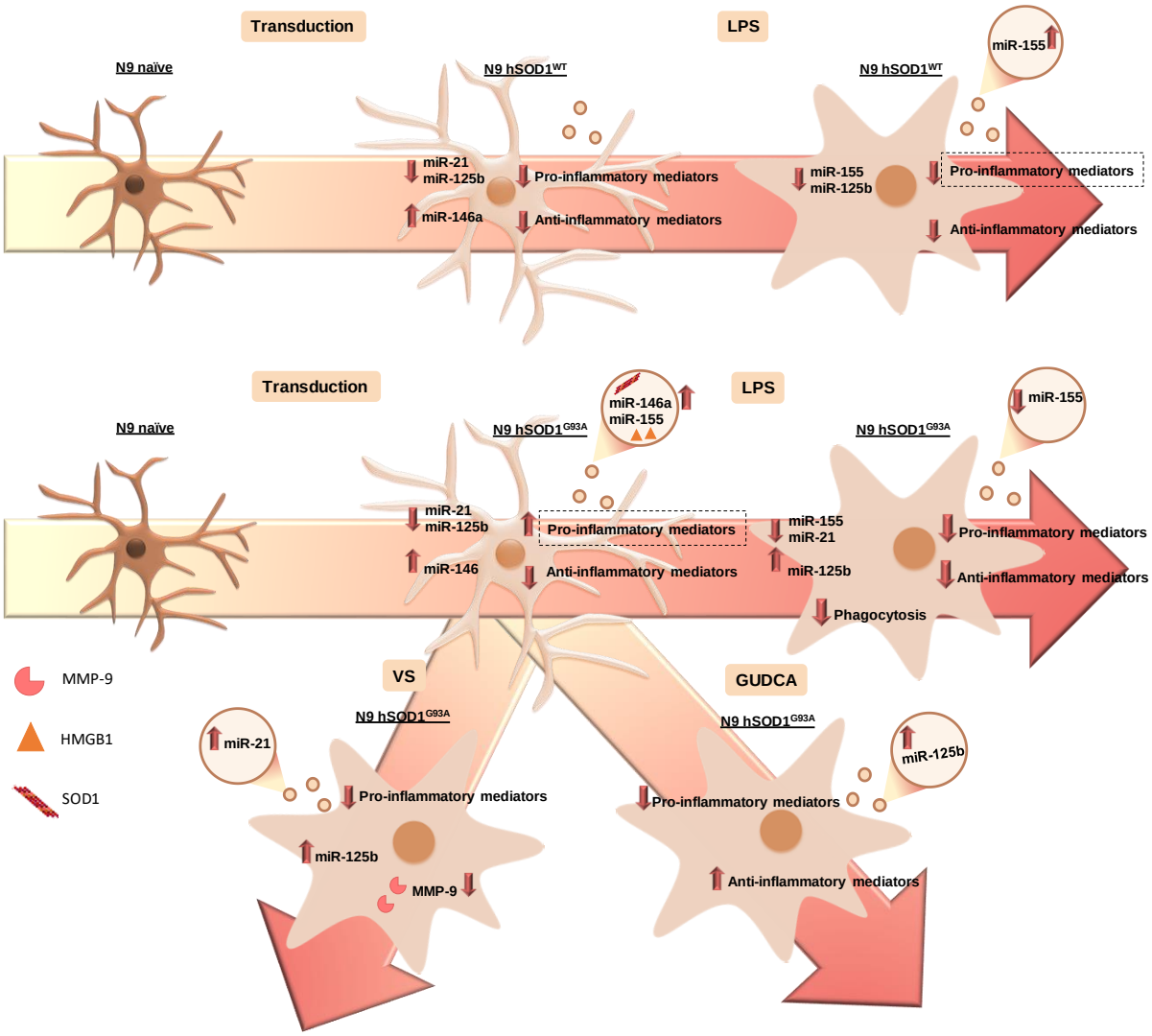


Figure IV. 1. Schematic representation of the major findings obtained in the present study. hSOD1^{WT} transduction causes pro- and anti-inflammatory gene expression mediators to decrease in N9 cells, although do not limit their capacity of response relatively to naïve cells under LPS exposure. On the other hand, hSOD1^{G93A} transduction produces an increase in the gene expression of pro-inflammatory mediators, while decreases some others, together with anti-inflammatory indicators, leading to a heterogeneous mixed population. Treatment of these transgenic cells with LPS produces a sustained reactive response similar to naïve cells, while decreases some pro- and anti-inflammatory mediators, and modifies the phagocytic capacity of the cells. GUDCA and VS treatment shows to modulate the gene expression of inflammatory-related molecules, with different consequences in N9 hSOD1^{G93A} cells and exosomal cargo for specific miRNAs. Also, treatment with VS decreased MMP-9 activation, thus contributing to a less inflammatory extracellular environment. **GUDCA,** Glycoursodeoxycholic Acid; **HMGB1,** High mobility group box protein 1; **LPS,** Lipopolysaccharide; **miR,** microRNA; **MMP,** Matrix Metalloproteinase; **SOD1,** Superoxide Dismutase 1; **VS,** Vinyl Sulfone.

Concluding remarks

- hSOD1^{WT} overexpression in N9 microglia causes downregulation of pro- and anti-inflammatory gene expression markers;
- N9 overexpressing hSOD1^{G93A} cells show a predominant reactive microglia with predominant elevation of pro- and anti-inflammatory gene expression markers;
- Upon LPS treatment, hSOD1^{WT} and hSOD1^{G93A} have some similarities with the LPS-treated naïve cells, although both show depressed anti-inflammatory markers and the hSOD1^{G93A} microglia a decreased reactivity;
- GUDCA and VS were able to downregulate some pro-inflammatory mediators and influence exosomal cargo, although acting in different pathways, showing promise in future combined approaches to ALS.

New perspectives and approaches

In the present study, we discuss and compare the effects of transduction with both hSOD1$^{WT/G93A}$ in microglia cells, the modifications in the cell responses upon stimulation with LPS and the capacity of GUDCA to rescue the effects of overexpressing hSOD1^{G93A} in the cells. Further in our work, it would be interesting to incubate GUDCA in LPS-treated hSOD1^{G93A} overexpressing cells, in order to evaluate the benefits of GUDCA after immunostimulation.

Moreover, co-cultures of both transduced cells with motor neurons alone and treated with LPS/GUDCA should be hypothesized in order to assess the effects of the overexpressing hSOD1$^{WT/G93A}$ in neurons, with and without stimulation, and how GUDCA can prevent possible damages caused by microglial cells in motor neurons. Given the importance of intercellular communication in the CNS, exosomes of these co-cultures media would provide us with essential information on how the cargo of these vesicles can affect and modulate another cell's behavior.

The final purpose with this approach, is to furtherly understand the role of glial cells in the disease and its progression, together with the effect of their reactivity to immunostimulation, and how and which pathways can be modulated in order to prevent inflammation and slow disease progression.

V. References

Abe K, Aoki M, Tsuji S, Itoyama Y, Sobue G, Togo M, Hamada C, Tanaka M, Akimoto M, Nakamura K, Takahashi F, Kondo K, Yoshino H, Abe K, Aoki M, Tsuji S, Itoyama Y, Sobue G, Togo M, Hamada C, Sasaki H, Yabe I, Doi S, Warita H, Imai T, Ito H, Fukuchi M, Osumi E, Wada M, Nakano I, Morita M, Ogata K, Maruki Y, Ito K, Kano O, Yamazaki M, Takahashi Y, Ishiura H, Ogino M, Koike R, Ishida C, Uchiyama T, Mizoguchi K, Obi T, Watanabe H, Atsuta N, Aiba I, Taniguchi A, Sawada H, Hazama T, Fujimura H, Kusaka H, Kunieda T, Kikuchi H, Matsuo H, Ueyama H, Uekawa K, Tanaka M, Akimoto M, Ueda M, Murakami A, Sumii R, Kudou T, Nakamura K, Morimoto K, Yoneoka T, Hirai M, Sasaki K, Terai H, Natori T, Matsui H, Kotani K, Yoshida K, Iwasaki T, Takahashi F, Kondo K, Yoshino H. 2017. Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. Lancet Neurol 16:505–512.

Abe M, Bonini NM. 2013. MicroRNAs and neurodegeneration: Role and impact. Trends Cell Biol 23:30–36.

Agostini M, Tucci P, Melino G. 2011. Cell death pathology: Perspective for human diseases. Biochem Biophys Res Commun 414:451–455.

Al-Chalabi A, Hardiman O. 2013. The epidemiology of ALS: a conspiracy of genes, environment and time. Nat Rev Neurol 9:617–628.

Alexander M, Hu R, Runtsch MC, Kagele DA, Mosbruger TL, Tolmachova T, Seabra MC, Round JL, Ward DM, O'Connell RM. 2015. Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. Nat Commun 6:7321.

Allen SP, Rajan S, Duffy L, Mortiboys H, Higginbottom A, Grierson AJ, Shaw PJ. 2014. Superoxide dismutase 1 mutation in a cellular model of amyotrophic lateral sclerosis shifts energy generation from oxidative phosphorylation to glycolysis. Neurobiol Aging 35:1499–1509.

Balendra R, Mizielinska S, Moens T, Niccoli T, Ridler C, Simone R, Woodling N, Parkinson G, Neidle S, Patani R, Partridge L, Isaacs A. 2016. Molecular mechanisms and therapeutic

strategies in amyotrophic lateral sclerosis caused by C9orf72 mutations. Lancet 387:S13.

Barber SC, Shaw PJ. 2010. Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. Free Radic Biol Med 48:629–41.

Baron R, Babcock AA, Nemirovsky A, Finsen B, Monsonego A. 2014. Accelerated microglial pathology is associated with Aβ plaques in mouse models of Alzheimer's disease. Aging Cell 13:584–595.

Basso M, Bonetto V. 2016. Extracellular vesicles and a novel form of communication in the brain. Front Neurosci 10.

Basso M, Pozzi S, Tortarolo M, Fiordaliso F, Bisighini C, Pasetto L, Spaltro G, Lidonnici D, Gensano F, Battaglia E, Bendotti C, Bonetto V. 2013. Mutant copper-zinc superoxide dismutase (SOD1) induces protein secretion pathway alterations and exosome release in astrocytes: Implications for disease spreading and motor neuron pathology in amyotrophic lateral sclerosis. J Biol Chem 288:15699–15711.

