THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

The Role of Complement System in Amyotrophic Lateral Sclerosis

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BSc (Hons)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2014

School of Biomedical Sciences

Abstract

There is increasing evidence that neuro-inflammation drives disease progression in many neurodegenerative conditions. Amyotrophic lateral sclerosis (**ALS**) is a debilitating, late onset neurodegenerative disorder that is characterized by the progressive death of upper and α-motor neurons within the central nervous system (**CNS**). Components of the innate immune complement system have been implicated in the pathogenesis of ALS. Within the complement signalling cascade C3a and C5a are regarded as potent inflammatory and immunomodulatory peptides with various biological functions. In the CNS their functions include chemotaxis and proliferation of microglia and astrocytes; generation of superoxide radicals; and induction of pro-inflammatory cytokine synthesis – all thought to be achieved via their main signalling receptors C3aR and CD88. Some of these functions have been observed in neurodegenerative disease, suggesting that these complement factors may play a role in ALS pathogenesis. However a comprehensive examination of complement expression and function of C3aR and CD88 in this disease has not been performed.

The initial aim of this thesis was to determine the expression of complement components (C1qB, C4, factor B, C3/C3b, C5, CD88 and C3aR) and regulators (CD55 and CD59a) in the lumbar spinal cord of the transgenic hSOD1^{G93A} mouse model of ALS. This was conducted during distinct disease stages, which were defined in this thesis. We found several early complement factors increased as disease progressed, whilst complement regulators decreased; suggesting overall increased complement activation through the classical or alternative pathways in hSOD1^{G93A} mice. CD88 and C3aR was also increased during disease progression, with immunolocalization demonstrating expression on motor neurons and increasing expression of CD88 on microglia and increasing expression of C3aR on astrocytes surrounding the regions of motor neuron death.

Our previous studies have demonstrated that hSOD1^{G93A} rats treated with the selective CD88 antagonist PMX205 had reduced gliosis and improvements in behavioural deficits, consistent with reduced neuropathology; suggesting CD88 has a pathogenic function in ALS. However, the contribution of C3aR to disease progression in ALS is still unknown. This thesis therefore next aimed to confirm the function of CD88, and determine the function of C3aR, in the disease progression of ALS in hSOD1^{G93A} mice. The function of CD88 signalling was investigated using two different approaches (pharmacological and genetic). Inhibition of CD88 using PMX205 (pharmacological approach) or hSOD1^{G93A} mice lacking CD88 (genetic approach) showed similarly extended survival when compared to vehicle and hSOD1^{G93A} mice. There was also a reduction in microglia, monocytes and cytokines (TNFα and IL-1β) transcripts in the spinal cord at the end-stage of disease. Taken together these results indicate that inhibition of CD88 significantly attenuates

disease progression potentially by reducing microglia/monocyte activation and generation of pro-inflammatory cytokines, which all have an important role in motor neuron death that contributes to pathology in the hSOD1^{G93A} mice.

Due to a lack of a selective and brain permeable C3aR antagonist, the function of C3aR signalling was investigated by using hSOD1^{G93A} mice lacking C3aR (**hSOD1^{G93A} x C3aR$^{-/-}$**) and comparing survival with hSOD1^{G93A} mice. By contrast with CD88, hSOD1^{G93A} x C3aR$^{-/-}$ mice showed significantly reduced survival relative to hSOD1^{G93A} mice and showed worsened behavioural deficits compared to hSOD1^{G93A} mice. There were also increased astrocyte and microglia transcripts but no change in cytokines (TNFα and IL-1β) transcript levels in the spinal cord at end-stage of disease. Taken together these results indicate that inhibition of C3aR significantly exacerbates disease progression potentially by increasing astrocytes and microglia activation, independent to inflammatory cytokines. This finding also opens a new window of opportunity to target C3a-C3aR signalling in astrocytes using selective agonists, to potentially slow ALS disease progression.

In summary, these results indicate that there is dysregulation of the complement system in the disease progression in a widely used ALS transgenic model. Complement peptides C3a and C5a generated during complement activation and their receptors C3aR and CD88 have two opposing roles in the progression of ALS in the hSOD1^{G93A} mouse. Hence therapeutic modulation of complement activation in ALS should be targeted towards downstream C5a inhibition, in order to avoid blocking any endogenous protective effects of upstream factors such as C3a in ALS progression.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed papers:

1. James W. Crane, Gilang P. Baiquni, Robert K.P. Sullivan, **John D. Lee**, Pankaj Sah, Stephen M. Taylor, Peter G. Noakes and Trent M. Woodruff (2009) The C5a anaphylatoxin receptor CD88 is expressed in presynaptic terminals of hippocampal mossy fibres**.** *Journal of Neuroinflammation* 16(6): 34 *[Impact factor 5.1]*

2. Frederik J. Steyn, Shyuan T. Ngo, **John D. Lee**, Joan W. Leong, Andrew J. Buckley, Johannes D. Veldhuis, Pamela A. McCombe, Chen Chen and Mark. C. Bellingham (2012) Impairments to the GH-IGF-I axis in hSOD1 (G93A) mice give insight into possible mechanisms of GH dysregulation in patients with amyotrophic lateral sclerosis. *Endocrinology*, 153(8): 3735-3746. *[Impact factor 4.8] *Cover Image*

3. Jenny N.T. Fung, Penny L. Jeffery, **John D. Lee**, Inge Seim, Deborah Roche, Andreas Obermair, Lisa K. Chopin and Chen Chen. (2013) Silencing of Ghrelin Receptor Expression Inhibits Endometrial Cancer Cell Growth *in vitro* and *in vivo*. *Am J Physiol Endocrinol Metab*. 305(2): 305-313. *[Impact factor 4.8]*

4. **John D. Lee**, Nur A. Kamaruzaman, Jenny N.T. Fung, Stephen M. Taylor, Bradley J. Turner, Julie D. Atkin, Trent M. Woodruff and Peter G. Noakes (2013) Dysregulation of complement cascade in the hSOD1^{G93A} transgenic mouse model of amyotrophic lateral sclerosis. *Journal of Neuroinflammation*. 10:119 *[Impact factor 5.1] *Cover Image, and Biomedical Central Highly Accessed Award*

5. Trent M. Woodruff, **John D. Lee** and Peter G. Noakes (2014) Role for terminal complement activation in amyotrophic lateral sclerosis disease progression. *Proceeding of the National Academy of Sciences of the United States of America*, 111 1: E3-E4. *[Impact factor 9.7]*

Book Chapter:

1. **John D. Lee**, Jia Y. Lee, Stephen M. Taylor, Peter G. Noakes and Trent M. Woodruff: Innate Immunity in ALS. In *Amyotrophic Lateral Sclerosis*. Edited by Maurer MH. InTech; 2012: 393-412

Conference abstracts:

1. Trent M. Woodruff, **John D. Lee**, Dale Pavolvski, Alex Widiapradja, Stephen M.Taylor, Peter G. Noakes (2009). Role of the Complement Factor C5a in Neurodegenerative Disease. *Proceedings of the Australian Society for Medical Research, Hobart, Nov 2009*

2. **John D. Lee**, Trent M. Woodruff, Stephen M. Taylor, Peter G. Noakes. Expression of Complement factors in the $SOD1^{G93A}$ mouse model of motor neuron disease (2010). *Proceedings of the 30th Annual Australian Neuroscience Society Meeting, Sydney, January 2010*

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4. **John D. Lee**, Trent M. Woodruff, Stephen M. Taylor, Peter G. Noakes. Up-regulation of complement components in the $hSOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis (2010). *40th Society for Neuroscience Meeting, San Diego, November 2010*

5. **John D. Lee**, Trent M. Woodruff, Stephen M. Taylor, Peter G. Noakes. Up-regulation of complement components in the $hSOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis (2011). *22nd International ALS/MND Symposium, Sydney, November 2011*

6. **John D. Lee**, Nur A. Kamaruzaman, Jenny N.T. Fung, Trent M. Woodruff, Stephen M. Taylor, Peter G. Noakes. The complement factor C5a contributes to the pathology in $hSOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis (2012). *International Motoneuron Meeting, Sydney, July 2012*

7. **John D. Lee**, Nur A. Kamaruzaman, Jenny N.T. Fung, Trent M. Woodruff, Peter G. Noakes. Distinct role for C3aR and CD88 in the disease progression of ALS in $hSOD1^{G93A}$ mice (2013). *43rd Society for Neuroscience Meeting, San Diego, November 2013*

Publications included in this thesis

John D. Lee, Jia Y. Lee, Stephen M. Taylor, Peter G. Noakes and Trent M. Woodruff: Innate Immunity in ALS. In *Amyotrophic Lateral Sclerosis*. Edited by Maurer MH. InTech; 2012: 393-412 – incorporated as part of Chapter 1.

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Trent M. Woodruff	Edited the paper (20%)

John D. Lee, Nur A. Kamaruzaman, Jenny N.T. Fung, Stephen M. Taylor, Bradley J. Turner, Julie D. Atkin, Trent M. Woodruff and Peter G. Noakes (2013) Dysregulation of complement cascade in the hSOD1^{G93A} transgenic mouse model of amyotrophic lateral sclerosis. *Journal of Neuroinflammation*. 10:119

- Incorporated as Chapter 3

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Trent M. Woodruff**, John D. Lee** and Peter G. Noakes (2014) Role for terminal complement activation in amyotrophic lateral sclerosis disease progression. *Proceeding of the National Academy of Sciences of the United States of America*, 111 1: E3-E4

- incorporated as part of chapter 4

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Contributions by others to the thesis

Apart from the contributions of the paper authors as listed above, I acknowledge the contributions of A/Prof. Trent Woodruff and A/Prof. Peter Noakes in editing this thesis. I would also like to acknowledge the staff at the University of Queensland Biological Resources (Otto and SCMB animal house) for their assistance with mice maintenance and breeding (Chapter 3, 4, and 5).

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

Acknowledgements

Throughout the good and the bad times during my PhD I have learnt so many valuable lessons and have received countless support and help from so many people to complete my PhD. Firstly I would like to thank my supervisors, Trent Woodruff and Peter Noakes. I would like to thank Trent for giving me the chance to experience a challenging PhD, for his academic input and the enthusiasm to push me through this difficult time. I would also like to thank him for the financial support to carry out this project. His patience, optimistic attitude and trust have made me working with him during my PhD a joy. I would also like to thank my supervisor Peter for helping me throughout my PhD with all the questions and strange ideas I had about the project. I greatly appreciate that your door was always open when I needed your help and your advice and guidance was priceless. Your inspiration and determination about research have always driven me to be better and always willing to take constructive criticism about my project.

I would like to acknowledge the financial support (Australian Postgraduate Awards scholarship) I received from the Australian government and the project funding resources NHMRC and MNDRIA. I would also like to acknowledge the fellowship I received from the Queensland Huntington's Disease Association.

Thanks to those past and present in the Woodruff and Noakes' laboratory for all the help, advice and assistance throughout this endeavour. Particularly I would like to acknowledge Nur Kamaruzaman, Athina Eu, Peppermint Lee, Haitao Wang and people I forgot to mention for all the help and fun in the laboratory. I would also like to acknowledge Mary and Maryam for all the genotyping of animals and small talks to steer me to a right path when everything seemed wrong.

The completion of this project would have never happened without the support of my friends outside and inside the laboratory. Special thank goes to Jenny for your endless support and love. Without you by my side I could not have survived and completed one of the toughest tasks in my life. I am blessed and feel lucky to have someone like you in my life. I would also like to thank Shu, Derik and Sean for all your talks and advice about life after PhD and what I should do to be more successful in science. Last but not least thanks to Lizzie, Angie, Kirat, Jerry and Cortina for all our drinks and talks to make my PhD enjoyable as possible.

My deepest gratitude goes to my parents and my sister for your endless love and support. I would like to take this opportunity to show my love and thanks to my parents who have sacrificed so much

in their life for me to study here in Australia. Without your sacrifice and effort I would not be the person I am today.

Keywords

complement system, C1q, C3, C5, CD88, C3aR, PMX205, amyotrophic lateral sclerosis, motor neuron disease, neuroinflammation

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 110903, Central Nervous System, 60%

ANZSRC code: 110707, Innate Immunity, 20%

ANZSRC code: 111501, Basic Pharmacology, 20%

Fields of Research (FoR) Classification

FoR code: 1109, Neurosciences, 60%

FoR code: 1107, Immunology, 20%

FoR code: 1115, Pharmacology and Pharmaceutical Sciences, 20%

“May the God of peace, who through the blood of the eternal covenant brought back from the dead our Lord Jesus, that great Shepherd of the sheep, equip you with everything good for doing his will, and may he work in us what is pleasing to him, through Jesus Christ, to whom be glory for ever and ever. Amen.”