Bell CW, Jiang W, Reich CF, Pisetsky DS. 2006. The extracellular release of HMGB1 during apoptotic cell death. AJP Cell Physiol 291:C1318–C1325.

Bellingham SA, Guo BB, Coleman BM, Hill AF. 2012. Exosomes: Vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? Front Physiol 3 MAY.

von Bernhardi R, Eugenín-von Bernhardi L, Eugenín J. 2015. Microglial cell dysregulation in brain aging and neurodegeneration. Front Aging Neurosci 7.

Biber K, Owens T, Boddeke E. 2014. What is microglia neurotoxicity (Not)? Glia 62:841–854.

Bishop NA, Lu T, Yankner BA. 2010. Neural mechanisms of ageing and cognitive decline. Nature 464:529–535.

Bondy SC, Lee DK. 1993. Oxidative stress induced by glutamate receptor agonists. Brain Res 610:229–233.

Brissette MJ, Lepage S, Lamonde AS, Sirois I, Groleau J, Laurin LP, Cailhier JF. 2012. MFG-E8 released by apoptotic endothelial cells triggers anti-inflammatory macrophage reprogramming. PLoS One 7.

Brites D. 2015. Glycoursodeoxycholic and Tauroursodeoxycholic Acids : From Basic Research to Clinical Applications in CNS Disorders. In: Aguilar Vilas MV, Otero Hernandez C, editors. Frontiers in Bioactive Compounds. . p 293–325.

Brites D, Fernandes A. 2015. Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation. Front Cell Neurosci 9.

Brites D, Vaz AR. 2014. Microglia centered pathogenesis in ALS: insights in cell interconnectivity. Front Cell Neurosci.

Brito MA, Lima S, Fernandes A, Falcão AS, Silva RFM, Butterfield DA, Brites D. 2008. Bilirubin injury to neurons: Contribution of oxidative stress and rescue by glycoursodeoxycholic acid. Neurotoxicology 29:259–269.

Bruce-Keller a J, Keeling JL, Keller JN, Huang FF, Camondola S, Mattson MP. 2000. Antiinflammatory effects of estrogen on microglial activation. Endocrinology 141:3646–3656.

Budnik V, Ruiz-Cañada C, Wendler F. 2016. Extracellular vesicles round off communication in the nervous system. Nat Rev Neurosci 17:160–172.

Bunton-Stasyshyn RKA, Saccon RA, Fratta P, Fisher EMC. 2015. SOD1 Function and Its Implications for Amyotrophic Lateral Sclerosis Pathology. Neurosci 21:519–529.

Butovsky O, Siddiqui S, Gabriely G, Lanser AJ, Dake B, Murugaiyan G, Doykan CE, Wu PM, Gali RR, Iyer LK, Lawson R, Berry J, Krichevsky AM, Cudkowicz ME, Weiner HL. 2012. Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. J Clin Invest 122:3063–87.

Buzas EI, György B, Nagy G, Falus A, Gay S. 2014. Emerging role of extracellular vesicles in inflammatory diseases. Nat Rev Rheumatol 10:356–364.

Caldeira C, Oliveira AF, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. 2014. Microglia change from a reactive to an age-like phenotype with the time in culture. Front Cell Neurosci 8:152.

Cannella D, Brenier-Pinchart MP, Braun L, vanRooyen JM, Bougdour A, Bastien O, Behnke MS, Curt RL, Curt A, Saeij JPJ, Sibley LD, Pelloux H, Hakimi MA. 2014. MiR-146a and miR-155 delineate a microRNA fingerprint associated with toxoplasma persistence in the host brain. Cell Rep 6:928–937.

Cardoso AL, Guedes JR, Pedroso De Lima MC. 2016. Role of microRNAs in the regulation of innate immune cells under neuroinflammatory conditions. Curr Opin Pharmacol 26:1–9.

Cardoso AL, Guedes JR, Pereira de Almeida L, Pedroso de Lima MC. 2012. miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. Immunology 135:73–88.

Cardoso FL, Herz J, Fernandes A, Rocha J, Sepodes B, Brito MA, McGavern DB, Brites D. 2015. Systemic inflammation in early neonatal mice induces transient and lasting neurodegenerative effects. J Neuroinflammation 12:82.

Chang SC, Kao MC, Fu MT, Lin CT. 2001. Modulation of No and cytokines in microglial cells by Cu/Zn-superoxide dismutase. Free Radic Biol Med 31:1084–1089.

Chaudhuri AA, So AY-L, Sinha N, Gibson WSJ, Taganov KD, O'Connell RM, Baltimore D. 2011. MicroRNA-125b Potentiates Macrophage Activation. J Immunol 187:5062–5068.

Chen H, Qian K, Du Z, Cao J, Petersen A, Liu H, Blackbourn LW, Huang C-L, Errigo A, Yin Y, Lu J, Ayala M, Zhang S-C. 2014. Modeling ALS with iPSCs Reveals that Mutant SOD1 Misregulates Neurofilament Balance in Motor Neurons. Cell Stem Cell 14:796–809.

Chen S, Zhang X, Song L, Le W. 2012a. Autophagy dysregulation in amyotrophic lateral sclerosis. In: Brain Pathology. Vol. 22. . p 110–116.

Chen Z, Jalabi W, Shpargel KB, Farabaugh KT, Dutta R, Yin X, Kidd GJ, Bergmann CC, Stohlman SA, Trapp BD. 2012b. Lipopolysaccharide-Induced Microglial Activation and Neuroprotection against Experimental Brain Injury Is Independent of Hematogenous TLR4. J Neurosci 32:11706–11715.

Chia R, Tattum MH, Jones S, Collinge J, Fisher EMC, Jackson GS. 2010. Superoxide dismutase 1 and tgSOD1 mouse spinal cord seed fibrils, suggesting a propagative cell death mechanism in amyotrophic lateral sclerosis. PLoS One 5:e10627.

Chiò A, Logroscino G, Traynor BJ, Collins J, Simeone JC, Goldstein LA, White LA. 2013. Global epidemiology of amyotrophic lateral sclerosis: a systematic review of the published literature. Neuroepidemiology 41:118–30.

Clement AM. 2003. Wild-Type Nonneuronal Cells Extend Survival of SOD1 Mutant Motor Neurons in ALS Mice. Science (80-) 302:113–117.

Colonna M, Butovsky O. 2017. Microglia Function in the Central Nervous System During Health and Neurodegeneration. Annu Rev Immunol 35:441–468.

Cortez L, Sim V. 2014. The therapeutic potential of chemical chaperones in protein folding diseases. Prion 8:197–202.

Cozzolino M, Carrì MT. 2012. Mitochondrial dysfunction in ALS. Prog Neurobiol 97:54–66.

Cui Y-HY-H, Le Y, Gong W, Proost P, Van Damme JJ, Murphy WJ, Wang JM. 2002. Bacterial Lipopolysaccharide Selectively Up-Regulates the Function of the Chemotactic Peptide Receptor Formyl Peptide Receptor 2 in Murine Microglial Cells. J Immunol 168:434–42.

Cunha C, Gomes C, Vaz AR, Brites D. 2016. Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization. Mediators Inflamm 2016.

Cunha C, Santos C, Gomes C, Fernandes A, Correia AM, Sebastião AM, Vaz AR, Brites D. 2017. Downregulated Glia Interplay and Increased miRNA-155 as Promising Markers to Track ALS at an Early Stage. Mol Neurobiol:1–18.

Curtale G, Mirolo M, Renzi TA, Rossato M, Bazzoni F, Locati M. 2013. Negative regulation of Toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. Proc Natl Acad Sci U S A 110:11499–504.

Dai X jing, Li N, Yu L, Chen Z yang, Hua R, Qin X, Zhang YM. 2015. Activation of BV2 microglia by lipopolysaccharide triggers an inflammatory reaction in PC12 cell apoptosis through a toll-like receptor 4-dependent pathway. Cell Stress Chaperones 20:321–331.

Devlin A-C, Burr K, Borooah S, Foster JD, Cleary EM, Geti I, Vallier L, Shaw CE, Chandran S, Miles GB. 2015. Human iPSC-derived motoneurons harbouring TARDBP or C9ORF72 ALS mutations are dysfunctional despite maintaining viability. Nat Commun 6:5999.

Dimayuga FO, Wang C, Clark JM, Dimayuga ER, Dimayuga VM, Bruce-Keller AJ. 2007. SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglial cells. J Neuroimmunol 182:89–99.

Drechsel DA, Estévez AG, Barbeito L, Beckman JS. 2012. Nitric oxide-mediated oxidative damage and the progressive demise of motor neurons in ALS. Neurotox Res 22:251–264.

Edbauer D, Haass C. 2016. An amyloid-like cascade hypothesis for C9orf72 ALS/FTD. Curr Opin Neurobiol 36:99–106.

Ellerman JE, Brown CK, De Vera M, Zeh HJ, Billiar T, Rubartelli A, Lotze MT. 2007. Masquerader: High mobility group box-1 and cancer. Clin Cancer Res 13:2836–2848.

Falcão AS, Carvalho LAR, Lidónio G, Vaz AR, Lucas SD, Moreira R, Brites D. 2017. Dipeptidyl Vinyl Sulfone as a Novel Chemical Tool to Inhibit HMGB1/NLRP3-Inflammasome and Inflamma-miRs in Aβ-Mediated Microglial Inflammation. ACS Chem Neurosci 8:89–99.

Falcão AS, Silva RFM, Pancadas S, Fernandes A, Brito MA, Brites D. 2007. Apoptosis and impairment of neurite network by short exposure of immature rat cortical neurons to unconjugated bilirubin increase with cell differentiation and are additionally enhanced by an inflammatory stimulus. J Neurosci Res 85:1229–1239.

Fang L, Teuchert M, Huber-Abel F, Schattauer D, Hendrich C, Dorst J, Zettlmeissel H, Wlaschek M, Scharffetter-Kochanek K, Kapfer T, Tumani H, Ludolph AC, Brettschneider J. 2010. MMP-2 and MMP-9 are elevated in spinal cord and skin in a mouse model of ALS. J Neurol Sci 294:51–56.