- Hebrews 13:20 ~ 21 -

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List of Abbreviations

AD – Alzheimer's Disease

ALS – Amyotrophic Lateral Sclerosis

BBB – Blood Brain Barrier

BDNF - Brain-derived nerurotrophic factor

BSA - Bovine serum albumin

CD11b - Cluster of differentiation molecule 11b

ChAT - Choline acetyltransferase

CNS – Central Nervous System

DAF - Decay accelerating factor

DAG - Diacylglycerol

DIG - Digoxygenin

DRG - Dorsal root ganglia

EAE - Experimental autoimmune encephalomyelitis

ERK1/2 - Extracellular signal-related kinase 1/2

ES - End-stage

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GDNF - Glial cell line-derived neurotrophic factor

GFAP - Glial fibrillary acidic protein

HD – Huntington's Disease

HRP - Horseradish peroxidase

$hSOD1^{G93A}$ x $C3aR^{-/-}$ - $hSOD1^{G93A}$ mice lacking C3aR

$hSOD1^{G93A}$ x $CD88^{-/-}$ - $hSOD1^{G93A}$ mice lacking CD88

Iba-1 - Ionised calcium binding adaptor molecule 1

IL-1β - Interleukin -1β

IP_3 - Inositol trisphosphate

LMC - Lateral motor column

LPS - Lipopolysaccharide

MAC – Membrane Attack Complex

MAPK - Mitogen-activated protein kinase

MBL 1 - Mannose binding lectin 1

MBL 2 - Mannose binding lectin 2

MND – Motor Neuron Disease

MS - Mid-symptomatic;

NADPH - Nicotinamide adenine dinucleotide phosphate

NF- κB - Nuclear factor – κB

NFL - Neurofilament

NGF - Nerve growth factor

NMDA - N-methyl-D-aspartate

NT-3 - Neurotrophin-3

OS - Onset

P – Postnatal

PB - Phosphate buffer

PBS - Phosphate buffered saline

PFA - Paraformaldehyde

PI3Kγ - Phosphatidylinositol-3-kinase/Akt

PIP_2 - Phosphoinositol-4, 5 bisphophate

PIP_3 - Phosphoinositol trisphosphate

PKC - Protein kinase C

PLC β_2 - Phospholipase β_2

PS - Pre-symptomatic

SOD1 – Superoxide Dismutase 1

TBS-T - Tris buffered solution- 0.1% Tween 20

TNFα - Tumour necrosis factor α

WT - Wild-type

CHAPTER 1

"General Introduction"

*** Part of this chapter was published as a book chapter in Amyotrophic Lateral Sclerosis** - **John D. Lee**, Jia Y. Lee, Stephen M. Taylor, Peter G. Noakes and Trent M. Woodruff: Innate Immunity in ALS. In *Amyotrophic Lateral Sclerosis*. Edited by Maurer MH. InTech; 2012: 393-412

1. INTRODUCTION

Long term activation of complement has been implicated in numerous neurodegenerative diseases including motor neuron disease (**MND**), Huntington's disease (**HD**), Alzheimer's disease (**AD**), and Parkinson's disease (Woodruff et al., 2008a, Woodruff et al., 2006, Fonseca et al., 2009, Loeffler et al., 2006). Within the complement signalling cascade C3a and C5a are regarded as potent inflammatory peptides with various biological functions. In the central nervous system (**CNS**) their functions include chemotaxis and proliferation of microglia and astrocytes; generation of superoxide radicals; and induction of pro-inflammatory cytokine synthesis – all thought to be achieved via their main signalling receptors C3aR and CD88 respectively (Wetsel, 1995a, Wetsel, 1995b, Gerard and Gerard, 1994, Crass et al., 1996, Ember and Hugli, 1997). Some of these functions have been observed in the above mentioned neurodegenerative diseases, suggesting that complement factors may play a role in the pathogenesis of these diseases.

1.1 Motor Neuron Disease

Amyotrophic lateral sclerosis (**ALS**), also known as Lou Gehrig's disease, is the most common form of MND. It is a debilitating, late onset neurodegenerative disorder that is characterized by the progressive death of upper and α-motor neurons within the CNS (Bruijn et al., 2004). This results in symptoms of muscle weakness and atrophy of skeletal muscles, leading to paralysis and eventual death due to failure of respiratory muscles (Cozzolino et al., 2008). ALS has a prevalence of approximately 1~2 per 100,000 worldwide with males being more susceptible than females (1.3 ~ 1.6: 1) (Strong, 2003, Woodruff et al., 2008b, Worms, 2001). The majority of ALS cases (~90%) are thought to be sporadic with unknown aetiology and no robust environmental risk factors, with the remaining 10% being familial. Of this 10%, approximately 20% have been linked to dominant mis-sense point mutations in the Copper/Zinc superoxide dismutase 1 (**SOD1**) gene, which results in a gain of unidentified deleterious properties (Rosen, 1993). Interestingly, the two aetiologies of ALS (i.e. sporadic and familial) are indistinguishable on the basis of their clinical and pathological features, including progressive muscle weakness, atrophy and spasticity, each of which reflects the degeneration and death of upper and α-motor neurons (Boillee et al., 2006). The mechanisms

leading to ALS are still unclear but theories have suggested that glutamate excitoxicity, oxidative stress, protein aggregation, mitochondrial dysfunction, cytoskeletal abnormalities and neuro-inflammation are all likely to play a role (Turner et al., 2013). Research into both the aetiology and mechanism of neurodegeneration may be helpful to identify potential therapeutic targets and treatments for ALS.

1.2 Innate immunity in neurodegenerative disease

Innate immunity is an evolutionary ancient system that provides the host with immediately available defence mechanisms. It is a rapid and coordinated cascade of reactions by host cells to protect them against foreign pathogens and insults (Akira et al., 2001, Nguyen et al., 2004). Until recently, the CNS was considered to be immunologically privileged because of its perceived inability to mount an immune response and process antigens. Recent studies have revealed that immune surveillance and differentiation between self and non-self does take place in the CNS, where glial cells, including microglia, astrocytes and oligodendrocytes, act as CNS immune effector cells (Hanisch et al., 2008, Lehnardt, 2010, Ricklin et al., 2010).

The role of innate immune system in the CNS is mainly to provide protection to neurons from foreign pathogens and injurious stimuli, and to maintain CNS homeostasis. It is also required for tissue modelling during development and following injury (Benard et al., 2008, Stevens et al., 2007, Mastellos et al., 2005, Rahpeymai et al., 2006). However sustained chronic inflammation can be harmful for neuronal integrity and may result in cellular dysfunction, which may drive neurodegeneration. There is increasing evidence that suggests an involvement of the innate immune system in the development of neuro-inflammation that underpins the progression of many neurodegenerative diseases including ALS. One of the major constituents of innate immune system is the complement cascade, which is the focus of this thesis, and is described in detail below.

1.3 The complement system in the CNS

The complement system participates in the recognition, trafficking and elimination of pathogens and unwanted host materials. It is an enzymatic cascade consisting of more than 30 plasma proteins and glycoproteins, and either soluble or membrane-bound receptors (Guo and Ward, 2005). Complement activation participates in host defence against pathogens primarily by cytotoxic and cytolytic activity through triggering formation of the membrane attack complex (**MAC** or **C5b-9**) on the target cell membrane (van Beek et al., 2003). It is activated via three canonical pathways: the classical, alternative, and lectin pathways; however importantly, it can also be activated by a fourth,

extrinsic protease pathway that does not require upstream complement activation (summarized in **Figure 1.1**) (Huber-Lang et al., 2006, Thoman et al., 1984).

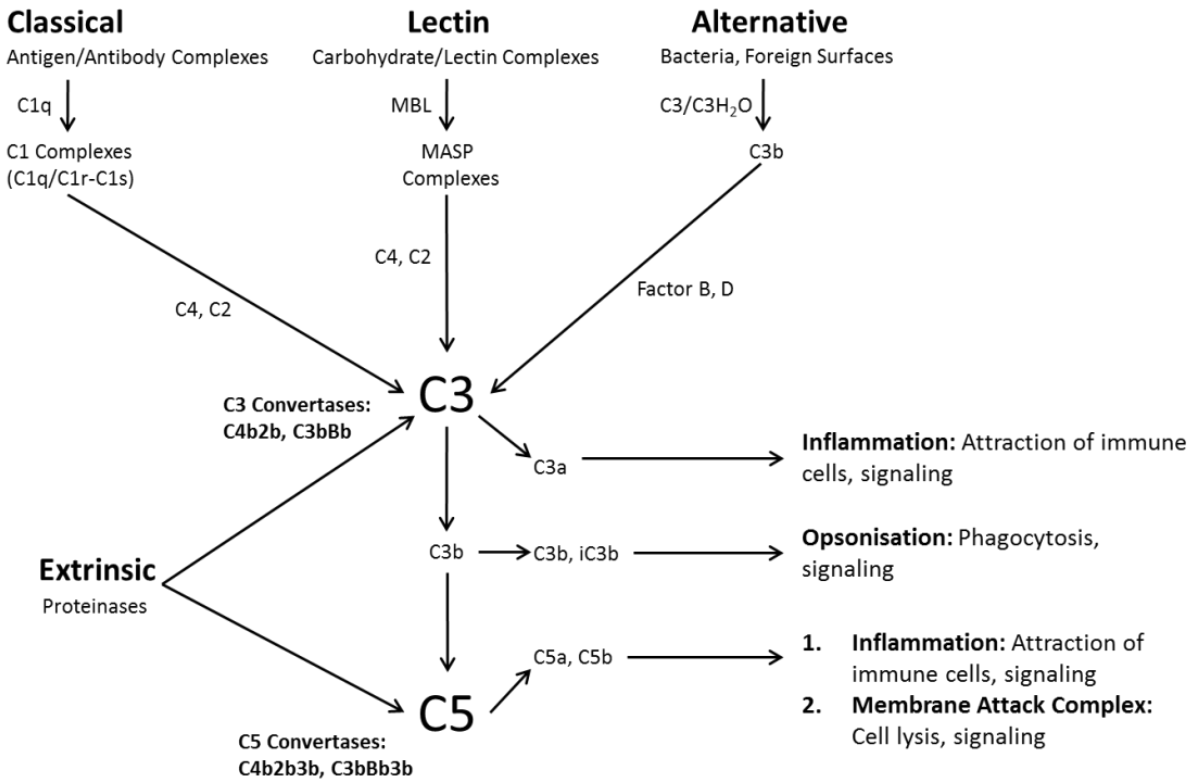

Figure 1.1: The Complement Cascade. *Complement is part of the innate immune system and can be activated via four different pathways: the classical pathway, an antigen-antibody complex; the alternative pathway, activated by bacteria and foreign surfaces; the lectin pathway activated by mannose binding lectin; and recently discovered extrinsic protease pathway involving direct cleavage of C3 and C5. Each pathway converges at C3 and leads to a common terminal point which involves the formation of the cytolytic membrane attack complex (**MAC**) leading to cell lysis. Formation of pro-inflammatory peptides C3a and C5a induces glial chemotaxis, proliferation, generation of superoxide radicals and release of inflammatory mediators. C3b and iC3b facilitates phagocytosis by opsonising foreign pathogens.*

The classical pathway is primarily activated in response to the recognition molecule C1q binding to antigen-antibody complexes such as immunoglobins (IgG and IgM) and pentraxins (such as C-reactive protein) bound to their targets (Ricklin et al., 2010, Woodruff et al., 2010). C1q may also bind directly to pathogen surfaces and to non-pathogen surfaces such as beta-amyloid and liposomes (Jiang et al., 1994, Marjan et al., 1994). The alternative pathway is activated by foreign surfaces that amplify the slow spontaneous hydrolysis of C3 which leads to the formation of C3 convertases (Pangburn et al., 1981, Ricklin et al., 2010). By contrast, the lectin pathway is initiated following the binding of mannose-binding lectin to carbohydrate groups on the surfaces of some pathogens (Woodruff et al., 2010). The activation of each of these pathways results in assembly of C3 and C5 convertase enzymes, which cleave their respective inactive complement factors C3 and C5 into their active fragments C3a, C3b, C5a and C5b. This leads to the formation of MAC through the non- enzymatic assembly of C5b with complement factors C6-C9, forming C5b-9 on the cell membrane, which creates a transmembrane pore, ultimately leading to cell lysis (Podack et al., 1982).

A recently identified fourth extrinsic pathway involves direct cleavage of C3 and C5 into C3a/C3b and C5a/C5b by proteolytic enzymes (serine proteases) such as kallikrein, thrombin and cell-derived proteases (Huber-Lang et al., 2002, Huber-Lang et al., 2006). As a result, synthesis of C5 by local inflammatory cells can produce C5a via cleavage of C5 with cell derived proteases, even when devoid of the complement cascade precursor, C3 (Huber-Lang et al., 2006). This pathway may provide a source of complement activation factors in the absence of upstream complement activation, and in a local tissue environment such as the CNS (Woodruff et al., 2010).

The primary function of complement activation is to provide a rapid response to infection and injury by initiating the production of opsonins C1q and C3b to opsonise pathogens, the production of the pro-inflammatory peptides C3a and C5a to recruit immune and inflammatory cells through ligand-receptor interactions with their corresponding receptors, C3aR and CD88, and the formation of cytolytic MAC, which ultimately leads to the destruction of invading organisms by cell apoptosis/necrosis (Liszewski et al., 1996).

Although the CNS does not receive the same composition of circulating complement factors synthesised in the liver by hepatocytes, due to the blood brain barrier (**BBB**), many studies have revealed that the CNS contains key components of complement cascade, where they are expressed by astrocytes, microglia, oligodendrocytes and neurons (Barnum, 1995, Gasque et al., 1997, Nataf et al., 2001, O'Barr et al., 2001, Stephan et al., 2012). Similar to the peripheral system, the role of

complement activation within the CNS is thought to primarily protect the neurons from foreign pathogens through activation of inflammatory and immune cascades by surrounding glial cells. In addition to their immune surveillance functions, recent studies have shown that complement molecules also have a role in adaptive immune response, nervous system development, regeneration and regulating CNS homeostasis by clearing cellular debris and also eliminating excess synapses (i.e. synaptic pruning) (Stevens et al., 2007, Stephan et al., 2012). Intriguingly, synaptic loss is not only a feature of neural development but is also a key pathological feature of neurodegenerative diseases (Schafer and Stevens, 2010, Woodruff et al., 2010). Hence it has been proposed that complement has multiple central roles in the CNS other than its canonical functions associated with host defence (Benard et al., 2008, Rahpeymai et al., 2006, Stevens et al., 2007, Stephan et al., 2012). Therefore dysregulation or imbalance of the complement system in the CNS can be harmful to the neurons and may lead to, or contribute to, neurodegenerative diseases including ALS.

1.4 Complement peptide C3a

One aspect of complement activation that is particular importance in the inflammatory response is the generation of biologically active peptides C3a and C5a. C3a is one of the complement peptides that are cleaved from C3 during complement activation. C3a is a 77-amino acid polypeptide that binds to and exerts its major effects through its high affinity receptor, the C3aR (Crass et al., 1996, Ember and Hugli, 1997). C3aR is a member of the rhodopsin family of seven transmembrane G-protein coupled receptors with smaller N-terminal domain and a greatly enlarged second extracellular domain (**Figure 1.2**) (Klos et al., 2013). Following the binding of C3a to C3aR, the primary signalling mechanism activated is through the pertussis toxin-sensitive G-protein $G_{\alpha i}$ and also through pertussis toxin-insensitive G-protein $G_{\alpha 12/13}$ and $G_{\alpha 16}$ (Langkabel et al., 1999, Crass et al., 1996, Klos et al., 2013, Li et al., 2008). Cellular activation of C3aR involves intracellular calcium mobilisation and activation of different signalling pathways including phosphatidylinositol-3-kinase/Akt (**PI3Kγ**), phospholipase C, mitogen-activated protein kinase (**MAPK**)/extracellular signal-related kinase 1/2 (**ERK1/2**), protein kinase C (**PKC**), nuclear factor - κB (**NF- κB**) and inhibition of MAPK/p38 and adenylate cyclase (summarised in **Figure 1.3**) (Klos et al., 1992, Li et al., 2008, Sayah et al., 2003, Gupta et al., 2012, Vibhuti et al., 2011). It is widely expressed on a variety of cells including glial cells, endothelial cells and neurons (Nataf et al., 1999), and its activation is known to have pro-inflammatory functions such as chemotaxis, degranulation, generation of cytokine and chemokine, superoxide production, smooth muscle contraction and cell apoptosis (Ember and Hugli, 1997, Gasque et al., 2002, Jacob et al., 2010, Legler et al., 1996, Kretzschmar et al., 1993, Sayah et al., 1999, Jauneau et al., 2003, Elsner et al., 1994a, Elsner et al., 1994b, Petering et al., 2000).

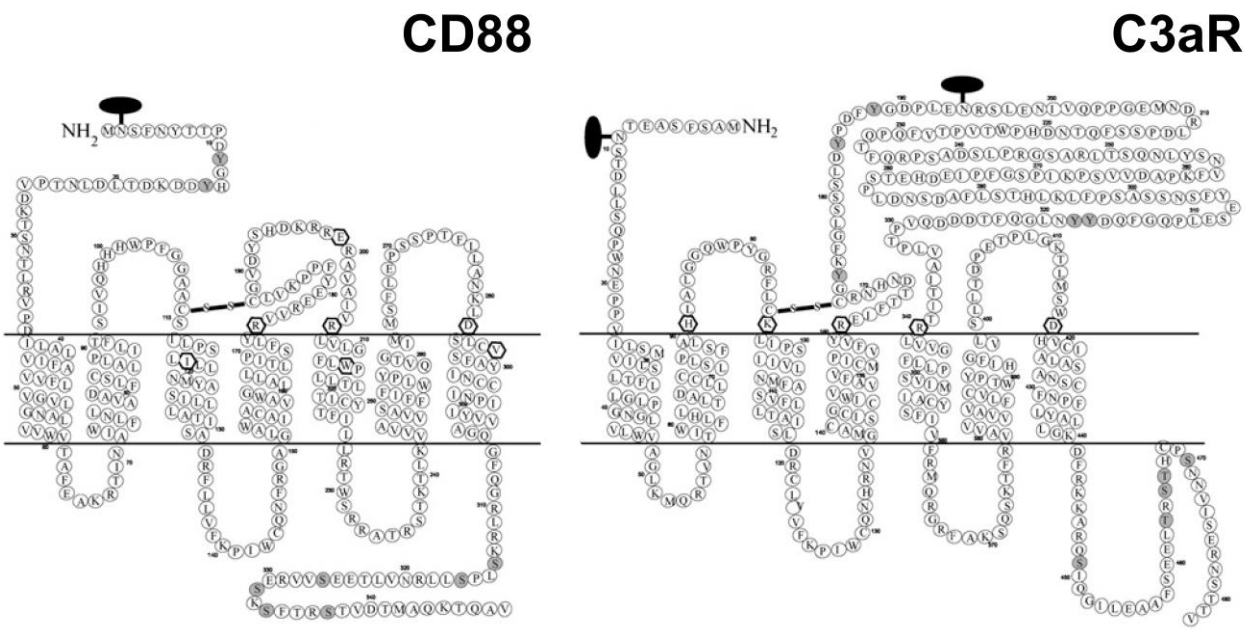

Figure 1.2: Complement peptide C5a and C3a receptors CD88 and C3aR. *Both receptors have overall sequence identity of 37%. Sequence and domain structures are shown for CD88 and C3aR. Glycosylation sites are indicated by the black ovals. Phosphorylation sites in intracellular domains and tyrosine sulfation sites in extracellular domains are indicated by grey circles. Unlike CD88, C3aR consists of smaller N-terminal domain and a greatly enlarged second extracellular domain. Adapted from (Klos et al., 2013).*

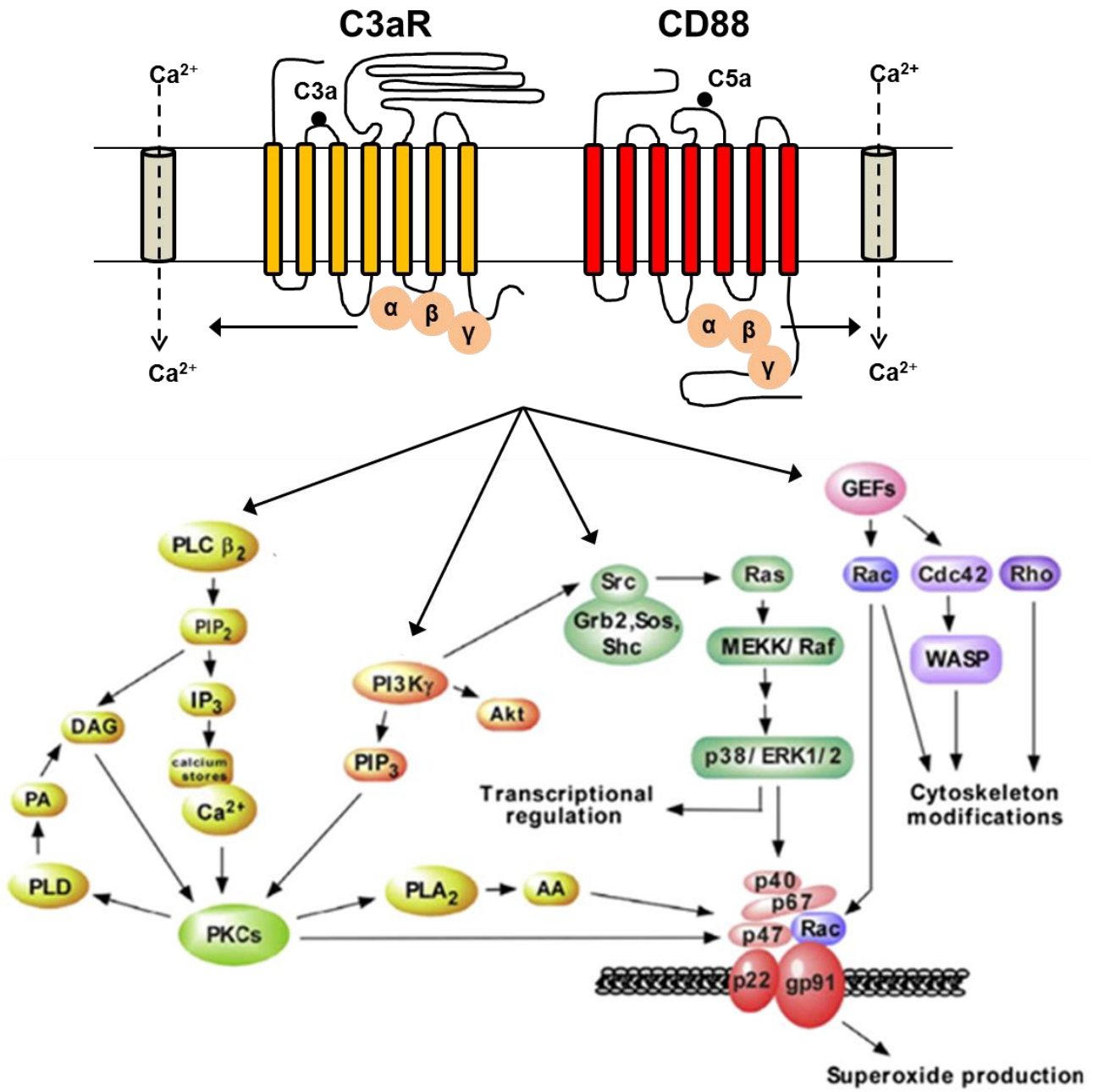

Figure 1.3: Schematic view showing the main signaling pathways initiated by C3aR and CD88 activation. *Cellular activation of C3aR and CD88 involves intracellular calcium mobilisation. Upon binding of C3a and C5a, receptor undergoes conformational change that enables dissociation of βγ complex from α subunit. Following its dissociation from α subunit, βγ complex of G-protein activates Phospholipase β_2 (PLC β_2) and PI3Kγ. PI3Kγ converts membrane phosphoinositol-4, 5 bisphophate (PIP_2) into phosphoinositol trisphosphate (PIP_3), which activates PKC. PLC β_2 hydrolyses PIP_2 into diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 causes the release of calcium from the intracellular calcium stores. Interplay between calcium and DAG activates the PKC. PKC is involved in the superoxide production by regulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Enhancing the activity of Src-like tyrosine kinases phophorylate docking proteins such as Shc adaptor protein, leads to the activation of Ras-Raf-MAPK-ERK pathway, which is involved in chemotaxis and superoxide production. Lastly, activation of low molecular weight G-proteins of the Rho family (Rho, Rac and Cdc42) via GEFs activation regulate many leukocyte functions, including adhesion to endothelial cells, chemotaxis and superoxide generation. Adapted from (Rabiet et al., 2007).*

1.5 Role of C3a in CNS diseases

Several studies have provided evidence indicating a pro-inflammatory role of C3a-C3aR signalling in the pathogenesis of CNS diseases including experimental lupus, traumatic brain injury and experimental autoimmune encephalomyelitis (**EAE**) (Jacob et al., 2010, Li et al., 2013, Wang et al., 2010, Boos et al., 2004). Specifically, they showed a significant up-regulation of C3aR and demonstrated that inhibiting C3aR using C3aR antagonist and decay accelerating factor (**DAF**) reduced pro-inflammatory cytokine release such as tumour necrosis factor α (**TNFα**) and interleukin -1β (**IL-1β**), astrogliosis, phosphorylated tau and improved BBB integrity in experimental lupus and traumatic brain injury (Jacob et al., 2010, Li et al., 2013). Ablation of C3aR in the brain attenuated EAE, whereas overexpression of C3a in the brain exacerbated EAE (Boos et al., 2004). Ultimately inhibition of C3aR in experimental lupus and traumatic brain injury lead to reduction in neuronal cell death while C3a-C3aR signalling developed more severe EAE, suggesting that C3a-C3aR has a pathogenic role in these CNS diseases.

By contrast, many studies have also demonstrated an anti-inflammatory role of C3a-C3aR signalling. C3a has been found to suppress the secretion of lipopolysaccharide (**LPS**)-induced pro-inflammatory cytokine TNF-α, IL-1β and interleukin-6 from peripheral blood mononuclear cells and modulate production of anti-inflammatory hormones (Fischer and Hugli, 1997, Takabayashi et al., 1998, Takabayashi et al., 1996, Francis et al., 2003). Mice lacking C3aR are more sensitive to LPS-induced lethality with increased IL-1β synthesis, suggesting that C3a-C3aR signalling plays a protective role by modulating pro-inflammatory cytokine synthesis (Kildsgaard et al., 2000). Furthermore it was also found that there was increased resistance to endotoxin induced lethality in mice that overexpress C3a in astrocytes (Boos et al., 2005). In support of these findings, a recent report also revealed marked neuroprotective properties for both C3a overexpression in the brain and administration of exogenous C3a peptide following neonatal ischemic brain injury (Jarlestedt et al., 2013). These combined studies suggest that C3a may also have anti-inflammatory functions, which could alleviate the progression of neurodegenerative diseases such as ALS.

1.6 Complement peptide C5a

C5a is considered to be the most potent inflammatory molecule generated upon complement activation and exhibits a broad range of functions. C5a is a 74-amino acid polypeptide that exerts its effect through two high affinity receptors, the classical C5a receptor (CD88), and the C5a-like receptor 2 (C5L2/GPR77). CD88 is a member of the rhodopsin family of seven transmembrane domain receptors coupled to the hetero-metric G proteins of the Gi subtype: pertussis toxin-sensitive $G_{\alpha i2}$, $G_{\alpha i3}$ or pertussis toxin-insensitive $G_{\alpha 16}$ (Amatruda et al., 1993, Johswich and Klos,

2007, Rollins et al., 1991). Cellular activation of CD88 involves intracellular calcium mobilization and activation of different signalling pathways including PI3Kγ (Perianayagam et al., 2002), Ras/B-Raf/MAPK/ERK (Buhl et al., 1994), phospholipase A_2, phospholipase D (Cockcroft, 1992, Mullmann et al., 1990), PKC (Buhl et al., 1994), p21-activated kinases, Rac GTPases (Huang et al., 1998), signal transducers and activators of transcription, sphingosine kinase (Melendez and Ibrahim, 2004) and NF-κB (Kastl et al., 2006)(summarised in **Figure 1.3**). It is widely expressed on variety of cells and tissues, and its activation is known to have pro-inflammatory functions such as chemotaxis, degranulation, superoxide production, and release of proteases, eicosanoids, cytokine and chemokine from inflammatory cells (Gomez-Cambronero et al., 2007, Melendez and Ibrahim, 2004, Torres and Forman, 1999, Tsai et al., 2004).

1.7 Role of C5a in CNS diseases

Despite the availability of studies demonstrating potential neuroprotective activities of C5a (Osaka et al., 1999, Mukherjee and Pasinetti, 2001, Mukherjee et al., 2008, O'Barr et al., 2001), the overall body of evidence accumulated to date suggest that C5a induced CD88 activation plays a pathogenic role in CNS diseases. In particular, CD88 activation seemed to be the driver of CNS pathology in numerous neurodegenerative diseases including AD, HD and ALS (Farkas et al., 2003, Fonseca et al., 2009, Fonseca et al., 2013, Singhrao et al., 1999, Woodruff et al., 2006, Woodruff et al., 2008a, Humayun et al., 2009). This was demonstrated by increased CD88 expression in human tissue and protection in animal models using a specific CD88 antagonist PMX205 (Fonseca et al., 2009, Woodruff et al., 2006, Woodruff et al., 2008a).

Specifically, they showed increased CD88 immunoreactivity concentrated around the β-amyloid plaque, which is a prominent feature of AD (Fonseca et al., 2013). In support of this pathogenic role for C5a in AD, previous study have shown that oral treatment with the CD88 antagonist PMX205 significantly attenuated AD-like pathology after 12 weeks of treatment in two distinct mouse models of AD. The reduction in pathology was correlative with improvements in contextual memory (Fonseca et al., 2009). CD88 is also up-regulated in the striatum of HD patients when compared with tissue from non-HD individuals (Singhrao et al., 1999). This is specifically on neurons and proliferating glia in the degenerating regions of HD brains, suggesting an involvement of CD88 activation in the progression of this disease. Previous study also showed that pre- or post-treatment of 3-nitropropionic acid induced striatal neurodegeneration in rats with CD88 antagonist PMX205 significantly reduced striatal lesion size, apoptosis, neutrophil infiltration, astrocyte proliferation and improved neurological deficits, indicating a pathogenic role for C5a in this disease model (Woodruff et al., 2006). Similar to AD and HD, there is significant up-regulation of CD88 on

proliferating astrocytes in the vicinity of degenerating motor neurons in the lumbar spinal cord of hSOD1^{G93A} rats (Woodruff et al., 2008a). Furthermore, CD88 antagonist PMX205 treatment in hSOD1^{G93A} rats reduced motor deficits and extended survival, indicating a pathogenic role for CD88 in ALS (Woodruff et al., 2008a). Taken together, these combined studies suggest that C5a has an important role in neuronal death and its activation could exacerbate the disease progression of neurodegenerative diseases such as ALS.