Fauré J, Lachenal G, Court M, Hirrlinger J, Chatellard-Causse C, Blot B, Grange J, Schoehn G, Goldberg Y, Boyer V, Kirchhoff F, Raposo G, Garin J, Sadoul R. 2006. Exosomes are released by cultured cortical neurones. Mol Cell Neurosci 31:642–648.

Feldman N, Rotter-Maskowitz A, Okun E. 2015. DAMPs as mediators of sterile inflammation in aging-related pathologies. Ageing Res Rev 24:29–39.

Fernandes A, Brites D. 2009. Contribution of inflammatory processes to nerve cell toxicity by bilirubin and efficacy of potential therapeutic agents. Curr Pharm Des 15:2915–2926.

Fernandes A, Vaz AR, Falcão AS, Silva RFM, Brito MA, Brites D. 2007. Glycoursodeoxycholic Acid and Interleukin-10 Modulate the Reactivity of Rat Cortical Astrocytes to Unconjugated Bilirubin. J Neuropathol Exp Neurol 66:789–798.

Ferraiuolo L, Higginbottom A, Heath PR, Barber S, Greenald D, Kirby J, Shaw PJ. 2011. Dysregulation of astrocyte-motoneuron cross-talk in mutant superoxide dismutase 1-related amyotrophic lateral sclerosis. Brain 134:2627–2641.

Ferraiuolo L, Meyer K, Sherwood TW, Vick J, Likhite S, Frakes A, Miranda CJ, Braun L, Heath PR, Pineda R, Beattie CE, Shaw PJ, Askwith CC, McTigue D, Kaspar BK. 2016. Oligodendrocytes contribute to motor neuron death in ALS via SOD1-dependent mechanism. Proc Natl Acad Sci 113:E6496–E6505.

Fleisher-Berkovich S, Filipovich-Rimon T, Ben-Shmuel S, Hulsmann C, Kummer MP, Heneka MT. 2010. Distinct modulation of microglial amyloid beta phagocytosis and migration by

neuropeptides. J Neuroinflammation 7:61.

Floeter MK, Traynor BJ, Farren J, Braun LE, Tierney M, Wiggs EA, Wu T. 2017. Disease progression in C9orf72 mutation carriers. Neurology 89:234–241.

Fujita K, Motoki K, Tagawa K, Chen X, Hama H, Nakajima K, Homma H, Tamura T, Watanabe H, Katsuno M, Matsumi C, Kajikawa M, Saito T, Saido T, Sobue G, Miyawaki A, Okazawa H. 2016. HMGB1, a pathogenic molecule that induces neurite degeneration via TLR4-MARCKS, is a potential therapeutic target for Alzheimer's disease. Sci Rep 6:31895.

Garden GA, La Spada AR. 2012. Intercellular (Mis)communication in Neurodegenerative Disease. Neuron 73:886–901.

Gascon E, Gao FB. 2012. Cause or effect: Misregulation of microRNA pathways in neurodegeneration. Front Neurosci:1–10.

Ghavami S, Shojaei S, Yeganeh B, Ande SR, Jangamreddy JR, Mehrpour M, Christoffersson J, Chaabane W, Moghadam AR, Kashani HH, Hashemi M, Owji AA, Łos MJ. 2014. Autophagy and apoptosis dysfunction in neurodegenerative disorders. Prog Neurobiol 112:24–49.

Ginhoux F, Lim S, Hoeffel G, Low D, Huber T. 2013. Origin and differentiation of microglia. Front Cell Neurosci 7.

Ginhoux F, Prinz M. 2015. Origin of Microglia: Current Concepts and Past Controversies. Cold Spring Harb Perspect Biol 7:a020537.

Godbout JP, Johnson RW. 2009. Age and Neuroinflammation: A Lifetime of Psychoneuroimmune Consequences. Immunol Allergy Clin North Am 29:321–337.

Gomes C, Keller S, Altevogt P, Costa J. 2007. Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis. Neurosci Lett 428:43–46.

Gordon PH. 2013. Amyotrophic Lateral Sclerosis: An update for 2013 Clinical Features, Pathophysiology, Management and Therapeutic Trials. Aging Dis 4:295–310.

Grad LI, Fernando SM, Cashman NR. 2015. From molecule to molecule and cell to cell: Prion-like mechanisms in amyotrophic lateral sclerosis. Neurobiol Dis 77:257–265.

Grad LI, Guest WC, Yanai A, Pokrishevsky E, O'Neill MA, Gibbs E, Semenchenko V, Yousefi M, Wishart DS, Plotkin SS, Cashman NR. 2011. Intermolecular transmission of superoxide dismutase 1 misfolding in living cells. Proc Natl Acad Sci 108:16398–16403.

Grad LI, Yerbury JJ, Turner BJ, Guest WC, Pokrishevsky E, O'Neill MA, Yanai A, Silverman JM, Zeineddine R, Corcoran L, Kumita JR, Luheshi LM, Yousefi M, Coleman BM, Hill AF, Plotkin SS, Mackenzie IR, Cashman NR. 2014. Intercellular propagated misfolding of wild-type Cu/Zn superoxide dismutase occurs via exosome-dependent and -independent mechanisms. Proc Natl Acad Sci 111:3620–3625.

Gurney M, Pu H, Chiu A, Dal Canto M, Polchow C, Alexander D, Caliendo J, Hentati A, Kwon

Y, Deng H, et al. 1994. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science (80-) 264:1772–1775.

Ha D, Yang N, Nadithe V, Pharmaceutica Sinica AB. 2016. Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. Acta Pharm Sin B 6:287–296.

Haidet-Phillips AM, Hester ME, Miranda CJ, Meyer K, Braun L, Frakes A, Song S, Likhite S, Murtha MJ, Foust KD, Rao M, Eagle A, Kammesheidt A, Christensen A, Mendell JR, Burghes AHM, Kaspar BK. 2011. Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. Nat Biotechnol 29:824–828.

Han-Xiang D, Hujun J, Ronggen F, Hong Z, Yong S, Erdong L, Makito H, Mauro CDC, Teepu S. 2008. Molecular dissection of ALS-associated toxicity of SOD1 in transgenic mice using an exon-fusion approach. Hum Mol Genet 17:2310–2319.

Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. 2002. Identification of a factor that links apoptotic cells to phagocytes. Nature 417:182–187.

He X, Jing Z, Cheng G. 2014. MicroRNAs: New regulators of toll-like receptor signalling pathways. Biomed Res Int 2014.

Heneka MT, Kummer MP, Latz E. 2014. Innate immune activation in neurodegenerative disease. Nat Rev Immunol 14:463–477.

Hensley K, Abdel-Moaty H, Hunter J, Mhatre M, Mou S, Nguyen K, Potapova T, Pye QN, Qi M, Rice H, Stewart C, Stroukoff K, West M. 2006. Primary glia expressing the G93A-SOD1 mutation present a neuroinflammatory phenotype and provide a cellular system for studies of glial inflammation. J Neuroinflammation 3:1–9.

Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, Kulik J, DeVito L, Psaltis G, DeGennaro LJ, Cleveland DW, Rothstein JD. 2002. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). Proc Natl Acad Sci 99:1604–1609.

Ibrahim A, Marbán E. 2016. Exosomes: Fundamental Biology and Roles in Cardiovascular Physiology. Annu Rev Physiol 78:67–83.

Ilieva H, Polymenidou M, Cleveland DW. 2009. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol 187:761–772.

Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. 2010. STAT3 Activation of miR-21 and miR-181b-1 via PTEN and CYLD Are Part of the Epigenetic Switch Linking Inflammation to Cancer. Mol Cell 39:493–506.

Jeyachandran A, Mertens B, McKissick EA, Mitchell CS. 2015. Type I Vs. Type II Cytokine Levels as a Function of SOD1 G93A Mouse Amyotrophic Lateral Sclerosis Disease Progression. Front Cell Neurosci 9:462.

Jiang M, Xiang Y, Wang D, Gao J, Liu D, Liu Y, Liu S, Zheng D. 2012. Dysregulated expression

of miR-146a contributes to age-related dysfunction of macrophages. Aging Cell 11:29–40.

Johann S, Heitzer M, Kanagaratnam M, Goswami A, Rizo T, Weis J, Troost D, Beyer C. 2015. NLRP3 inflammasome is expressed by astrocytes in the SOD1 mouse model of ALS and in human sporadic ALS patients. Glia 63:2260–2273.

Kabashi E, Valdmanis PN, Dion P, Spiegelman D, McConkey BJ, Velde C Vande, Bouchard J-P, Lacomblez L, Pochigaeva K, Salachas F, Pradat P-F, Camu W, Meininger V, Dupre N, Rouleau GA. 2008. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. Nat Genet 40:572–574.

Kabir ME, Safar JG. 2014. Implications of prion adaptation and evolution paradigm for human neurodegenerative diseases. Prion 8:1–6.

Kang SH, Li Y, Fukaya M, Lorenzini I, Cleveland DW, Ostrow LW, Rothstein JD, Bergles DE. 2013. Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. Nat Neurosci 16:571–579.

Kaplan A, Spiller KJ, Towne C, Kanning KC, Choe GT, Geber A, Akay T, Aebischer P, Henderson CE. 2014. Neuronal matrix Metalloproteinase-9 is a determinant of selective Neurodegeneration. Neuron 81:333–348.

Karkeni E, Astier J, Tourniaire F, El Abed M, Romier B, Gouranton E, Wan L, Borel P, Salles J, Walrand S, Ye J, Landrier JF. 2016. Obesity-associated inflammation induces microRNA-155 expression in adipocytes and adipose tissue: Outcome on adipocyte function. J Clin Endocrinol Metab 101:1615–1626.

Keene DC, Rodrigues CM, Eich T, Chhabra MS, Steer CJ, Low WC. 2002. Tauroursodeoxycholic acid, a bile acid, is neuroprotective in a transgenic animal model of Huntington's disease. Proc Natl Acad Sci U S A 99:10671–10676.

Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, Burrell JR, Zoing MC. 2011. Amyotrophic lateral sclerosis. Lancet 377:942–955.