1.8 Clinical evidence of complement involvement in ALS

Several studies have been conducted on ALS patients in an attempt to identify whether complement components are up-regulated in disease progression (**Table 1.1**). It has been proposed that the classical complement pathway is involved in the pathophysiology of ALS, as studies have shown that activation fragments of complement components C3 and C4 are increased in the serum, cerebrospinal fluid (**CSF**), and neurological tissue (including spinal cord and motor cortex) of ALS patients (Annunziata and Volpi, 1985, Apostolski et al., 1991, Goldknopf et al., 2006, Kawamata et al., 1992, Trbojevic-Cepe et al., 1998). The first of these studies examined C3 immunofluorescence in spinal cord and motor cortex of 16 ALS patients and demonstrated significant C3 deposition, which appeared to be on astrocyte-like cells with no apparent neuronal staining (Donnenfeld et al., 1984). Subsequent studies measured C3c, C4, C1 inactivator and C3 activator fractions in the serum and CSF of 13 ALS patients but only detected increased levels of C3c in the CSF of ALS patients compared to normal individuals (Annunziata and Volpi, 1985). Furthermore Apopstolski and colleagues (1991) measured serum C4, C3 and Factor B levels in 33 ALS patients and found an increase in C4 levels when compared to normal individuals (Apostolski et al., 1991). Increased clusters of C3d and C4d coated fibers on oligodendroglia and degenerating neurites in spinal cord and motor cortex was also found in 8 ALS patients compared to 5 normal individuals (Kawamata et al., 1992). Two separate studies also investigated C1q, C4d and C4 levels in the serum and CSF of ALS patients and found C4d levels significantly increased in 15 ALS patients which also correlated with disease severity (Tsuboi and Yamada, 1994); another study also detected up-regulation of C4 in ALS patients (Trbojevic-Cepe et al., 1998). Studies by Grewal and colleagues (1999) and Jiang and colleagues (2005) have identified increased mRNA of upstream classical pathway complement components (C1q and C2) in the spinal cord of ALS patients (Grewal et al., 1999, Jiang et al., 2005). Recently, Sta and colleagues (2011) have found increased levels of C1q, C3c, C3d and C5b-9 in the spinal cord and motor cortex of ALS patients compared to normal individuals (Sta et al., 2011). The expression of these complement components was observed in glial cells rather than neurons (Sta et al., 2011). Lastly, complement component C3 was also found to be up-regulated in the CSF of 71 ALS patients when compared to 40 normal individuals (Ganesalingam et al., 2011).

These findings of up-regulated complement components and activation fragments, predominantly composing the classical pathway, in the serum, CSF, and neurological tissue in ALS patients strongly suggest that the classical complement pathway may be involved in the progression of disease in ALS. However it is currently unknown where these complement factors originate, and what initiates their activation. Complement factors can be produced by various cells within the CNS suggesting local production in response to disturbance in CNS homeostasis due to immunoglobulin deposits and auto-antibodies in the CNS of ALS patients (Donnenfeld et al., 1984, Niebroj-Dobosz et al., 2006). Also the circulation could be a source of these complement factors as there is BBB breakdown in the end stages of ALS (Apostolski et al., 1991). Overall, evidence from these clinical studies helps us to propose that complement system activation occurs in ALS patients, and may play a role in the disease pathology. This is also supported by evidence of studies showing involvement of complement factors in animal models of ALS.

Table 1.1 Clinical evidence of complement activation in ALS patients

Complement factors	mRNA/Protein	Sample	Methods
C3	Protein	Spinal Cord, Motor cortex	Immunofluorescence
C3c	Protein	Serum, CSF	Single radial immuno-diffusion
C4	Protein	Serum	Single radial immuno-diffusion
C3d, C4d	Protein	Spinal Cord, Motor cortex	Immunohistochemistry
C4d	Protein	CSF	Sandwich ELISA
C4	Protein	CSF	Laser nephelometry
C1q	mRNA	Spinal Cord, Motor cortex	Northern blot, *In situ* hybridization
C2	mRNA	Spinal Cord	Microarray
C3c, C3dg, Factor H	Protein	Serum	2D gel electrophoresis
C1q, C3c, C3d, C5b-9	mRNA/Protein	Spinal Cord, Motor cortex	qPCR, immunohistochemistry
C3	Protein	CSF	Sandwich ELISA

1.9 Experimental evidence of complement involvement in ALS

Many studies in animal models of ALS have shown the involvement of the complement system during disease progression, supporting findings in ALS patients (**Table 1.2**). Although the SOD1 gene mutation only accounts for 2% of total ALS cases, mouse models carrying over-expression of mutant human SOD1 enzyme are widely used, as it leads to progressive symptoms, which are very similar to the human condition.

The first study to demonstrate experimentally the involvement of complement factors in a SOD1 transgenic mouse model was performed by Perrin and colleagues in 2005. They isolated ventral motor neurons from the lumbar spinal cord of $hSOD1^{G93A}$ transgenic mouse using laser-capture micro-dissection and then using microarray analysis they detected increased levels of all subcomponents of C1q in these mice at early symptomatic and end stage when compared to motor neurons from wild-type (**WT**) mice (~5 and ~8 fold respectively) (Perrin et al., 2005).

Subsequent studies in two distinct hSOD1 transgenic mouse models also used laser-capture micro-dissection to isolate lumbar motor neurons from $hSOD1^{G37R}$ and $hSOD1^{G85R}$ transgenic mice which showed up-regulation of genes for all three C1q subcomponents when compared to $hSOD1^{WT}$ mice 2 months prior to clinical onset (Postnatal (**P**) 105 days) (Lobsiger et al., 2007). In addition, this group demonstrated that the complement regulatory molecule, DAF also decreased at this time point (Lobsiger et al., 2007). Furthermore they showed that C1q protein was expressed by motor neurons using immunohistochemistry on spinal cord sections of both $hSOD1^{G37R}$ and $hSOD1^{G85R}$ transgenic mice but absent in the age-matched control mice (Lobsiger et al., 2007).

A separate group also used laser-capture micro-dissection to isolate the lumbar motor neurons from $SOD1^{G93A}$ transgenic mice. Using microarray analysis and real time quantitative PCR, they showed there were increased levels of C1q (subcomponent B) and C4 mRNA at disease onset (P90) and late-stage disease (P120) (~7 and ~8 fold respectively) (Ferraiuolo et al., 2007). A similar study also used microarray analysis in a separate hSOD1 transgenic mouse model using whole lumbar spinal cord homogenate (Fukada et al., 2007). This study used $hSOD1^{L126delTT}$ transgenic mice and showed elevated levels of C1q (subcomponent B) mRNA in post-symptomatic (P154) mice compared to WT mice. A very recent study has shown increased levels of C1q in the neuromuscular junction of $hSOD1^{G93A}$ transgenic mice compared to WT mice (Heurich et al., 2011).

By contrast to the above studies, which indicates a role for the classical complement pathway in the progression of pathology of the hSOD1 transgenic mouse, recent studies have demonstrated that

when hSOD1 transgenic mice were bred onto a background deficient in complement C1q, C3 and C4, there was no difference in the onset of motor symptoms and survival when compared to hSOD1 transgenic mice (Chiu et al., 2009, Lobsiger et al., 2013). These studies indicate that blocking upstream complement activation pathways does not alter the disease course in hSOD1 transgenic mice. It is possible instead, that downstream complement factors, potentially generated by the fourth "extrinsic pathway", may play a role in immune activation and macrophage recruitment in these mice.

To further validate the involvement of downstream components of the complement cascade in the disease progression of ALS, up-regulation of C5a receptor CD88 mRNA and protein was observed in mice deficient in the low molecular weight neurofilament (**NFL**) subunit protein, a mouse model of motor neuron degeneration in which neurofilament aggregates in a similar fashion to that in ALS patients (Humayun et al., 2009). This study showed that there was a 4 and 3 fold increase in CD88 mRNA expression level at 2 and 3 months respectively, a time which is early in the disease process (Humayun et al., 2009). There was also an increased immunoreactivity of CD88 in motor neurons of NFL deficient mice when compared to WT mice at 3, 4 and 5 months. Our own findings also support a pathogenic role for C5a in ALS (Woodruff et al., 2008a). Chronic administration of a specific C5a receptor antagonist, developed in our laboratories (Wong et al., 1998) in hSOD1^{G93A} transgenic rats, markedly delayed the onset of motor symptoms and increased survival, compared to untreated animals (Woodruff et al., 2008a). We also showed up-regulation of CD88 in the lumbar spinal cord of hSOD1^{G93A} transgenic rats, which increased as disease progressed (Woodruff et al., 2008a).

These findings of up-regulated complement components in different animal models of ALS suggest that the activation of complement system is critically linked with disease progression in ALS. Whilst inhibition of classical, lectin and alternative complement pathways (C1q, C3 and C4) failed to ameliorate disease in hSOD1 transgenic mice, inhibition of the classical receptor for C5a, CD88, reduced disease pathology and extended survival in hSOD1^{G93A} transgenic rats. It should be noted that C5a is expressed following activation of all complement pathways (**Figure 1.1**), and can also be generated in the absence of C3 and other upstream complement factors (Huber-Lang et al., 2006). Hence inhibiting central components of the complement system, at the C5 level, may have benefits in slowing disease progression in ALS, as opposed to inhibiting an individual activation pathway. Specifically, our studies suggest that inhibiting the pro-inflammatory C5 activation fragment, C5a, which is central to, and generated by, all complement pathways, may be a novel therapeutic strategy to treat ALS.

Table 1.2 Experimental evidence of complement activation in animal models of ALS

Complement factors	mRNA/Protein	Transgenic model	Reference
C1q	mRNA	Mouse SOD1 ^{G93A}	(Perrin et al., 2005)
C1q, DAF	mRNA	Mouse SOD1 ^{G37R} and SOD1 ^{G85R}	(Lobsiger et al., 2007)
C1q, C4	mRNA/Protein	Mouse SOD1 ^{G93A}	(Ferraiuolo et al., 2007)
C1q	mRNA	Mouse SOD1 ^{L126delTT}	(Fukada et al., 2007)
CD88	mRNA/Protein	Rat SOD1 ^{G93A}	(Woodruff et al., 2008a)
CD88	mRNA/Protein	Mouse NFL -/-	(Humayun et al., 2009)
C1q, C3	mRNA/Protein	Mouse SOD1 ^{G93A}	(Heurich et al., 2011)

1.10 Rationale, Hypothesis and Aims

There is increasing evidence that implicates the involvement of the innate immune system in the progression of ALS. In particular, the inappropriate activation or dysregulation of the complement system may play a role in ALS pathology. Evidence for this includes elevated levels of complement activation fragments in the serum, CSF, spinal cord and motor cortex of ALS patients. This has also been supported with elevated levels of complement activation fragments in various animal models of ALS. Moreover, inhibition of the C5a receptor using a specific C5a receptor antagonist ameliorated disease symptoms in a rat model of ALS. Collectively, these studies suggest that complement activation at the level of C5, may play a crucial role in the progression of ALS. Hence reducing complement-induced inflammation using inhibitors to target the C5-C5a receptor axis, could be an important therapeutic strategy to treat ALS.

Based on the current knowledge of complement activation in ALS, I hypothesised that downstream complement components including CD88 and C3aR may be the key point at which complement mediated neurotoxicity occurs in the hSOD1^{G93A} ALS mouse model. More specifically I propose that inhibition of CD88 will have improved disease symptoms and extended survival whereas inhibition of C3aR will have worsened disease symptoms with reduced survival. In order to investigate these hypotheses I aimed to:

1) Examine the expression and cellular localisation of C3, C5 and their receptors C3aR and CD88 in hSOD1^{G93A} mice during disease progression,

2) Elucidate the function and mechanism of CD88 using the CD88 selective antagonist PMX205 in hSOD1^{G93A} mice and follow these pharmacological studies by utilising hSOD1^{G93A} mice lacking CD88.

3) Understand the role of C3aR in the disease progression of ALS using hSOD1^{G93A} mice lacking C3aR.

CHAPTER 2

"Methods"

2.1 Ethical Statement

All experimental procedures were approved by the University of Queensland Animal Ethics Committee (Permit Number: 227-09, 433-12, 047-11, 033-12), and complied with the policies and regulations regarding animal experimentation and other ethical matters (Drummond and Sorenson, 2009). They were conducted in accordance with the Queensland Government Animal Research Act 2001, associated Animal Care and Protection Regulations (2002 and 2008), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition (National Health and Medical Research Council, 2004). ARRIVE guidelines have been followed in the preparation of this thesis.

2.2 Animals

Transgenic hSOD1^{G93A} mice (B6-Cg-Tg (SOD1-G93A) 1Gur/J) were obtained from Jackson laboratory (Bar Harbor, ME, USA). Homozygous C5aR deficient (C5aR$^{-/-}$) and C3aR deficient (C3aR$^{-/-}$) mice were kindly provided by Dr. Wetsel and described previously (Hollmann et al., 2008, Kildsgaard et al., 2000). These animals were maintained at the University of Queensland Biological Resources Animal Facilities under specific pathogen free conditions. All animals were housed in a 12h light/dark cycle with free access to food and water.

hSOD1^{G93A} transgenic mice express high levels of the human SOD1 mutant protein (~25 copies)(Alexander et al., 2004), in which glycine residue is replaced with an alanine residue at position 93 (Gurney, 1994). The SOD1 mutation causes a toxic gain-of-function rather than diminished SOD1 activity (Wong et al., 1995). It is considered to be a reliable model for human ALS as transgenic mice carrying mutant forms of SOD1 develop progressive muscle deficits very similar in phenotype (tremor and eventual paralysis) and histopathological hallmarks (selective grey matter degeneration) to human ALS (Gurney, 1997). hSOD1^{G93A} mice at four predefined stages of ALS were used throughout the thesis (**Table 2.1**). By the end stage of ALS, hSOD1^{G93A} mice display significant signs of hind-limb weakness, paralysis and loss of the righting reflex.