Kim YJ, Hwang SY, Oh ES, Oh S, Han IO. 2006. IL-1??, an immediate early protein secreted by activated microglia, induces iNOS/NO in C6 astrocytoma cells through p38 MAPK and NF-??B pathways. J Neurosci Res 84:1037–1046.

King A, Troakes C, Smith B, Nolan M, Curran O, Vance C, Shaw CE, Al-Sarraj S. 2015. ALS-FUS pathology revisited: singleton FUS mutations and an unusual case with both a FUS and TARDBP mutation. Acta Neuropathol Commun 3:62.

Kokkola R, Andersson Å, Mullins G, Östberg T, Treutiger CJ, Arnold B, Nawroth P, Andersson U, Harris RA, Harris HE. 2005. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. Scand J Immunol 61:1–9.

Komine O, Yamanaka K. 2015. Neuroinflammation in motor neuron disease. Invit Rev Artic Nagoya J Med Sci 77.

Könnecke H, Bechmann I. 2013. The role of microglia and matrix metalloproteinases involvement in neuroinflammation and gliomas. Clin Dev Immunol 2013.

Krämer-Albers EM, Bretz N, Tenzer S, Winterstein C, Möbius W, Berger H, Nave KA, Schild H, Trotter J. 2007. Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons? Proteomics - Clin Appl 1:1446–1461.

Lasiene J, Yamanaka K. 2011. Glial cells in amyotrophic lateral sclerosis. Neurol Res Int 2011:718987.

Lee J, Hyeon SJ, Im H, Ryu H, Kim Y, Ryu H. 2016. Astrocytes and Microglia as Non-cell Autonomous Players in the Pathogenesis of ALS. Exp Neurobiol 25:233–240.

Lee JK, Shin JH, Lee JE, Choi E-J. 2015. Role of autophagy in the pathogenesis of amyotrophic lateral sclerosis. BBA - Mol Basis Dis 1852:2517–2524.

Lee S, Kwak M, Kim S, Shin J. 2014. The role of high mobility group box 1 in innate immunity. Yonsei Med J 55:1165–1176.

Lin MT, Beal MF. 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443:787–795.

Liu J, Lillo C, Jonsson PA, Velde C Vande, Ward CM, Miller TM, Subramaniam JR, Rothstein JD, Marklund S, Andersen PM, Brännström T, Gredal O, Wong PC, Williams DS, Cleveland DW. 2004. Toxicity of familial ALS-linked SOD1 mutants from selective recruitment to spinal mitochondria. Neuron 43:5–17.

Liu S, Stolz DB, Sappington PL, Macias CA, Killeen ME, Tenhunen JJ, Delude RL, Fink MP. 2006. HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers. Am J Physiol Cell Physiol 290:C990-9.

Liu Y, Pattamatta A, Zu T, Reid T, Bardhi O, Borchelt DR, Yachnis AT, Ranum LPW. 2016. C9orf72 BAC Mouse Model with Motor Deficits and Neurodegenerative Features of ALS/FTD. Neuron 90:521–534.

Lotze MT, Tracey KJ. 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331–342.

Lu B, Wang C, Wang M, Li W, Chen F, Tracey KJ, Wang H. 2014. Molecular mechanism and therapeutic modulation of high mobility group box 1 release and action: an updated review. Expert Rev Clin Immunol 10:713–727.

Ma X, Becker Buscaglia LE, Barker JR, Li Y. 2011. MicroRNAs in NF-kappaB signaling. J Mol Cell Biol 3:159–66.

Maciotta S, Meregalli M, Torrente Y. 2013. The involvement of microRNAs in neurodegenerative diseases. Front Cell Neurosci 7.

Mackenzie IRA, Ansorge O, Strong M, Bilbao J, Zinman L, Ang LC, Baker M, Stewart H, Eisen

A, Rademakers R, Neumann M. 2011. Pathological heterogeneity in amyotrophic lateral sclerosis with FUS mutations: Two distinct patterns correlating with disease severity and mutation. Acta Neuropathol 122:87–98.

Mackenzie IRA, Bigio EH, Ince PG, Geser F, Neumann M, Cairns NJ, Kwong LK, Forman MS, Ravits J, Stewart H, Eisen A, McClusky L, Kretzschmar HA, Monoranu CM, Highley JR, Kirby J, Siddique T, Shaw PJ, Lee VMY, Trojanowski JQ. 2007. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. Ann Neurol 61:427–434.

Mattiazzi M, D'Aurelio M, Gajewski CD, Martushova K, Kiaei M, Flint Beal M, Manfredi G. 2002. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. J Biol Chem 277:29626–29633.

Meyer K, Ferraiuolo L, Miranda CJ, Likhite S, McElroy S, Renusch S, Ditsworth D, Lagier-Tourenne C, Smith RA, Ravits J, Burghes AH, Shaw PJ, Cleveland DW, Kolb SJ, Kaspar BK. 2014. Direct conversion of patient fibroblasts demonstrates non-cell autonomous toxicity of astrocytes to motor neurons in familial and sporadic ALS. Proc Natl Acad Sci 111:829–832.

Mollica L, De Marchis F, Spitaleri A, Dallacosta C, Pennacchini D, Zamai M, Agresti A, Trisciuoglio L, Musco G, Bianchi ME. 2007. Glycyrrhizin Binds to High-Mobility Group Box 1 Protein and Inhibits Its Cytokine Activities. Chem Biol 14:431–441.

Mori K, Weng S-M, Arzberger T, May S, Rentzsch K, Kremmer E, Schmid B, Kretzschmar HA, Cruts M, Van Broeckhoven C, Haass C, Edbauer D. 2013. The C9orf72 GGGGCC Repeat Is Translated into Aggregating Dipeptide-Repeat Proteins in FTLD/ALS. Science (80-) 339:1335–1338.

Mosley RL, Gendelman HE. 2010. Control of neuroinflammation as a therapeutic strategy for amyotrophic lateral sclerosis and other neurodegenerative disorders. Exp Neurol 222:1–5.

Munch C, O'Brien J, Bertolotti A. 2011. Prion-like propagation of mutant superoxide dismutase-1 misfolding in neuronal cells. Proc Natl Acad Sci 108:3548–3553.

Murakami K, Kondo T, Epstein CJ, Chan PH. 1997. Overexpression of CuZn-Superoxide Dismutase Reduces Hippocampal Injury After Global Ischemia in Transgenic Mice. Stroke 28:1797–1804.

Musumeci D, Roviello GN, Montesarchio D. 2014. An overview on HMGB1 inhibitors as potential therapeutic agents in HMGB1-related pathologies. Pharmacol Ther 141:347–357.

Myszczynska M, Ferraiuolo L. 2016. New In Vitro Models to Study Amyotrophic Lateral Sclerosis. Brain Pathol 26:258–65.

Nagai M, Aoki M, Miyoshi I, Kato M, Pasinelli P, Kasai N, Brown RH, Itoyama Y. 2001. Rats

expressing human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. J Neurosci 21:9246–9254.

Nagai M, Re DB, Nagata T, Chalazonitis A, Jessell TM, Wichterle H, Przedborski S. 2007. Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. Nat Neurosci 10:615–622.

Nakamura Y, Si QS, Kataoka K. 1999. Lipopolysaccharide-induced microglial activation in culture: Temporal profiles of morphological change and release of cytokines and nitric oxide. Neurosci Res 35:95–100.

Nakao N, Frodl EM, Widner H, Carlson E, Eggerding FA, Epstein CJ, Brundin P. 1995. Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. Nat Med 1:226–31.

Nardo G, Trolese MC, Tortarolo M, Vallarola A, Freschi M, Pasetto L, Bonetto V, Bendotti C. 2016. New Insights on the Mechanisms of Disease Course Variability in Als From Mutant Sod1 Mouse Models . Brain Pathol 26:237–47.

Nave K-A. 2010. Myelination and support of axonal integrity by glia. Nature 468:244–252.

Niebroj-Dobosz I, Rafałowska J, Fidziańska A, Gadamski R, Grieb P. 2007. Myelin composition of spinal cord in a model of amyotrophic lateral sclerosis (ALS) in SOD1G93A transgenic rats. Folia Neuropathol 45:236–41.

Nonaka T, Masuda-Suzukake M, Arai T, Hasegawa Y, Akatsu H, Obi T, Yoshida M, Murayama S, Mann DMA, Akiyama H, Hasegawa M. 2013. Prion-like Properties of Pathological TDP-43 Aggregates from Diseased Brains. Cell Rep 4:124–134.

Orihuela R, McPherson CA, Harry GJ. 2016. Microglial M1/M2 polarization and metabolic states. Br J Pharmacol 173:649–665.

Paez-Colasante X, Figueroa-Romero C, Sakowski SA, Goutman SA, Feldman EL. 2015. Amyotrophic lateral sclerosis: mechanisms and therapeutics in the epigenomic era. Nat Rev Neurol 11:266–279.

Pandolfi F, Altamura S, Frosali S, Conti P. 2016. Key Role of DAMP in Inflammation, Cancer, and Tissue Repair. Clin Ther 38:1017–1028.

Parisi C, Arisi I, D'Ambrosi N, Storti AE, Brandi R, D'Onofrio M, Volont? C. 2013. Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation. Cell Death Dis 4:e959.

Parisi C, Napoli G, Amadio S, Spalloni A, Apolloni S, Longone P, Volonté C. 2016. MicroRNA-125b regulates microglia activation and motor neuron death in ALS. Cell Death Differ 23:531–541.

Park J, Min JS, Kim B, Chae U Bin, Yun JW, Choi MS, Kong IK, Chang KT, Lee DS. 2015. Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells

by regulating MAPK and NF-κB pathways. Neurosci Lett 584:191–196.

Pasinelli P, Brown RH. 2006. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci 7:710–723.

Pegoraro V, Angelini C. 2017. Role of MicroRNA in Amyotrophic Lateral Sclerosis:evidence for gender and age of onset difference (P4.112). Neurology 88:Supplement P4.112.

Pereira DM, Simões AES, Gomes SE, Castro RE, Carvalho T, Rodrigues CMP, Borralho PM. 2014. MEK5/ERK5 signaling inhibition increases colon cancer cell sensitivity to 5-fluorouracil through a p53-dependent mechanism. Oncotarget.

Perez MJ, Britz O. 2009. Bile-acid-induced cell injury and protection. World J Gastroenterol 15:1677–1689.