To generate hSOD1^{G93A} mice lacking C5aR (hSOD1^{G93A} x C5aR$^{-/-}$) and C3aR (hSOD1^{G93A} x C3aR$^{-/-}$), transgenic heterozygous hSOD1^{G93A} male were first cross-bred with C5aR$^{-/-}$ and C3aR$^{-/-}$ females to generate F1 progeny (hSOD1^{G93A} x C5aR$^{+/-}$ and hSOD1^{G93A} x C3aR$^{+/-}$). hSOD1^{G93A} x C5aR$^{+/-}$

and hSOD1^{G93A} x C3aR$^{+/-}$ males were cross bred with C5aR$^{-/-}$ and C3aR$^{-/-}$ females to obtain F2 progeny (hSOD1^{G93A} x C5aR$^{-/-}$ and hSOD1^{G93A} x C3aR$^{-/-}$) (**Figure 2.1**).

All mice were anaesthetized with intraperitoneal injection of zolazapam (50mg/kg; Zoletil, Lyppard, Australia) and xylazine (10mg/kg; Xylazil, Lyppard, Australia) prior to the collection of tissue samples.

Table 2.1: Different stages defined in ALS

Stage	Age	Phenotype
Pre-symptomatic	30 days postnatal	No signs of motor deficit
Onset	70 days postnatal	Initial signs of motor deficit (grip strength)
Mid-symptomatic	130 days postnatal	Weakness in hind-limb and tremor when suspended by the tail
End	150 to 175days postnatal	Full paralysis of lower limbs and loss of righting reflex

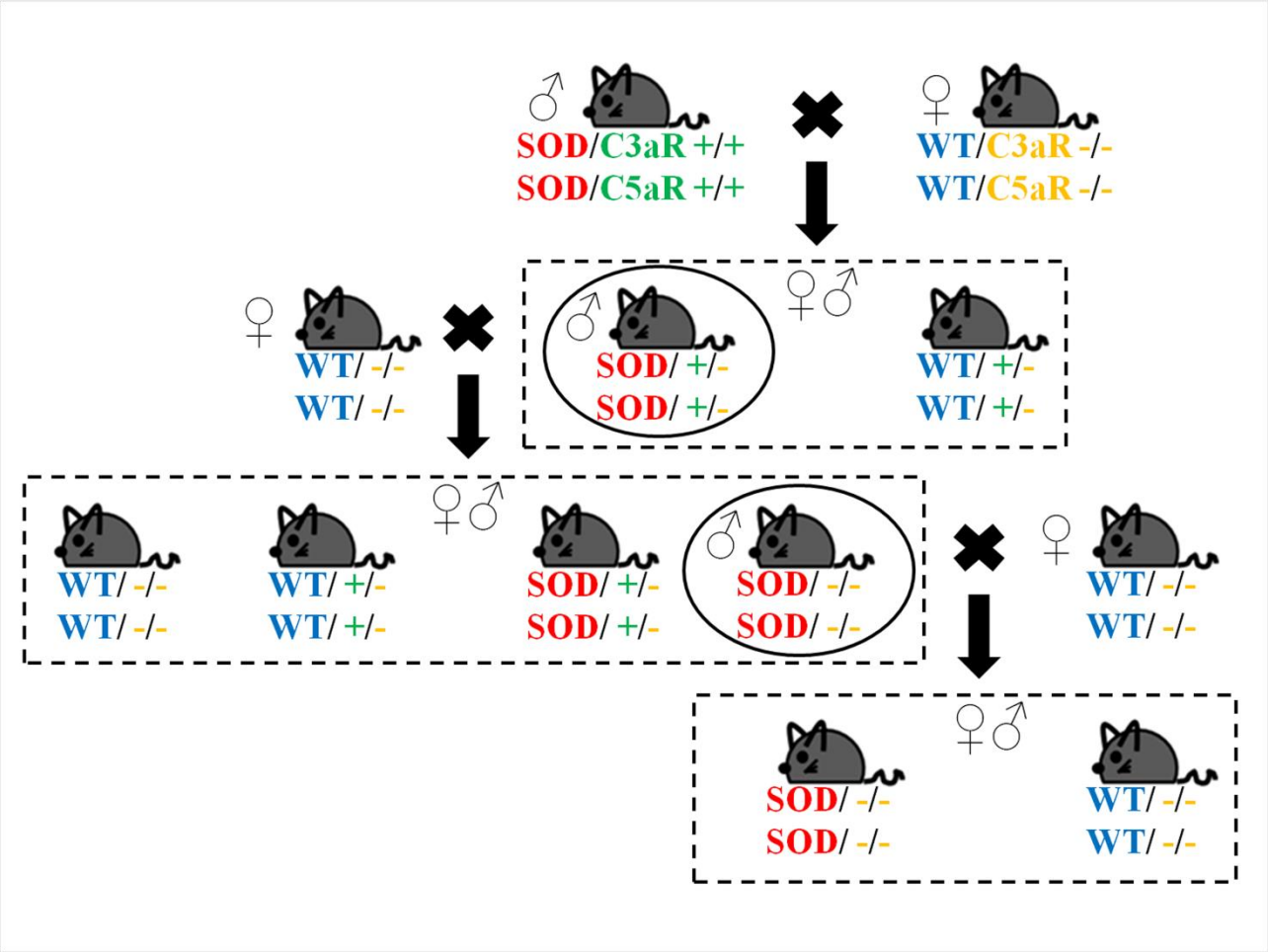

Figure 2.1: The breeding scheme to obtain hSOD1^{G93A} mice lacking C5aR and C3aR. *To generate hSOD1^{G93A} mice lacking C5aR (hSOD1^{G93A} x C5aR$^{-/-}$) and C3aR (hSOD1^{G93A} x C3aR$^{-/-}$), transgenic heterozygous hSOD1^{G93A} male were first cross-bred with C5aR$^{-/-}$ and C3aR$^{-/-}$ females to generate F1 progeny (hSOD1^{G93A} x C5aR$^{+/-}$ and hSOD1^{G93A} x C3aR$^{+/-}$). hSOD1^{G93A} x C5aR$^{+/-}$ and hSOD1^{G93A} x C3aR$^{+/-}$ males were cross bred with C5aR$^{-/-}$ and C3aR$^{-/-}$ females to obtain F2 progeny (hSOD1^{G93A} x C5aR$^{-/-}$ and hSOD1^{G93A} x C3aR$^{-/-}$).*

2.3 Drug Treatment and experimental design

2.3.1 PMX205

PMX205 (hydrocinnamate-[OP (D-Cha) WR]) is an orally active cyclic hexapeptide developed in our laboratory at the University of Queensland (March et al., 2004). This compound is based on the linear C5aR antagonist, Me-FKPdChaWR and the eventual cyclisation to induce structural and metabolic stability (March et al., 2004). This drug has been shown to reduce pathology in numerous rodent models of inflammatory disease including rheumatoid arthritis (Woodruff et al., 2002), ischemic reperfusion injuries (Arumugam et al., 2004) and inflammatory bowel disease (Woodruff et al., 2003), as well as acute neurotrauma (Beck et al., 2010). PMX205 is more lipophilic than the previous C5aR antagonist PMX53, which results in increased CNS penetrance across the blood brain barrier (Woodruff et al., 2006). Hence, it has been shown to be more efficacious to reduce disease severity and prolong survival in animal models of neural degeneration (Woodruff et al., 2008a, Fonseca et al., 2009, Ager et al., 2010, Woodruff et al., 2006).

2.3.2 Drug Regime

PMX205 was administered orally in drinking water at a dose of 3mg/kg/day and 9mg/kg/day. Litter-matched female hSOD1^{G93A} transgenic mice were administered PMX205 from 35 days postnatal, which is approximately 35 days before initial motor deficit symptoms hence termed "Pre-Onset". PMX205 was also administered from 91 days postnatal at 9mg/kg/day which is approximately 21 days after initial motor deficit symptoms hence termed "Post-Onset". These drug treatments were continued till end-stage of disease progression in these mice. These treatments were performed blinded to the researcher.

2.4 Disease progression and survival analysis

The rate of disease progression in all these mice was determined by age at which maximal grip strength decline at 25, 50, 75 and 100% throughout disease progression. Survival (or rather "death") was determined by the inability of the animal to right itself within 30 seconds if laid on either side. This is a widely accepted and published endpoint for life span studies in ALS mice and guarantees that euthanasia occurs prior to the mice being unable to reach food or water (Ludolph et al., 2007, Scott et al., 2008).

2.5 Weight Measurements and Behavioural Tests

All mice (including hSOD1^{G93A}, wild-type (**WT**), hSOD1^{G93A} x C3aR$^{-/-}$ and C3aR$^{-/-}$) were weighed weekly at the same time of day (4 p.m.), from 42 days of age until end-stage when they lose their

righting reflex. Two neuromotor tests the rota-rod and hind-limb grip strength test, were conducted on these mice (detailed below). These tests were performed blinded to the genotype.

2.5.1 Rota-rod Test

Mice were tested for their motor coordination from 42 days of age using a Rota-rod apparatus (Ugo Basile Rota-rod, Italy) at a constant speed of 20 rpm. Each mouse was given three attempts and the longest latency to fall was recorded; 180s was chosen as the arbitrary cut off time. One week prior to the test, mice were trained twice to remain on the Rota-rod apparatus to exclude differences in motivation and motor learning. In the training phase, mice were placed on the Rota-rod at a constant speed of 20 rpm for a maximum duration of 240s (Zhou et al., 2007).

2.5.2 Hind-limb grip strength Test

A digital force gauge (Ugo Basile) was used to measure maximal muscle grip strength. The mice were held by their tail and lowered until the mice grasped the T-bar connected to the digital force gauge with their hind limbs. The tail is lowered until the body is horizontal and the mice are pulled away from the T-bar with a smooth steady pull until both of their hind limbs released the bar. The digital force gauge will remain fixed at its maximum deflection and the strength of the grip was measured in gram force. Each mouse was given ten attempts and the maximum grip strength was recorded.

2.6 Immunohistochemistry

hSOD1^{G93A} and WT mice were fixed by transcardiac perfusion with 2% sodium nitrite buffer (Ajax Finechem Pty Ltd, Australia) followed by 4% paraformaldehyde (**PFA**; Sigma, MO, USA) in 0.1M Phosphate buffer pH7.4 (**PB**). The lumbar spinal cords were collected and then placed in 4% PFA for another 2 hours at 4°C. The lumbar spinal cords were embedded in OCT (Sakura, CA, USA) and snap frozen in liquid nitrogen. Serial transverse cryosections (16µm) were collected on Superfrostplus slides (Menzel-Glaser, Braunschweig, DEU) for estimation of motor neuron numbers and fluorescence immunohistochemistry.

For the motor neuron numbers in the spinal cord, the sections are stained using 0.1% thionin (v/v) in acetic acid buffer solution (Sigma) for 3 minutes. The sections were rinsed in distilled water before being mounted with xylene-soluble mounting medium (ProSciTech, Australia). The lumbar lateral motor column (**LMC**) extending from the 2nd lumbar dorsal root ganglia (**DRG**) to the 6th lumbar DRG was selected from our serial spinal sections, with the aid of mouse brain atlases (Watson, 2009). α - motor neurons within the lumbar LMC were identified as a large motor neurons

that showed a dark stained cytoplasm, a distinct pale nucleus and dark stained nucleoli (Banks et al., 2001, Banks et al., 2005, Clarke and Oppenheim, 1995). They were counted on both sides of the spinal cord in every tenth sections. The total number of motor neurons counted was divided by the number of sections counted and multiplied by the total number of sections containing the lumber LMC. The mouse genotypes were not made available to the researcher conducting the counts until it was completed.

Fluorescence double labelling was performed to localise the expression of C1q, C3b, C3, C5 and its receptors C3aR and CD88 with specific cell-type markers for motor neurons (choline acetyltransferase (**ChAT**)), astrocytes (glial fibrillary acidic protein (**GFAP**)) and microglia (ionised calcium binding adaptor molecule 1 (**Iba-1**) and cluster of differentiation molecule 11b (**CD11b**)). All sections were rehydrated in phosphate buffered saline pH 7.4 (**PBS**) and blocked in PBS containing 3% bovine serum albumin (**BSA**) or 3% donkey serum and 1% BSA for 1 hour at room temperature. Next, sections were incubated overnight at 4°C with a combination of antibodies outlined in **table 2.2**. All primary antibodies were diluted in PBS containing 1% BSA or 1% donkey serum. These sections were then washed 3 x 10 mins with PBS prior to incubation with an appropriate Alexa conjugated secondary cocktail: Alexa 555 goat anti-rat, Alexa 555 goat anti-mouse, Alexa 555 goat anti-chicken, Alexa 594 donkey anti-rat, Alexa 555 donkey anti-mouse, Alexa 555 donkey anti-chicken, Alexa 488 goat anti-mouse, Alexa 488 goat anti-rabbit, Alexa 488 goat anti-rat and Alexa 488 donkey anti goat. All secondary antibodies were diluted in PBS containing 1% BSA or 1% donkey serum (1:1000 for Alexa 555/594 and 1:600 for Alexa 488). Following 3 x 5min washes in PBS, the sections were incubated for 5min in 4, 6-diamidino-2-phenylindole (Life Technologies, USA). All sections were mounted with Prolong Gold Anti-Fade medium (Invitrogen). IgG negative controls (mouse IgG2a, Serotec; rat IgG2a, Serotec; chicken IgY, Promega) were used in place of primary antibodies to give a measure of non-specific background staining. These IgG and IgY control antibodies were used at the same concentrations and were of the same species to the primary antibodies listed above. Images were taken with a Zeiss LSM Meta 510 upright confocal microscope using a Plan-Apochromat 63x oil objective (Carl Zeiss Inc; Oberkochen, Germany).

Quantification of immunofluorescence for C1q and C3b was performed on ~ 25 to 35 lumbar spinal cord sections (per animal; n=3) spaced 160µm apart and expressed as percentage immuno-reactive area per section. Staining procedures and image exposures were all standardized between genotype and between sections.