Perry VH, Holmes C. 2014. Microglial priming in neurodegenerative disease. Nat Rev Neurol 10:217–224.

Philips T, Bento-Abreu A, Nonneman A, Haeck W, Staats K, Geelen V, Hersmus N, Küsters B, Van Den Bosch L, Van Damme P, Richardson WD, Robberecht W. 2013. Oligodendrocyte dysfunction in the pathogenesis of amyotrophic lateral sclerosis. Brain 136:471–482.

Philips T, Rothstein JD. 2014. Glial cells in amyotrophic lateral sclerosis. Exp Neurol 262:111–120.

Pinto S, Cunha C, Barbosa M, Vaz AR, Brites D. 2017. Exosomes from NSC-34 cells transfected with hSOD1-G93A are enriched in mir-124 and drive alterations in microglia phenotype. Front Neurosci 11.

Pitt JM, Kroemer G, Zitvogel L. 2016. Extracellular vesicles: masters of intercellular communication and potential clinical interventions. J Clin Invest 126:1139–43.

Potolicchio I, Carven GJ, Xu X, Stipp C, Riese RJ, Stern LJ, Santambrogio L. 2005. Proteomic Analysis of Microglia-Derived Exosomes: Metabolic Role of the Aminopeptidase CD13 in Neuropeptide Catabolism. J Immunol 175:2237–2243.

Ramalho RM, Viana RJS, Low WC, Steer CJ, Rodrigues CMP. 2008. Bile acids and apoptosis modulation: an emerging role in experimental Alzheimer's disease. Trends Mol Med 14:54–62.

Ramesh N, Pandey UB. 2017. Autophagy Dysregulation in ALS: When Protein Aggregates Get Out of Hand. Front Mol Neurosci 10.

Ramesh T, Lyon AN, Pineda RH, Wang C, Janssen PML, Canan BD, Burghes AHM, Beattie CE. 2010. A genetic model of amyotrophic lateral sclerosis in zebrafish displays phenotypic hallmarks of motoneuron disease. Dis Model Mech 3:652–662.

Ren W, Zhu L, Hua F, Jin J, Cai Y. 2010. [The effect of lipopolysaccharide on gene expression of TLR4 and MD-2 in rat alveolar macrophage and its secretion of inflammation cytokines]. Zhonghua jie he he hu xi za zhi = Zhonghua jiehe he huxi zazhi = Chinese J

Tuberc Respir Dis 33:367–371.

Renton AE, Chiò A, Traynor BJ. 2013. State of play in amyotrophic lateral sclerosis genetics. Nat Neurosci 17:17–23.

Righi M, Mori L, Libero G De, Sironi M, Biondi A, Mantovani A, Donini SD, Ricciardi-Castagnoli P. 1989. Monokine production by microglial cell clones. Eur J Immunol 19:1443–1448.

Robberecht W, Philips T. 2013. The changing scene of amyotrophic lateral sclerosis. Nat Rev Neurosci 14:248–264.

Robbins PD, Morelli AE. 2014. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol 14:195–208.

Rodrigues CM, Fan G, Wong PY, Kren BT, Steer CJ. 1998. Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. Mol Med 4:165–178.

Rotunno MS, Bosco DA. 2013. An emerging role for misfolded wild-type SOD1 in sporadic ALS pathogenesis. Front Cell Neurosci 7:253.

Rudolph G, Kloeters-Plachky P, Sauer P, Stiehl A. 2002. Intestinal absorption and biliary secretion of ursodeoxycholic acid and its taurine conjugate. Eur J Clin Invest 32:575–580.

Rutherford NJ, Zhang Y-J, Baker M, Gass JM, Finch NA, Xu Y-F, Stewart H, Kelley BJ, Kuntz K, Crook RJP, Sreedharan J, Vance C, Sorenson E, Lippa C, Bigio EH, Geschwind DH, Knopman DS, Mitsumoto H, Petersen RC, Cashman NR, Hutton M, Shaw CE, Boylan KB, Boeve B, Graff-Radford NR, Wszolek ZK, Caselli RJ, Dickson DW, Mackenzie IR, Petrucelli L, Rademakers R. 2008. Novel Mutations in TARDBP (TDP-43) in Patients with Familial Amyotrophic Lateral Sclerosis. PLoS Genet 4:e1000193.

Saba R, Gushue S, Huzarewich RLCH, Manguiat K, Medina S, Robertson C, Booth SA. 2012. MicroRNA 146a (miR-146a) is over-expressed during prion disease and modulates the innate immune response and the microglial activation state. PLoS One 7.

Sábado J, Casanovas A, Tarabal O, Hereu M, Piedrafita L, Calderó J, Esquerda JE. 2014. Accumulation of misfolded SOD1 in dorsal root ganglion degenerating proprioceptive sensory neurons of transgenic mice with amyotrophic lateral sclerosis. Biomed Res Int 2014.

Sargsyan SA, Blackburn DJ, Barber SC, Grosskreutz J, De Vos KJ, Monk PN, Shaw PJ. 2011. A comparison of in vitro properties of resting SOD1 transgenic microglia reveals evidence of reduced neuroprotective function. BMC Neurosci 12:91.

Sargsyan SA, Blackburn DJ, Barber SC, Monk PN, Shaw PJ. 2009. Mutant SOD1 G93A microglia have an inflammatory phenotype and elevated production of MCP-1. Neuroreport 20:1450–5.

Sargsyan SA, Monk PN, Shaw PJ. 2005. Microglia as potential contributors to motor neuron injury in amyotrophic lateral sclerosis. Glia 51:241–253.

Sarko DK, McKinney CE. 2017. Exosomes: Origins and therapeutic potential for neurodegenerative disease. Front Neurosci 11.

Sathasivam S, Shaw PJ. 2005. Apoptosis in amyotrophic lateral sclerosis--what is the evidence? Lancet Neurol 4:500–509.

Schneider A, Simons M. 2013. Exosomes: Vesicular carriers for intercellular communication in neurodegenerative disorders. Cell Tissue Res 352:33–47.

Sheedy FJ. 2015. Turning 21: Induction of miR-21 as a key switch in the inflammatory response. Front Immunol 6.

Sheller-Miller S, Urrabaz-Garza R, Saade G, Menon R. 2017. Damage-Associated molecular pattern markers HMGB1 and cell-Free fetal telomere fragments in oxidative-Stressed amnion epithelial cell-Derived exosomes. J Reprod Immunol 123:3–11.

Sierra A, Gottfried-Blackmore AC, Mcewen BS, Bulloch K. 2007. Microglia derived from aging mice exhibit an altered inflammatory profile. Glia 55:412–424.

Silva SL, Vaz AR, Barateiro A, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2010. Features of bilirubin-induced reactive microglia: From phagocytosis to inflammation. Neurobiol Dis 40:663–675.

Silva SL, Vaz AR, Diógenes MJ, Van Rooijen N, Sebastião AM, Fernandes A, Silva RFM, Brites D. 2012. Neuritic growth impairment and cell death by unconjugated bilirubin is mediated by NO and glutamate, modulated by microglia, and prevented by glycoursodeoxycholic acid and interleukin-10. Neuropharmacology 62:2397–2407.

Silverman JM, Fernando SM, Grad LI, Hill AF, Turner BJ, Yerbury JJ, Cashman NR. 2016. Disease Mechanisms in ALS: Misfolded SOD1 Transferred Through Exosome-Dependent and Exosome-Independent Pathways. Cell Mol Neurobiol 36:377–381.

Simões AES, Pereira DM, Gomes SE, Brito H, Carvalho T, French A, Castro RE, Steer CJ, Thibodeau SN, Rodrigues CMP, Borralho PM. 2015. Aberrant MEK5/ERK5 signalling contributes to human colon cancer progression via NF-κB activation. Cell Death Dis 6:e1718.

Simons M, Raposo G. 2009. Exosomes--vesicular carriers for intercellular communication. Curr Opin Cell Biol 21:575–581.

Sreedharan J, Blair IP, Tripathi VB, Hu X, Vance C, Rogelj B, Ackerley S, Durnall JC, Williams KL, Buratti E, Baralle F, de Belleroche J, Mitchell JD, Leigh PN, Al-Chalabi A, Miller CC, Nicholson G, Shaw CE. 2008. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science 319:1668–72.

Stansley B, Post J, Hensley K. 2012. A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. J Neuroinflammation 9:577.

Streit WJ. 2006. Microglial senescence: does the brain's immune system have an expiration date? Trends Neurosci 29:506–510.

Tan W, Pasinelli P, Trotti D. 2014. Role of mitochondria in mutant SOD1 linked amyotrophic lateral sclerosis. Biochim Biophys Acta 1842:1295–301.

Tang Y, Le W. 2016. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol 53:1181–1194.

Taylor AR, Robinson MB, Gifondorwa DJ, Tytell M, Milligan CE. 2007. Regulation of heat shock protein 70 release in astrocytes: Role of signaling kinases. Dev Neurobiol 67:1815–1829.

Thome AD, Harms AS, Volpicelli-Daley LA, Standaert DG. 2016. microRNA-155 Regulates Alpha-Synuclein-Induced Inflammatory Responses in Models of Parkinson Disease. J Neurosci 36:2383–2390.

Thompson AG, Gray E, Heman-Ackah SM, Mäger I, Talbot K, Andaloussi S El, Wood MJ, Turner MR. 2016. Extracellular vesicles in neurodegenerative disease — pathogenesis to biomarkers. Nat Rev Neurol 12:346–357.

Tili E, Michaille J-J, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA, Croce CM. 2007. Modulation of miR-155 and miR-125b Levels following Lipopolysaccharide/TNF-{alpha} Stimulation and Their Possible Roles in Regulating the Response to Endotoxin Shock. J Immunol (Baltimore, Md 1950) 179:5082–5089.

Tkach M, Théry C. 2016. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. Cell 164:1226–1232.

Tsung A, Tohme S, Billiar TR. 2014. High-mobility group box-1 in sterile inflammation. J Intern Med 276:425–443.

Turner BJ, Talbot K. 2008. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. Prog Neurobiol 85:94–134.

Urbanelli L, Magini A, Buratta S, Brozzi A, Sagini K, Polchi A, Tancini B, Emiliani C. 2013. Signaling pathways in exosomes biogenesis, secretion and fate. Genes (Basel) 4:152–170.

Urushitani M, Ezzi SA, Matsuo A, Tooyama I, Julien J-P. 2008. The endoplasmic reticulum-Golgi pathway is a target for translocation and aggregation of mutant superoxide dismutase linked to ALS. FASEB J 22:2476–2487.

Urushitani M, Shimohama S. 2001. The role of nitric oxide in amyotrophic lateral sclerosis. Amyotroph Lateral Scler Other Mot Neuron Disord 2:71–81.

Vaz AR, Cunha C, Gomes C, Schmucki N, Barbosa M, Brites D. 2015. Glycoursodeoxycholic Acid Reduces Matrix Metalloproteinase-9 and Caspase-9 Activation in a Cellular Model of Superoxide Dismutase-1 Neurodegeneration. Mol Neurobiol 51:864–877.

Vaz AR, Delgado-Esteban M, Brito MA, Bolaños JP, Brites D, Almeida A. 2010. Bilirubin selectively inhibits cytochrome c oxidase activity and induces apoptosis in immature cortical neurons: Assessment of the protective effects of glycoursodeoxycholic acid. J Neurochem 112:56–65.

Vehviläinen P, Koistinaho J, Gundars G. 2014. Mechanisms of mutant SOD1 induced mitochondrial toxicity in amyotrophic lateral sclerosis. Front Cell Neurosci 8.

Vinsant S, Mansfield C, Jimenez-Moreno R, Moore VDG, Yoshikawa M, Hampton TG, Prevette D, Caress J, Oppenheim RW, Milligan C. 2013. Characterization of early pathogenesis in the SOD1G93A mouse model of ALS: Part II, results and discussion. Brain Behav 3:431–457.

Volonte C, Apolloni S, Parisi C. 2015. MicroRNAs: newcomers into the ALS picture. CNS Neurol Disord Drug Targets 14:194–207.

Vucic S, Kiernan MC. 2009. Pathophysiology of neurodegeneration in familial amyotrophic lateral sclerosis. Curr Mol Med 9:255–272.

Vucic S, Rothstein JD, Kiernan MC. 2014. Advances in treating amyotrophic lateral sclerosis: Insights from pathophysiological studies. Trends Neurosci 37:433–442.

Waisman A, Ginhoux F, Greter M, Bruttger J. 2015. Homeostasis of Microglia in the Adult Brain: Review of Novel Microglia Depletion Systems. Trends Immunol 36:625–636.

Wang J, Slunt H, Gonzales V, Fromholt D, Coonfield M, Copeland NG, Jenkins NA, Borchelt DR. 2003. Copper-binding-site-null SOD1 causes ALS in transgenic mice: Aggregates of non-native SOD1 delineate a common feature. Hum Mol Genet 12:2753–2764.

Wang L, Zhang X, Liu L, Yang R, Cui L, Li M. 2010. Atorvastatin protects rat brains against permanent focal ischemia and downregulates HMGB1, HMGB1 receptors (RAGE and TLR4), NF-??B expression. Neurosci Lett 471:152–156.

Watson MR, Lagow RD, Xu K, Zhang B, Bonini NM. 2008. A drosophila model for amyotrophic lateral sclerosis reveals motor neuron damage by human SOD1. J Biol Chem 283:24972–81.

Wegorzewska I, Baloh RH. 2011. TDP-43-based animal models of neurodegeneration: New insights into ALS pathology and pathophysiology. Neurodegener Dis 8:262–274.

Wolf SA, Boddeke HWGM, Kettenmann H. 2017. Microglia in Physiology and Disease. Annu Rev Physiol 79:619–643.

Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, Price DL. 1995. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. Neuron 14:1105–1116.

Xiao Q, Zhao W, Beers DR, Yen AA, Xie W, Henkel JS, Appel SH. 2007. Mutant SOD1 ^{G93A} microglia are more neurotoxic relative to wild-type microglia. J Neurochem 102:2008–2019.

Yamanaka K, Boillee S, Roberts EA, Garcia ML, McAlonis-Downes M, Mikse OR, Cleveland DW, Goldstein LSB. 2008. Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS mice. Proc Natl Acad Sci

105:7594–7599.

Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Wang H, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ. 2004. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc Natl Acad Sci USA 101:296–301.

Yang H, Tracey KJ. 2010. Targeting HMGB1 in inflammation. Biochim Biophys Acta - Gene Regul Mech 1799:149–156.

Yang H, Wang H, Chavan SS, Andersson U. 2015. High Mobility Group Box Protein 1 (HMGB1): The Prototypical Endogenous Danger Molecule. Mol Med 21 Suppl 1:S6–S12.

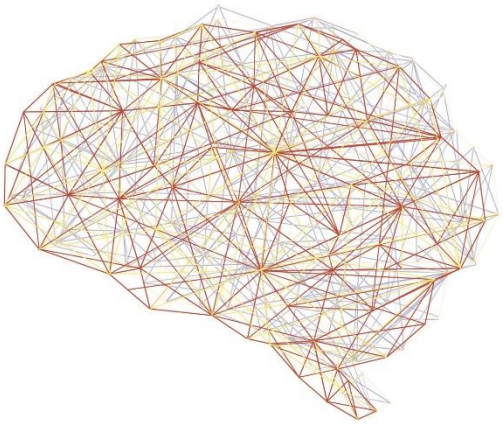
Zappulli V, Pagh Friis K, Fitzpatrick Z, Maguire CA, Breakefield XO. 2016. Extracellular vesicles and intercellular communication within the nervous system. J Clin Invest 126:1198–1207.

Zhang X, Dong H, Zhang S, Lu S, Sun J, Qian Y. 2015. Enhancement of LPS-induced microglial inflammation response via TLR4 under high glucose conditions. Cell Physiol Biochem 35:1571–1581.

Zhang Y, Cook A, Kim J, Baranov S V., Jiang J, Smith K, Cormier K, Bennett E, Browser RP, Day AL, Carlisle DL, Ferrante RJ, Wang X, Friedlander RM. 2013. Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progression in a mouse model of amyotrophic lateral sclerosis. Neurobiol Dis 55:26–35.

VI. Annex

Annex VI.1



XV MEETING OF THE
PORTUGUESE SOCIETY
FOR NEUROSCIENCE

MAY 25-26, 2017 | Braga

Session theme: Neurodegeneration

SOD1G93A transduced microglia show a depressed inflammatory response to LPS

Catarina Ezequiel (1), Carolina Cunha (1), Ana Rita Vaz (1,2), Dora Brites (1,2)

(1) Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal; (2) Department of Biochemistry and Human Biology, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal.

Immune unbalance plays a crucial role in Amyotrophic Lateral Sclerosis (ALS) and microglia dysfunction was shown to be associated with neuronal injury and to influence the onset and progression of the disease [1]. Indeed, there are evidences that microglia can either be highly reactive in early stages or irresponsive to stress stimuli [2]. However, it is not known the signaling pathways that are affected in the mutated ALS microglia that may be responsible for the dual microglia signatures, mainly when stimulated by a proinflammatory stimulus as LPS. Our previous data showed that LPS stimulation of microglial N9 cells switch to a prevalent M1 polarization [3]. With this is mind we proposed to evaluate the resultant effects of overexpressing human SOD1G93A mutation, one of the most common in ALS, in the reactivity of microglia towards LPS.

For that, we used mouse N9 microglial cell line, expressing WT human SOD1 (WT-MG) or containing the G93A mutation (mSOD1-MG), either incubated or not with 300 ng/ml of LPS for 48 h. Upon incubation, phagocytic capacity was evaluated by quantifying the number of ingested beads and mRNA was isolated to evaluate the expression of different M1/M2-associated cell polarization markers.

We observed that mSOD1-MG significantly lost the arginase-1 associated M2 phenotype as well as the expression of the IL-10 anti-inflammatory cytokine, an effect that was exacerbated in the presence of LPS. In addition, the mSOD1-MG showed an increased expression of the stress-related HMGB1 and lower capacity to upregulate S100B levels or MHCII expression upon interaction with LPS. Intriguingly, mSOD1-MG still sustained a moderate ability to increase some markers of M1 microglial phenotype, either in the absence or in the presence of LPS, such as TNFα, microRNA (miR)-155 and miR-146a. In terms of the phagocytic ability, the mSOD1-MG was able to ingest an increased number of beads than the WT-MG, but not in the presence of LPS.

Overall, our study provides a model to characterize microglial heterogeneity in ALS, and data indicate that ALS microglia, although sustaining a moderate inflammatory response to LPS, show increased levels of the alarmin HMGB1 and low expression of MHCII expression that may lead to suboptimal Th cell response during neuroinflammation along the progression of the disease (4).

Chapter VI. Annex

References:

1. Boillée, S., et al., Onset and progression in inherited ALS determined by motor neurons and microglia*. Science*, 2006. **312**(5778): p. 1389-92.

2. Brites, D. and A.R. Vaz, Microglia centered pathogenesis in ALS: insights in cell interconnectivity. Front Cell Neurosci, 2014. **8**: p. 117.

3. Cunha, C., et al., Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization*. Mediators Inflamm*, 2016. **2016**: p. 6986175.

4. Chen, Y., et al., Low-Level MHC class II expression leads to suboptimal Th cell response, increased autoaggression, and heightened cytokine inducibility. *J Immunol*, 2017. **198**(5): p.1928-43.

Funded by Santa Casa da Misericórdia de Lisboa [Project ELA-2015-002 (DB) and Research Fellowship (MB)], and Fundação para a Ciência e Tecnologia: SFRH/BD/91316/2012 PhD grant (CC) and SFRH/BPD/76590/2011 Post-Doctoral grant (ARV)].