Table 2.2 Summary of antibodies used for immunohistochemistry

Antibody	Manufacturer	Dilution	In combination with
Rat anti-mouse C1q	Hycult Biotechnology	1:1,000	GFAP, Iba-1, ChAT
Rat anti-mouse C3b	Hycult Biotechnology	1:50	GFAP, Iba-1, ChAT
Mouse anti-mouse C5	Hycult Biotechnology	1:1,000	GFAP, CD11b, ChAT
Rat anti-mouse CD88	AbD Serotec	1:250	GFAP, Iba-1, ChAT
Rat anti-mouse C3	Hycult Biotechnology	1:50	GFAP, Iba-1, ChAT
Chicken anti-mouse C3aR	BMA Biomedicals	1:1,000	GFAP, Iba-1, ChAT
Mouse anti-mouse GFAP	BD Biosciences	1:1,000	C1q, C3b, C5, CD88, C3, C3aR
Rabbit anti-mouse Iba-1	Wako	1:400	C1q, C3b, CD88, C3, C3aR
Rat anti-mouse CD11b	Abcam	1:300	C5
Goat anti-mouse ChAT	Chemicon	1:100	C1q, C3b, C5, CD88, C3, C3aR

2.7 Real time quantitative PCR

Lumbar spinal cords from all the mice (including hSOD1^{G93A}, WT, hSOD1^{G93A} x C5aR$^{-/-}$, C5aR$^{-/-}$, hSOD1^{G93A} x C3aR$^{-/-}$ and C3aR$^{-/-}$) were collected into RNA*later* (Ambion, NY, USA) and stored at -20°C for subsequent quantitative PCR analysis. Total RNA was isolated using RNeasy Lipid Tissue extraction kit according to manufacturer's instructions (QIAGEN, CA, USA). After the total RNA was purified using Turbo DNAse treatment (Ambion), cDNA was synthesized using Stratagene RT kit (Agilent Technologies, CA, USA). Commercially available gene specific TaqMan probes and SYBR green (Applied Biosystems, USA) were used to amplify target gene of interest. All probes and primers used are listed in **table 2.3** and **2.4** respectively. Relative target gene expression to glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) was determined using this formula: $2^{-\Delta CT}$ where ΔCt = (Ct target gene – Ct GAPDH) (Livak and Schmittgen, 2001). Final measures are presented as relative levels of gene expression in hSOD1^{G93A}, hSOD1^{G93A} x C5aR$^{-/-}$, C5aR$^{-/-}$, hSOD1^{G93A} x C3aR$^{-/-}$ and C3aR$^{-/-}$ mice compared with expression in WT controls.

Table 2.3: Taqman probes used for quantitative PCR

Gene of interest	Catalogue number
C1qB	Mm01179619_m1
C4	Mm00437896_g1
Factor B	Mm00433909_m1
C3	Mm01232779_m1
CD55	Mm00438377_m1
CD59a	Mm00483149_m1
C5	Mm00439275_m1
CD88	Mm00500292_s1
C3aR	Mm01184110_m1
Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Mannose binding lectin 1	Mm00495413_m1
Mannose binding lectin 2	Mm00487623_m1
Tumour Necrosis Factor	Mm00443258_m1
Interleukin-1β	Mm00434228_m1
Entpd1	Mm00515447_m1
Ly6C 1/2	Mm03009946_m1

Table 2.4: List of primers used for SYBR Green quantitative PCR

Gene of interest	Primer Sequence	Product Length
Iba-1	Forward: 5` - ACAGCAATGATGAGGATCTGC - 3`	146
	Reverse: 5` - CTCTAGGTGGGTCTTGGGAAC - 3`	
GFAP	Forward: 5` - TCCTGGAACAGCAAAACAAG - 3`	224
	Reverse: 5` - CAGCCTCAGGTTGGTTTCAT - 3`	
GAPDH	Forward: 5` - CCAGGAGCGAGACCCCACTAACA - 3`	150
	Reverse: 5` - TCGGCAGAAGGGGCGGAG - 3`	

2.8 Western blot Analysis

Lumbar spinal homogenates from hSOD1^{G93A} and WT mice were resolved on a 10% sodium dodecyl sulphate polyacrylamide gel and electro transferred onto nitrocellulose membranes. Membranes were blocked in 2.5% skim-milk-Tris buffered solution- 0.1% Tween 20 (**TBS-T**) for CD88 and 5% BSA-TBS-T for CD55 and incubated overnight with one of the following antibodies; anti-CD88 (1:1000; BMA Biomedical, Switzerland), or anti-CD55 (1:1000; Hycult Biotechnology, Netherlands). All primary antibodies were diluted in 5% BSA-TBS-T. Anti-CD88 was detected with goat anti-chicken horseradish peroxidase (**HRP**; 1:15,000; GE Healthcare, PA, USA) and anti-CD55 was detected with goat anti-rat HRP (1:10,000; GE Healthcare). These secondary antibodies were detected by enhanced chemiluminescence (Amersham, PA, USA). Blots were stripped and re-probed with anti-GAPDH, (1:15,000; Millipore, MA, USA) and detected with sheep anti-mouse HRP (1:4,000; GE Healthcare) to ensure equal protein loading. Densitometric analyses of immuno-reactive bands were performed by deducting background pixels from grayscale pixel density of bands multiplied by its area value using Image J software (Abramoff, 2004). The integrated pixel value for each band was normalized to its corresponding anti-GAPDH band. The normalized integrated pixel values of hSOD1^{G93A} bands were compared to WT bands.

2.9 *In situ* hybridisation

hSOD1^{G93A} transgenic mice and WT mice were euthanized by cervical dislocation. The lumbar spinal cord were collected and then fixed overnight in 4% PFA in 1 x PBS at 4°C. Following this incubation, lumbar spinal cord was washed 3 x 5min in 1 x PBS , followed by submersion overnight in sucrose solution at 10% and 25% in 1 x PBS . The lumbar spinal cords was embedded in a cryoprotectant mounting medium (optimal cutting temperature; OCT, Sakura Fineteck, USA) and snap frozen in liquid nitrogen. Lumbar spinal cords were sectioned using a cryostat (Leica 3050n) into 16μm thick transverse sections and dry mounted onto a Superfrost (+) slides for *in situ* hybridization detailed below.

Digoxygenin (**DIG**)-labelled RNA probes will be generated from PCR derived templates using T3 and T7 RNA polymerases by adding T3 (5’ – AATTAACCCTCACTAAAGGG – 3’) and T7 (5’ – TAATACGACTCACTATAGGG – 3’) RNA polymerase promoter sequences to the primers specific for CD88;Forward –TAATACGACTCACTATAGGGATCATCTACTCGGTGGTGTTCC and Reverse - AATTAACCCTCACTAAAGGGGAGAGACCTTAGGAGTCGTCCA. A standard PCR is performed and the PCR product is run on a 1% agarose gel. The band corresponding to my gene of interest will be excised and purified using Min-elute gel extraction kit (Qiagen) according

to manufacturer's instructions. DIG – labelled RNA probes will be synthesized using DIG RNA labelling mix (Roche) according to manufacturer's instructions.

Sections will be re-hydrated in 1 x PBS for 5 minutes and post fixed in 4% PFA in 1 x PBS for 15 minutes and treated with Proteinase K (30µg/ml) for 10 minutes at room temperature. Sections will be acetylated for 10 minutes (acetic anhydride, 0.25%; Sigma) and hybridized with DIG-labelled probes overnight at 65°C. Hybridisation buffer contains 1 x salts (200mM sodium chloride, 13mM tris, 5mM sodium phosphate monobasic, 5mM sodium phosphate dibasic, 5mM EDTA), 50% formamide, 10% dextran sulfate, 1mg/ml yeast tRNA (Roche), 1 x Denhardt's (1% w/v bovine serum albumin, 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone) and DIG-labelled probe (1:2000). Sections will be washed in post-hybridisation buffer (1 x SSC, 50% formamide, 0.1% Tween-20) for 2 x 10 minutes at 65°C followed by 2 x 15 minutes washes in 1 x MABT (150mM sodium chloride, 100mM maleic acid, 0.1% Tween-20, pH 7.5) at room temperature, then will be followed by RNAse treatment (400mM sodium chloride, 10mM tris pH 7.5, 5mM EDTA, 20ug/ml RNAse A) for 30 minutes at 37°C. Sections will be blocked in blocking solution (1 x MABT, 2% blocking reagent (Roche), 20% hear inactivated goat serum) for 1 hour at room temperature and incubated overnight at 4°C with anti-DIG antibody (1:2500 in blocking solution). Sections will be then washed in 1 x NTMT (100mM sodium chloride, 50mM magnesium chloride, 100mM tris pH 9.5, 0.1% Tween-20), followed by incubation in NBT/BCIP in 1 x NTMT according to manufacturer's instructions (Promega). Slides will be counterstained with nuclear fast, dehydrated and cleared in xylene and mounted in cytoseal mounting medium (VWR).

2.10 ELISA

96-well plates (Greiner Bio-One, Frickenhausen, Germany) were pre-coated with monoclonal rat anti-mouse C5a capture antibody (Clone I52 – 1486; BD Pharmingen, San Diego, CA, USA) diluted in coating buffer (100µM, $NaHCO_3$, 34µM Na_2CO_3, pH 9.5) overnight at 4°C in a sealed humidified container. This capture antibody is specific for a neo-epitope exposed only in mouse C5a/C5a desArg and does not cross-react with C5 (Pavlovski et al., 2012, Wu et al., 2013). Following the plate being blocked for 1 hour at room temperature with assay diluent (10% FCS/PBS), C5a standard and lumbar spinal cord homogenates was incubated for 2 hours at room temperature. The plates were subsequently incubated with biotinylated rat anti-mouse C5a detection antibody (clone I52-278; BD Pharmingen) for 1 hour at room temperature, and then incubated with Streptavidin-HRP conjugate for 30 minutes at room temperature. Tetramethylbenzidine (Sigma) substrate was used as the chromogen and the plate was read at 450nm. Levels of C5a in lumbar

spinal cord samples were adjusted to micrograms per protein and expressed as nanograms of C5a per microgram of protein.

2.11 Statistical Analysis

All measures were performed using GraphPad Prism 5.0 (San Diego, CA, USA). The statistical differences between different cohort of animals for body weight, rota-rod test and hind-limb grip strength test, were analysed using two-tailed Student's *t*-test at each time point. For the results from motor neuron counts, quantitative real time PCR, western blotting, ELISA, statistical differences between different cohorts of animals was analysed using two-tailed *t*-test at each stage of disease progression. All data are presented as mean ± SEM and differences were considered significant when $P \leq 0.05$.

CHAPTER 3

"Dysregulation of the complement cascade in the hSOD1^{G93A} transgenic mouse model of amyotrophic lateral sclerosis"

*** This chapter was published in Journal of Neuroinflammation** - **John D Lee**, Nur A Kamaruzaman, Jenny NT Fung, Stephen M Taylor, Bradley J Turner, Julie D Atkin, Trent M Woodruff and Peter G Noakes (2013) Dysregulation of complement cascade in the hSOD1^{G93A} transgenic mouse model of amytrophic lateral sclerosis. *Journal of Neuroinflammation*. 10:119

3.1 INTRODUCTION

Amyotrophic lateral sclerosis (**ALS**) is a late onset neurodegenerative disorder that is characterized by selective loss of upper motor neurons within the motor cortex, and α-motor neurons of the spinal cord and brainstem (Bruijn et al., 2004). This results in symptoms of muscle weakness and atrophy of skeletal muscles, leading to paralysis and eventual death due to failure of respiratory muscles (Cozzolino et al., 2008). The mechanisms leading to ALS are still unclear but there are compelling data that suggest neuro-inflammation may contribute to the disease progression of ALS (Bruijn et al., 2004, Woodruff et al., 2008b, Lee et al., 2012). These include the presence of reactive microglia and astrocytes, infiltration of T lymphocytes and up-regulation of cyclooxygenase 2 and prostaglandin E_2 in the spinal cord of ALS patients and animal models (Almer et al., 2001, Almer et al., 2002, McGeer and McGeer, 2002, Henkel et al., 2013, Zhao et al., 2012). The classical complement system is also implicated in ALS pathology, as studies have shown activation fragments of complement components C1q, C3 and C4 are increased in the serum, cerebrospinal fluid and neurological tissue (including spinal cord and motor cortex) of ALS patients (Lee et al., 2012).

In addition to evidence suggesting complement involvement in human ALS pathology, several studies have demonstrated the involvement of complement factors in animal models of ALS. The up-regulation of the classical pathway complement components C1q and C4, as well as of the central factor C3, has been shown in human SOD1 transgenic rodent models of ALS (Lee et al., 2012). Other studies have also shown the up-regulation of the major pro-inflammatory C5a receptor, CD88, during disease progression (Humayun et al., 2009, Woodruff et al., 2008a). In addition, our group has shown that chronic administration of a specific CD88 antagonist in hSOD1^{G93A} transgenic rats delayed the onset of motor symptoms and increased survival compared to untreated animals (Woodruff et al., 2008a). Overall, these studies indicate that over-activation of

complement system, and increased C5a-CD88 signalling contributes to the progression of disease in these animal models of ALS.

In the present study, we examined the expression and cellular location of major complement factors and regulators during defined disease stages in hSOD1^{G93A} mice in order to provide a comprehensive overview of complement's involvement in ALS. Additionally, given the importance of C5a in disease pathology in ALS models (Humayun et al., 2009, Woodruff et al., 2008a) we also examined mRNA, protein levels, and the cellular localisation of C5, C5a and its cognate receptor, CD88 during disease progression. Our findings demonstrate a global dysregulation of complement, as disease progressed in these murine models of human ALS.

3.2 RESULTS

3.2.1 Motor deficits in hSOD1^{G93A} mice correlate with lumbar motor neuron loss during disease progression.

To monitor the decline in neuromotor performance and loss of motor neurons during ALS progression in hSOD1^{G93A} mice, body weights, motor behavioural tests and motor neuron counts were performed. The onset of disease was defined as a stage in which a neuromotor deficit was measurable. In this study, hSOD1^{G93A} mice showed a decrease in their body weight, hind-limb grip strength and rota-rod performance when compared to wild-type (**WT**) mice. The weight of the hSOD1^{G93A} mice reached the maximum at 133 days of age and was significantly decreased when compared to WT mice at 140 days (mean body weight, hSOD1^{G93A} = 20.4 ± 0.23g and WT = 22.3 ± 0.25g, n = 9, ***** $P < 0.05$**,** # $P < 0.001$; arrow in **Figure 3.1A**).