Chapter VI. Annex

Annex VI.2

GRUPO DE ESTUDOS DE ENVELHECIMENTO CEREBRAL E DEMÊNCIA

31ª REUNIÃO DO GEECD

30 DE JUNHO E 1 DE JULHO DE 2017, MUSEU DO ORIENTE, LISBOA

PROGRAMA FINAL

DIA 30 DE JUNHO, SEXTA-FEIRA

8h30-9h30 - Abertura do secretariado e Registo

9h00 -10h30 - **Comunicações orais**

Moderação: Maria José Diógenes e Isabel Santana

7 min apresentação + 3min discussão

09.00 - In Search of a Biomarker: Comparison between PIB and CSF in Alzheimer's disease (Diogo Reis Carneiro, Inês Baldeiras, João Castelhano, Miguel Castelo-Branco, Isabel Santana)

09.10 - Upregulation of miR-155 in hSOD1G93A microglia is depicted in their derived-exosomes (Carolina Cunha, Marta Barbosa, Catarina Ezequiel, Ana Rita Vaz, Dora Brites)

09.20 - Progressão de Défice Cognitivo Ligeiro para Doença de Alzheimer: Efeito da Butirilcolinesterase (António José Gabriel, Maria Rosário Almeida, Maria Helena Ribeiro, Diogo Carneiro, Daniela Valério, Ana Cristina Pinheiro, Rui Pascoal, Isabel Santana, Inês Baldeiras)

09.30 - Dissecting deregulated cell-to-cell communication in an in vitro AD model (Ana Rita Ribeiro, Carolina Cunha, Ana Rita Vaz, Adelaide Fernandes, Dora Brites)

09.40 - A exposição ambiental a elementos tóxicos como fator de risco para o declínio cognitivo. (Sandra Freitas, Marina Cabral-Pinto, Agostinho Almeida, Paula Moreira, Paula Marinho-Reis, Teresa Condesso de Melo, Paula Pinto, Mário Rodrigues Simões, Carlos Ordens)

09.50 – Está a apatia após o AVC relacionada com a cefaleia? (Lara Caeiro, Mariana Carvalho-Dias, Teresa Guerreiro Martins, José M. Ferro, Ana Verdelho)

10.00 - Alterações neuropsicológicas em doentes que apresentam cefaleias após o AVC (Lara Caeiro, Mariana Carvalho-Dias, Teresa Guerreiro Martins, José M. Ferro, Ana Verdelho)

10.10 - Neuropsychological Predictors of Long-Term MCI stability (Luísa Alves, Sandra Cardoso, Alexandre Mendonça, Manuela Guerreiro, Dina Silva)

Upregulation of miR-155 in hSOD1^{G93A} microglia is depicted in their derived-exosomes

Carolina Cunha*, Marta Barbosa, Catarina Ezequiel, Ana Rita Vaz, Dora Brites

*jcarolinacunha@ff.ulisboa.pt

Background: Motor neuron (MN) degeneration in amyotrophic lateral sclerosis (ALS) starts focally and spreads to neighboring areas indicating that neuroinflammation and intercellular communication are crucial for disease propagation. The transfer of molecules through exosomes, including microRNAs (miRs), modulates the function of recipient cells. We recently showed that miR-155 is increased in early stages of the disease in the transgenic SOD1^{G93A} mouse model (familiar ALS) and maintained over disease progression (Cunha et al. Mol Neurobiol, 2017).

Aims: We aimed to study (i) microglia activation in ALS; (ii) the transport of inflammatory microRNAs (inflamma-miRs) in exosomes; (iii) the distribution of exosomes in microglia-MNs co-culture.

Methods: To study microglia activation we used: (i) N9 microglia transduced with hSOD1^{WT} or hSOD1^{G93A}, and (ii) primary microglia obtained from the spinal cord of 7 day-old SOD1^{G93A} mice and WT littermates. Phenotype markers and inflamma-miRs were assessed by qRT-PCR (Cunha et al. Med Inflam, 2016). Exosomes were isolated from supernatants by differential ultracentrifugation. Exosomes were stained with the PKH67 fluorescent dye to assess their internalization by the recipient cell (Pinto et al. Front Neurosci, 2017).

Results: The expression of hSOD1^{G93A} in N9 microglia induced the upregulation of MHC-II, IL-1β, IL-10 and miR-155, a major regulator of pro-inflammatory responses. Conversely, miR-124 and miR-146a, linked to neuroprotection, were decreased. Primary SOD1^{G93A} microglia showed a similar pro-inflammatory status with upregulation of MHC-II, iNOS and IL-1β. Again, miR-155 was highly expressed, now only accompanied by miR-124 downregulation. Interestingly, N9 microglia-derived exosomes recapitulated the cell of origin carrying high miR-155 levels. Finally, we observed that exosomes were preferentially internalized by microglia relatively to motor neurons.

Conclusions: Our data show that microglia acquire a pro-inflammatory phenotype in ALS and suggest that miR-155 release in exosomes may account for the propagation of inflammatory responses in an autocrine/paracrine manner.

Keywords: Microglia activation in ALS; miR-155; Exosomal cargo; Inflamma-miRs transfer

Funded by FCT [PTDC/SAU-FAR/118787/2010 (DB), iMed.ULisboa-UID/DTP/04138/2013, PhD/Post-Doctoral grants SFRH/BD/91316/2012 (CC), SFRH/BPD/76590/2011 (ARV)] and Santa Casa da Misericórdia de Lisboa [Project ELA-2015-002 (DB)].

Annex VI.3


9th
iMed.ULisboa Postgraduate
STUDENTS' MEETING
& 2nd i3DU Meeting
July 13th-14th 2017
DRUG DISCOVERY
DRUG DESIGN
DRUG DEVELOPMENT
DRUG USAGE
BOOK OF
ABSTRACTS

Microglia Acquire a Pro- inflammatory Phenotype in ALS and Release miR-155 through Exosomes

Cunha C.* (1), Barbosa M. (1), Ezequiel C. (1), Vaz A.R. (1,2), Brites D. (1,2)

(1) Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal; (2) Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.

* **Email**: jcarolinacunha@ff.ulisboa.pt

Motor neuron (MN) degeneration in amyotrophic lateral sclerosis (ALS) starts focally and spreads to neighboring areas indicating that neuroinflammation and intercellular communication are crucial for disease propagation. The transfer of molecules through exosomes, including microRNAs (miRs), modulates the function of recipient cells. We recently showed that miR-155 is increased in early stages of the disease in the transgenic SOD1G93A mouse model (familiar ALS) and maintained over disease progression. [1]
We aimed to study (i) microglia activation in ALS; (ii) the transport of inflammatory microRNAs (inflamma-miRs) in exosomes; (iii) the distribution of exosomes in microglia-MNs co-cultures.

To study microglia activation we used: (i) primary microglia obtained from the spinal cord of 7 day-old SOD1G93A mice and WT littermates; and (ii) N9 microglia transduced with hSOD1WT or hSOD1G93A. (ii) Phenotype markers were assessed by qRT-PCR and immunocytochemistry and inflamma-miRs by qRT-PCR. Exosomes were isolated from cell culture supernatants by differential ultracentrifugation. Exosomes were stained with the PKH67 fluorescent dye to assess their internalization by recipient cells. [2]

Analysis of inflamma-miRs expression showed that miR-155, a major regulator of pro-inflammatory responses, was upregulated in spinal SOD1G93A microglia while miR-124, linked to neuroprotection, was decreased. Upregulation of CD80, MHC-II and iNOS along with SOCS1 and arginase 1 (Arg1) downregulation further suggest an M1-polarization of SOD1G93A microglia. Importantly, iNOS/Arg1 double-immunostaining showed that different populations coexist and 48% of microglia have a typical pro-inflammatory phenotype (iNOShigh/Arg1low). Accordingly, NF-kB activation, IL-1beta upregulation and nitric oxide (NO) release were also observed. The overexpression of hSOD1G93A in N9 microglia induced an activation profile similar to spinal SOD1G93A microglia including the upregulation of miR-155, MHC-II and IL-1β as well as downregulation of miR-124 and miR-146a. Interestingly, N9 microglia-derived exosomes recapitulated the cell of origin carrying high miR-155 levels.

Finally, we observed that exosomes were preferentially internalized by microglia relatively to motor neurons.

Our data show that microglia acquire a pro-inflammatory phenotype in ALS and suggest that miR-155 release in exosomes may account for the propagation of inflammatory responses in an autocrine/paracrine manner.

Funding: Funded by Fundacao para a Ciencia e a Tecnologia [PTDC/SAU-FAR/118787/2010 (DB), iMed.ULisboa- UID/DTP/04138/2013, PhD/Post-Doctoral grants SFRH/BD/91316/2012 (CC), SFRH/BPD/76590/2011 (ARV)] and Santa Casa da Misericordia de Lisboa [Project ELA-2015-002 (DB) and research fellowship (MB)]

Keywords: Microglia Activation in ALS; Pro-inflammatory Phenotype; miR-155; Exosomal Cargo

References:

1. Cunha C, Santos C, Gomes C, Fernandes A, Correia AM, Sebastiao AM, et al. Downregulated Glia Interplay and Increased miRNA-155 as Promising Markers to Track ALS at an Early Stage. Mol Neurobiol 2017 Jun 13.

2. Pinto S, Cunha C, Barbosa M, Vaz AR, Brites D. Exosomes from NSC-34 Cells Transfected with hSOD1-G93A Are Enriched in miR-124 and Drive Alterations in Microglia Phenotype. Front Neurosci 2017; 11:273.

Expression of hSOD1G93A causes microglia activation, but unresponsiveness to LPS, while hSOD1wt has a calming effect and sustains reactivity to LPS

Catarina Ezequiel (1), Carolina Cunha (1), Ana Rita Vaz (1,2), Dora Brites (1,2)

(1) Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal; (2) Department of Biochemistry and Human Biology, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal.

Neuroinflammation is one of the main hallmarks of Amyotrophic Lateral Sclerosis (ALS), and activation of microglia cells, the immune resident cells in the Central Nervous System (CNS), play a key role in disease onset and progression.[1] It was showed that stable overexpression of $SOD1^{WT}$ in mouse microglia cells decreases production of ROS and release of proinflammatory cytokines.[2] Inversely, the overexpression of $SOD1^{G93A}$ in microglia stimulated ROS production and neurotoxicity, while decreased microglia response towards LPS inflammatory stimulus.[3] Our recent data indicate an induction of M1 polarization with a specific signature in terms of either pro- and anti-inflammatory associated markers in N9-microglia exposed to lipopolysaccharide (LPS).[4]

We aimed to investigate the role of $hSOD1^{WT}$ (MGWT) and $hSOD1^{G93A}$ (MGG93A) in microglia and their reactivity upon LPS stimulation, by using the mouse N9-microglia cell line, transduced with MGWT or MGG93A. Cells were incubated or not with 300 ng/ml LPS for 48h. Non-transduced N9 (naïve) were used as controls. Upon incubation, mRNA was isolated to evaluate the expression of different markers associated with microglia pro- and anti-inflammatory phenotype.