Rota-rod is a widely used measure of neuromotor performance in hSOD1^{G93A} mice (Mead et al., 2011). In our study, both hSOD1^{G93A} and WT mice remained on the Rota-rod for the full duration of the test until 119 days when hSOD1^{G93A} mice showed ~30% reduction in the time remained on the rota-rod (n = 12, * $P < 0.05$, # $P < 0.001$; arrow in **Figure 3.1B**). Next, we measured maximal hind-limb grip strength as an alternate measure of neuromotor function (Lepore et al., 2007, Schafer and Hermans, 2011). At 70 days, hSOD1^{G93A} mice showed a significant reduction in grip strength (~35% reduction; n = 12, * $P < 0.05$, + $P < 0.01$, # $P < 0.001$) when compared to WT mice (arrow in **Figure 3.1C**). Our results suggest that the hind-limb grip strength test is a more sensitive measure of detecting motor deficit symptoms in hSOD1^{G93A} mice compared to weight loss and rota-rod performance (Schafer and Hermans, 2011). The Rota-rod test mainly evaluates balance and co-ordination, and does not necessarily reflect muscle denervation (i.e. loss of muscle function; (Weydt

et al., 2003)). By contrast, the decline in grip strength in the hSOD1^{G93A} mice closely correlated with the onset of lumbar motor neuron loss at 70 days (n = 6, *** P < 0.001; **Figure 3.1D**). Post 70 days, we observed further declines in hind-limb grip strength presumably resulting from a progressive drop in lumbar motor neuron numbers up to end-stage (**Figure 3.1C and D**).

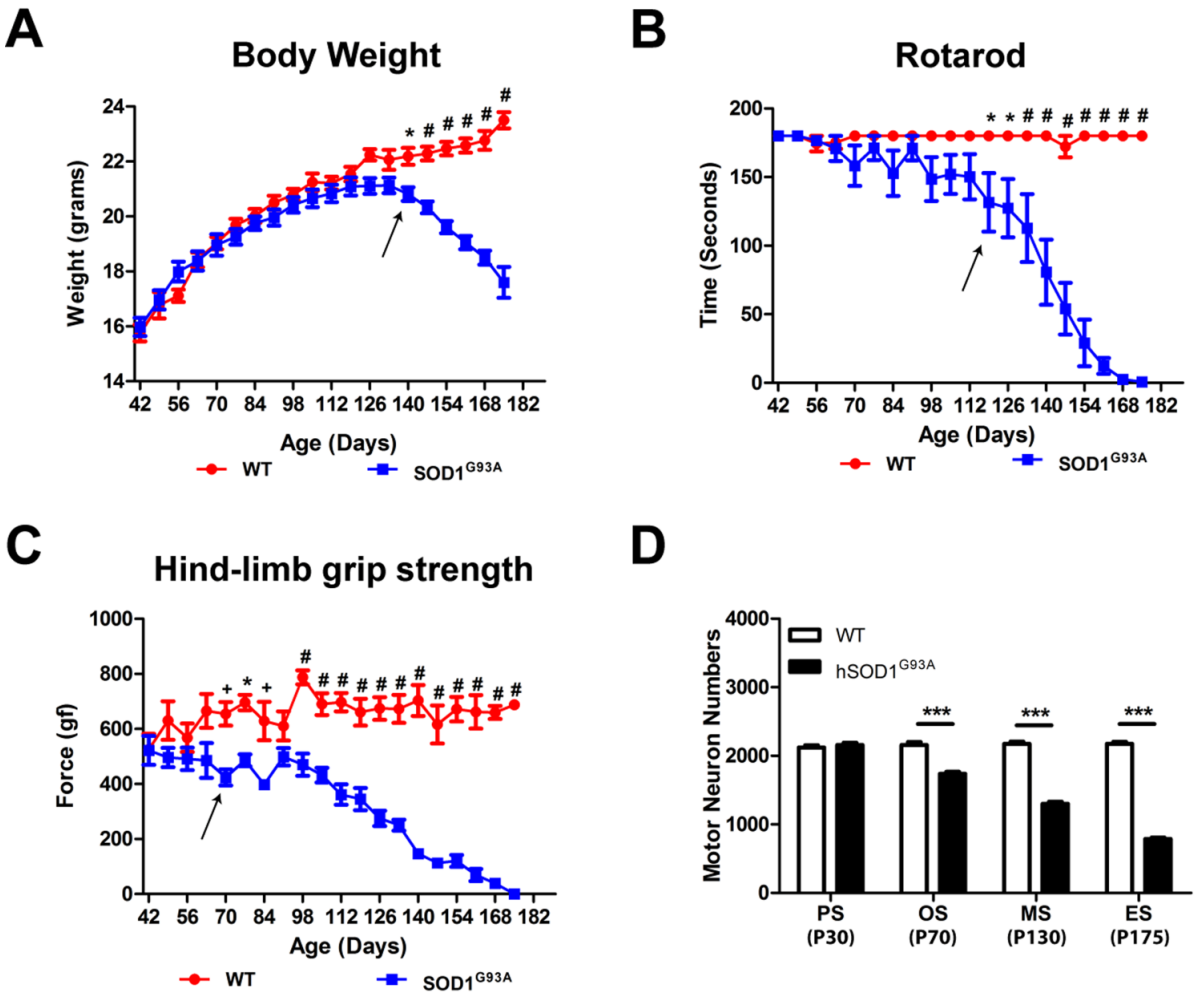

Figure 3.1: Decline in motor performance during ALS progression correlates with lumbar motor neuron loss in the lumbar spinal cord of hSOD1^{G93A} mice. ***A*** *shows significant weight loss of hSOD1^{G93A} mice when compared to wild type (**WT**) control mice at 140 days of age (arrow, n = 12, * P < 0.05, # P < 0.001, student t-test).* ***B*** *and* ***C*** *show significant reduction in time spent on rota-rod and hind-limb grip strength for hSOD1^{G93A} versus WT mice, at 119 days and 70 days respectively (arrows, n = 12, * P<0.05, + P<0.01, # P<0.001, student t-test).* ***D*** *shows lumbar motor neuron loss in hSOD1^{G93A} mice when compared to WT control mice at 70 days of age onwards (n = 6, *** P<0.001, student t-test). The decline in motor neuron number at 70 days correlates with the onset of loss of hind limb muscle strength at this same age (**C**). Data expressed as mean ± SEM. PS = pre-symptomatic (30 days postnatal [**P30**]); OS = onset (70 days postnatal [**P70**]); MS = mid-symptomatic (130 days postnatal [**P130**]) and ES = end-stage (175 days postnatal [**P175**]).*

3.2.2 Components of the classical and alternate pathways of complement are up regulated along with decreased expression levels of complement regulators in hSOD1^{G93A} mice.

Previous studies have identified various members of the complement system are up regulated in ALS and in ALS animal models, however it is unclear which of the major complement pathways are being activated. To investigate this further, we examined the mRNA and protein levels for some of the key initiators of the complement pathways, the classical, alternate and lectin pathway, as well as the major complement regulators CD55 and CD59a (Woodruff et al., 2010).

The expression levels of initiating components of the classical pathway (C1qB and C4), the alternative pathway (factor B), the lectin pathway (mannose binding lectin 1 and 2 (**MBL1** and **MBL2**)), the central component common to all pathways - C3, and the complement regulators CD55 and CD59a were measured in the lumbar spinal cord of hSOD1^{G93A} mice during disease progression of ALS (30 days to 175 days). This was achieved using one or combination of the following: quantitative real time PCR, immunofluorescence and/or western blotting. C1qB and C4 transcripts were significantly increased by 1.2 fold and 1.3 fold at onset, 1.7 fold and 2.9 fold at mid-symptomatic, and 13.1 fold and 10.7 fold by end stage of disease in hSOD1^{G93A} mice when compared to WT mice respectively (n = 6, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; **Figure 3.2A** and **Figure 3.4D**). Up-regulation of C1q at end-stage was confirmed using immunofluorescence where there was marked increase in hSOD1^{G93A} mice compared to WT mice (**Figure 3.2B**). We also examined that marked increase of C1q in hSOD1^{G93A} mice was localised to motor neurons and activated microglia (white arrows in **Figure 3.2L-N** [detailed in **3.2U**], and white arrows in **Figure 3.2R-T** [detailed in **3.2W**]), compared to WT, where little to no C1q was observed (**Figure 3.2C-E** and **Figure 3.2I-K**). We did not observe C1q on GFAP expressing astrocytes in either hSOD1^{G93A} or WT mice (**Figure 3.2O-Q** [detailed in **3.2V**] for hSOD1^{G93A} mice; **Figure 3.2F-H** for WT mice). Factor B showed a similar activation profile to that of C1qB and C4, namely there was a 2.2 fold increase in mRNA at mid-symptomatic and by end-stage of disease, there was a 6.0 fold increase in hSOD1^{G93A} mice respectively compared with WT mice (n=6, * $P < 0.05$, *** $P < 0.001$; **Figure 3.4E**).

The central component of complement system C3 was also increased in hSOD1^{G93A} mice, however its expression profile only dramatically increased by end stage in hSOD1^{G93A} mice when compared to WT. Specifically, we observed 1.8 and 1.6 fold increases at onset and mid-symptomatic with a dramatic 10.2 fold increase in C3 mRNA by end-stage of disease when compared to WT mice (n=6, * $P < 0.05$ and *** $P < 0.001$; **Figure 3.3A**). We then examined the expression and localisation of its activation fragment C3b in hSOD1^{G93A} and WT mice at end stage. Increased immunolabeling for

C3b was observed in the lumbar spinal cords of hSOD1^{G93A} mice compared to WT mice, at end-stage (**Figure 3.3B**). In hSOD1^{G93A} mice, C3b deposition appeared primarily on motor neurons and microglia (white arrows in **Figure 3.3L-N** [detailed in **3.3U**], and white arrows in **Figure 3.3R-T** [detailed in **3.3W**]), compared to WT, where there was little to no C3b staining (**Figure 3.3C-E**, and white arrows in **Figure 3.3I -K**). We did not observe C3b staining on GFAP expressing astrocytes in either hSOD^{G39A} or WT mice (**Figure 3.3O-Q** [detailed in **3.3V**] for hSOD1^{G93A} mice, and **Figure 3.3F-G** for WT mice). Changes in mRNA expression level of MBL1 and MBL2, which are the initiating components of the lectin pathway, were not detectable in either hSOD1^{G93A} or WT mice (*data not shown*), suggesting this pathway plays a minor role in this model.

The regulators of complement system CD55 and CD59a were also investigated, as they are important in maintaining homeostasis and keeping the complement system in its proper physiological state. Specifically, CD55 and CD59a negatively regulate complement activation by accelerating C3 convertase decay and inhibiting the assembly of membrane attack complex respectively (Spendlove et al., 2006, Kimberley et al., 2007). CD55 mRNA expression was initially increased by 1.4 fold at pre-symptomatic, and decreased at mid-symptomatic and end stage of disease by 0.4 fold and 0.5 fold respectively when compared to WT (n=6, * $P < 0.05$ and *** $P < 0.001$; **Figure 3.4A**). This was confirmed at protein level using western blotting, where a 41kDa CD55 immuno-reactive band was observed in all stages of hSOD1^{G93A} mice and their respective WT mice (**Figure 3.4B**, upper panel). Semi-quantitative densitometry analyses of these bands with respect to glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) loading controls (**Figure 3.4B**, lower panel), revealed increased CD55 protein in the lumbar spinal cord of hSOD1^{G93A} mice by 3.2 fold at pre-symptomatic and decreased by 0.7 fold at mid-symptomatic and 0.6 fold at end stage respectively, when compared to WT mice (n=4, * $P < 0.05$ and ** $P < 0.01$; **Figure 3.4C**). CD59a mRNA was also increased initially at onset by 1.3 fold and decreased at end stage of disease by 0.2 fold when compared to WT mice (n=6, * $P < 0.05$ and ** $P < 0.01$; **Figure 3.4F**).

Collectively, the above results suggest that regulation of complement system is perturbed, which leads to activation of classical and alternate pathways of complement system in the lumbar spinal cord of hSOD1^{G93A} mice, which may contribute to the disease progression of ALS.