We observed that MGWT are in a less reactive state when compared to naïve N9, as demonstrated by decreased pro-inflammatory (HMGB1, S100B, IL-1β, MHCII and RAGE) and anti-inflammatory (IL-10, Arginase 1) markers. However, upon LPS stimulation, both cell types (naïve and MGWT) similarly switched to a more pro-inflammatory state, as indicated by increased levels of HMGB1, S100B, IL-1β, MHCII and RAGE.

On the other hand, MGG93A cells had a more heterogeneous phenotype, indicated by increased expression of either pro- (HMGB1, S100B, IL-1β, MHCII, RAGE) and anti-inflammatory (Arginase 1) markers. In addition, we observed miR-146a/-21 downregulation and miR-155 upregulation in MGG93A. However, upon LPS exposure, MGG93A became less responsive than MGWT, with a less activated phenotype demonstrated by the decreased expression of S100B, RAGE and IL-1β.

Overall, our study provides information on the beneficial action of hSOD1^{WT} overexpression in microglia and on the irresponsiveness caused by the presence of hSOD1^{G93A} upon an inflammatory stimulus. A better knowledge on the SOD1 function in microglia will contribute to understand risk-associated mutations and how SOD1 targeting may promote neuroprotection.

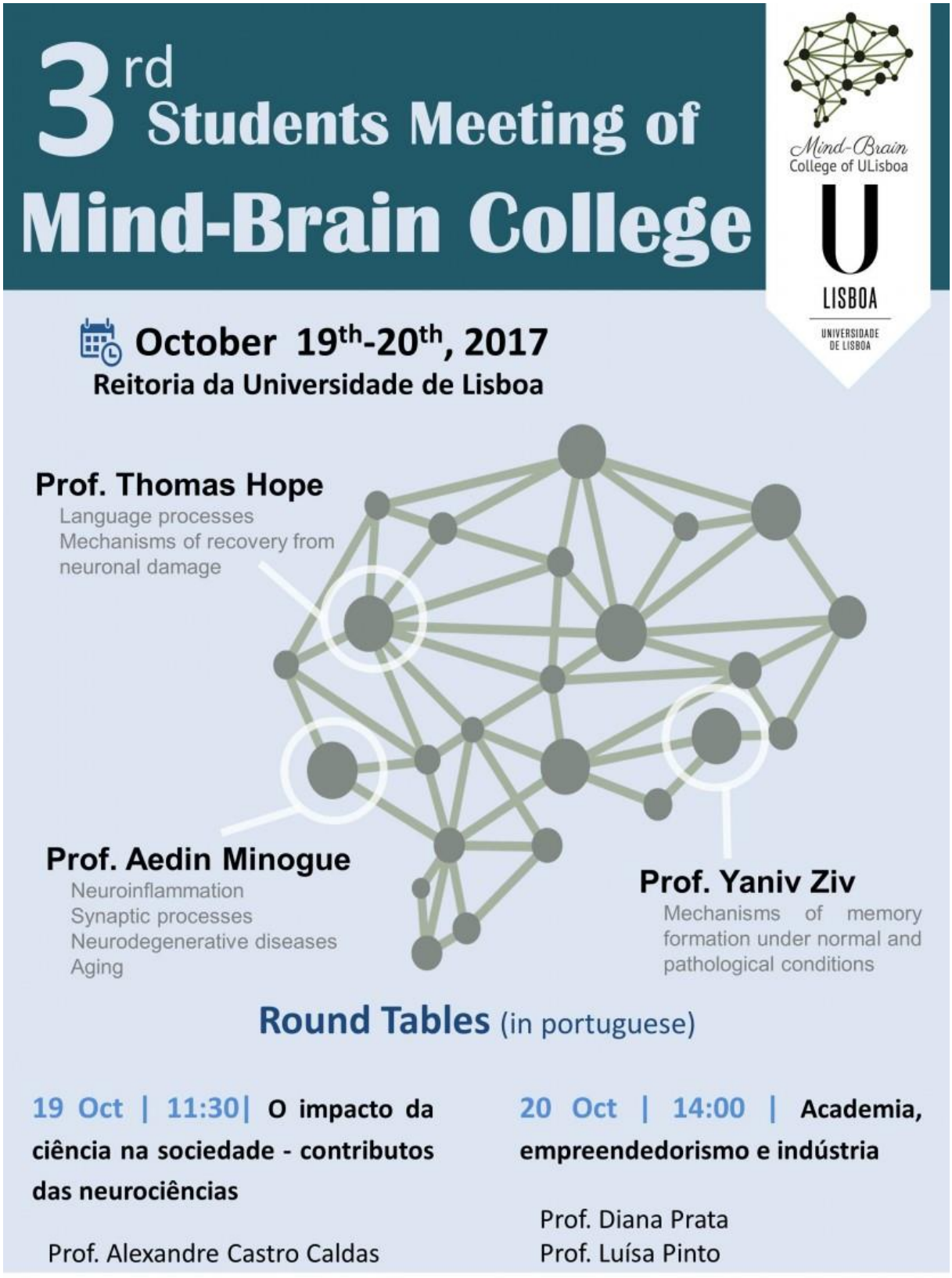
Keywords: Microglia-hSOD1 overexpression; hSOD1G93A mutation; LPS-stimulated microglia

References:

1. Boillée S., Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G et al. *Onset and progression in inherited ALS determined by motor neurons and microglia.* Science.2006; **312**(5778): p. 1389-92.
2. Dimayuga FO, Wang C, Clark JM, Dimayunga ER, Dimayuga VM, Bruce-Keller AJ. *SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglia cells*. *J Neuroimmunol*. 2007; **182**(1-2): 89-99.
3. Xiao Q, Zhao W, Beers DR, Yen AA, Xie W, Henkel JS et al. Mutant SOD1^{G93A} microglia are more neurotoxic relative to wild-type microglia. *J Neurochem*. 2007; **102**(6):2008-19.
4. Cunha C, Gomes C, Vaz AR, Brites D. *Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization.* Mediators Inflamm. 2016; **2016**: 6986175.

Funded by Santa Casa da Misericórdia de Lisboa [Project ELA-2015-002 (DB)], and Fundação para a Ciência e Tecnologia: SFRH/BD/91316/2012 PhD grant (CC) and SFRH/BPD/76590/2011 Post-Doctoral grant (ARV)] and iMed.ULisboa project (Pest-UID/DTP/04138/2013).

Annex VI.4


3rd Students Meeting of Mind-Brain College
Mind-Brain College of ULisboa
LISBOA
UNIVERSIDADE DE LISBOA
October 19th-20th, 2017
Reitoria da Universidade de Lisboa
Prof. Thomas Hope
Language processes
Mechanisms of recovery from neuronal damage
Prof. Aedin Minogue
Neuroinflammation
Synaptic processes
Neurodegenerative diseases
Aging
Prof. Yaniv Ziv
Mechanisms of memory formation under normal and pathological conditions
Round Tables (in portuguese)
19 Oct | 11:30| O impacto da ciência na sociedade - contributos das neurociências
Prof. Alexandre Castro Caldas
20 Oct | 14:00 | Academia, empreendedorismo e indústria
Prof. Diana Prata
Prof. Luísa Pinto
Academia
Sociedade Portuguesa de Neurociências
LISBOA
Sea4Us
justNews

Transfection of N9 microglia with hSOD1G93A inhibits their response to LPS and triggers M1 polarization, which is reversed by GUDCA

Catarina Ezequiel (1), Carolina Cunha (1), Ana Rita Vaz (1,2), Dora Brites (1,2)

(1) Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal; (2) Department of Biochemistry and Human Biology, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal.

Microglia are key players in the pathogenic mechanisms of Amyotrophic Lateral Sclerosis (ALS), the third most common neurodegenerative disease. SOD1 mutation is highly common in the familial cases of ALS and the SOD1G93A mouse model revealed an increased number of $Arg1^+$ and $iNOS^+$ microglia in the spinal cord. In contrast, microglia overexpressing wtSOD1 have attenuated levels of ROS and NO. Here, we transfected N9 microglia with wtSOD1 and SOD1G93A to assess cell phenotypic changes and differences in response to 300 ng/ml lipopolysaccharide (LPS). We also used 50 μM glycoursodeoxycholic acid (GUDCA), known for its anti-inflammatory effects, to test its regenerative ability over dysfunctional microglia.

Considering the gene expression of *M1* and M2 selected *markers,* N9 cells transfected with SOD1 showed lower M1 (HMGB1, S100B, IL-1β; $p<0.01$) and M2 /Arg1 and IL-10; $p<0.01$) levels than the naïve cells. However, cells did not respond differently to the 48 h incubation with LPS (similar HMGB1, S100B and IL-1β levels). In contrast, cells transfected with SOD1G93A showed a decreased inflammatory response to LPS (reduced S100B and IL-1β; $p<0.01$). However, these mutated microglia were found activated with a marked elevation of inflammatory markers (HMGB1, S100B, IL-1β and miR-155; at least $p<0.05$), together with decreased anti-inflammatory ones (Arg1, miR-21 and miR-146a; at least $p<0.05$). When we treated cells for 48 h with GUDCA we observed the recovery of the steady state of microglia, with a reduction of M1 polarized microglia (HMGB1, S100B and IL-1β, $p<0.05$) and an increase of M2 anti-inflammatory markers (miR-21, miR-146a and IL-10; at least $p<0.05$).

Data highlight that SOD1G93A transfection lead to a dysfunctional and activated microglia phenotype that is rescued by GUDCA.

Keywords: ALS; M1/M2 polarization; miRNAs; LPS/GUDCA

Funded by Santa Casa da Misericórdia de Lisboa [Project ELA-2015-002 (DB)] and FCT [SFRH/BD/102718/2014 (CC), SFRH/BPD/76590/2011 (ARV), and Pest-UID/DTP/04138/2013 (iMed.ULisboa)].