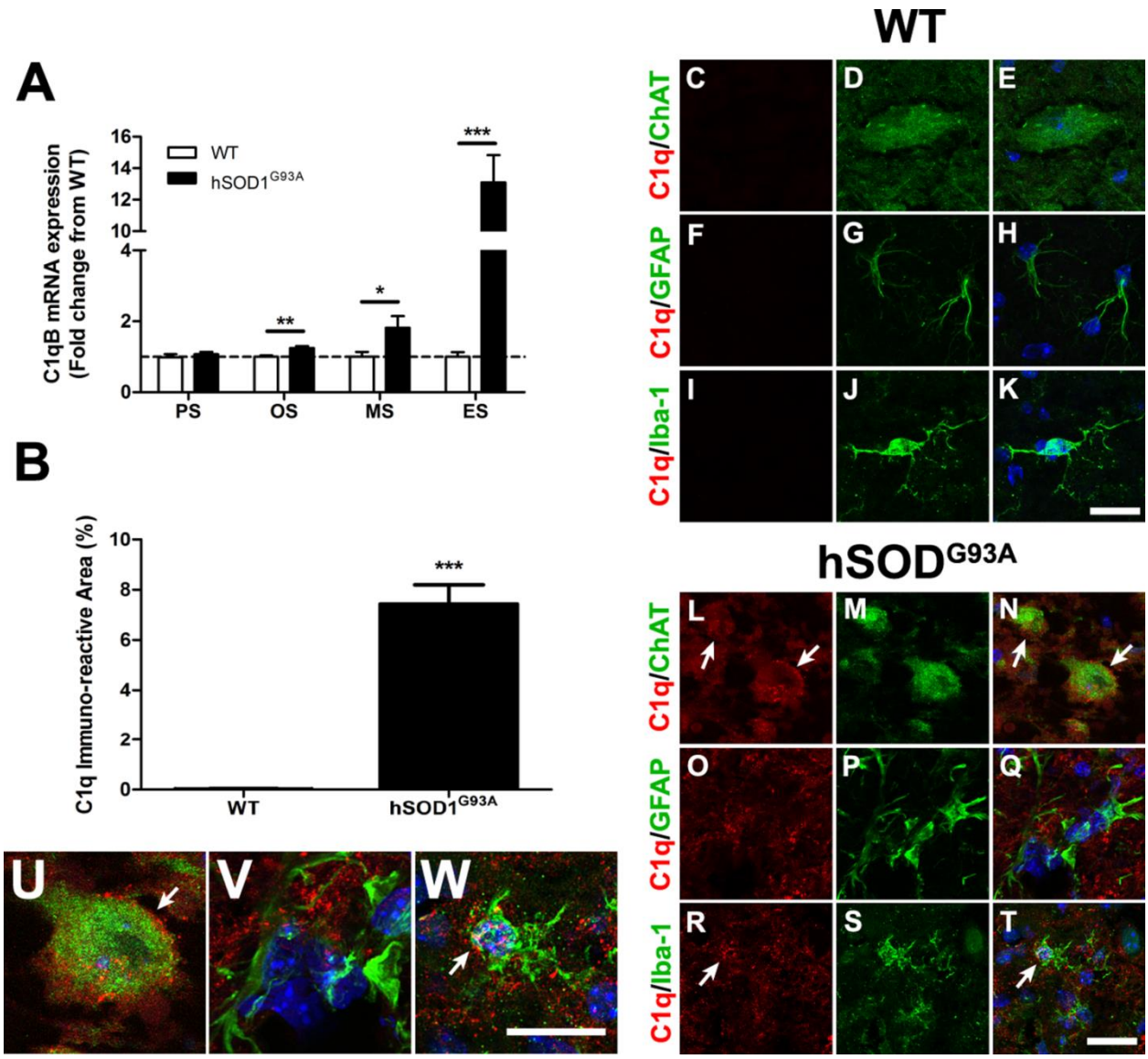

Figure 3.2: Expression and localisation of C1q in hSOD1^{G93A} and wild-type mice during disease progression. ***(A)*** *mRNA expression profile of C1qB in lumbar spinal cord of hSOD1^{G93A} mice relative to wild-type (**WT**) mice. Dashed line, baseline expression in WT controls at each time point.* ***(B)*** *Degree of immunolabelling for C1q significantly increased in the lumbar spinal cord of hSOD1^{G93A} mice at end-stage when compared with WT mice. (**A**, **B**) Data expressed as mean ± SEM (n = 6 mice/group; *P <0.05, **P <0.01, ***P <0.001, Student t test).* ***(C)*** *to* ***(T)*** *Double immunolabelling of C1q (red) with cellular markers (green) for motor neurons (ChAT; (**C**) to (**E**) WT mice, (**L**) to (**N**) hSOD1^{G93A} mice), astrocyte (GFAP; (**F**) to (**H**) WT mice, (**O**) to (**Q**) hSOD1^{G93A} mice), and microglia (Iba-1; (**I**) to (**K**) WT mice, (**R**) to (**T**) hSOD1^{G93A} mice) in the ventral lumbar spinal cord of WT and hSOD1^{G93A} mice (end stage). There was minimal expression of C1q in WT (**C**, **F** and **I**) with marked increase in hSOD1^{G93A} mice (**L**, **O** and **R**). In hSOD1^{G93A} mice, C1q was co-localised with ChAT-positive motor neurons (white arrows in (**L**) and (**N**) (detailed in **U**)). There was little to no co-localisation of C1q with GFAP-positive astrocytes (**Q** (detailed in **V**)), and minimal co-localisation with Iba-1-labelled microglia (white arrows in **R** and **T** (detailed in **W**)). PS, pre-symptomatic; OS, onset; MS, mid-symptomatic; ES, end-stage. Scale bar for all panels = 20 μm.*

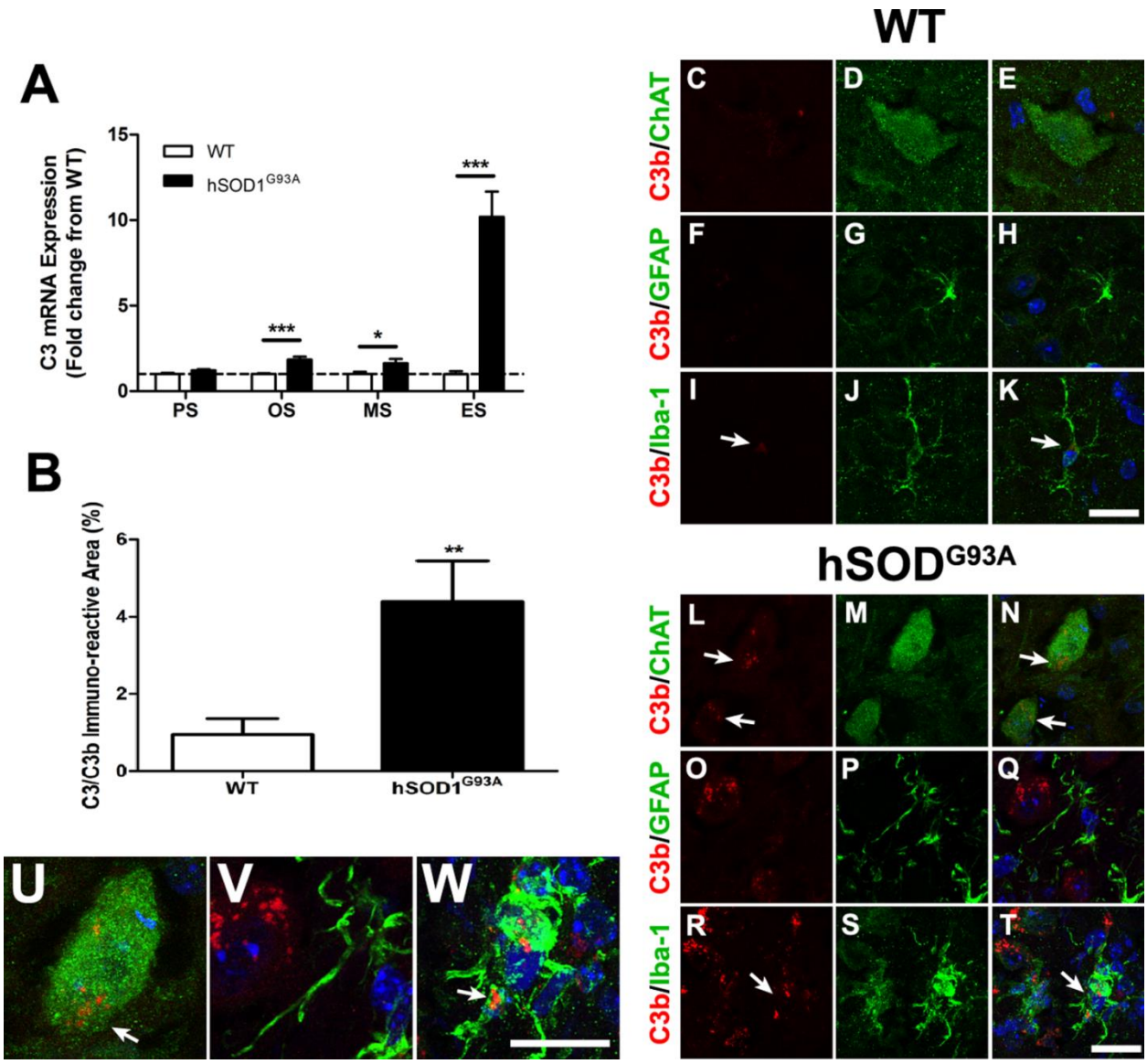

Figure 3.3: Localisation and expression of C3/C3b in hSOD1^{G93A} and wild-type mice during disease progression. ***(A)*** *mRNA expression profile of C3 in lumbar spinal cord of hSOD1^{G93A} mice relative to wild-type (WT) mice. Dashed line, baseline expression in WT controls at each time point.* ***(B)*** *Degree of immunolabelling for C3b significantly increased in the lumbar spinal cord of hSOD1^{G93A} mice at end-stage when compared with WT mice. (**A**, **B**) Data expressed as mean ± SEM (n = 6 mice/group; *P <0.05, **P <0.01, ***P <0.001, Student t test).* ***(C)*** *to* ***(T)*** *Double immunolabelling of C3b (red) with cellular markers (green) for motor neurons (ChAT; (**C**) to (**E**) WT mice, (**L**) to (**N**) hSOD1^{G93A} mice), astrocyte (GFAP; (**F**) to (**H**) WT mice, (**O**) to (**Q**) for hSOD1^{G93A} mice), and microglia (Iba-1; (**I**) to (**K**) WT mice, (**R**) to (**T**) hSOD1^{G93A} mice) in the ventral lumbar spinal cord of WT and hSOD1^{G93A} mice (end stage). C3b immunolabelling was absent on motor neurons in WT mice (**C** to **E**), but was present on motor neurons in hSOD1^{G93A} mice (white arrows in **L** and **N** (detailed in **U**)). There was minimal co-localisation of C3b with Iba-1-labelled microglia in WT (white arrows in **I** and **K**). In hSOD1^{G93A} mice immunolabelling of C3b was evident in Iba-1-labelled microglia (white arrows, **R** and **T** (detailed in **W**)). There was no co-localisation with C3b and GFAP-positive astrocytes in WT and hSOD1^{G93A} mice (**F** to **H** for WT, **O** to **Q** (detailed on **V**) for hSOD1^{G93A} mice). PS, pre-symptomatic; OS, onset; MS, mid-symptomatic and ES, end-stage. Scale bars for all panels = 20 μM.*

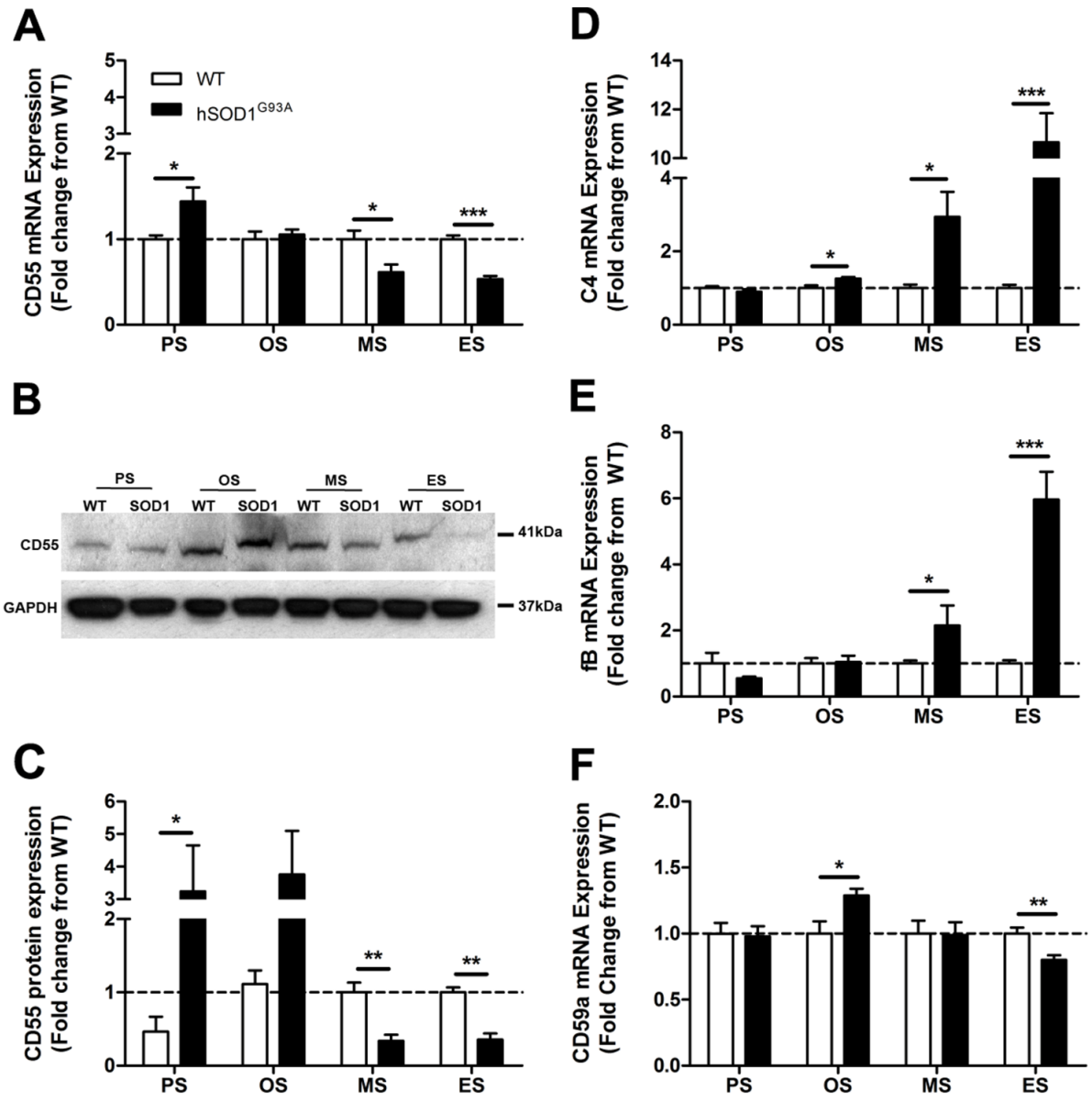

Figure 3.4: Altered expression of complement components in hSOD1^{G93A} and wild-type mice at different ages of disease progression. ***(A)*** *mRNA expression of CD55 in the lumbar spinal cord of hSOD1^{G93A} transgenic mice relative to age-matched wild-type (**WT**) mice at four different ages.* ***(B)*** *Representative western blot of CD55 with glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) in the lumbar spinal cord of hSOD1^{G93A} mice (SOD1) relative to age-matched WT mice, at different ages of disease progression.* ***(C)*** *Protein expression of CD55 determined by semi-quantitative densitometry in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched WT mice at four different ages.* ***(D)*** *to* ***(F)*** *mRNA expressions of C4 (classical pathway, **D**), factor B (alternative pathway, **E**) and CD59a (regulator, **F**) in lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched WT mice at four different ages. (**A**, **C**, **D**, **E**, **F**) Dashed lines, baseline expressions in WT controls at each time point. Data expressed as mean ± SEM (n = 6 mice/group; *P <0.05, **P <0.01, ***P <0.001, Student t test). PS, pre-symptomatic; OS, onset; MS, mid-symptomatic and ES, end-stage.*

3.2.3 C5 is expressed by motor neurons but is not altered in hSOD1^{G93A} mice

C5a, the ligand for CD88, is rapidly generated from its precursor protein C5 following complement activation (Woodruff et al., 2011). We therefore examined the mRNA expression of C5 and protein levels of C5a in hSOD1^{G93A} and WT mice by quantitative real time PCR and ELISA respectively. C5 mRNA expression levels did not change in hSOD^{G93A} mice when compared to WT mice over the four ages examined (**Figure 3.5A**). Intriguingly, when we examined the protein expression levels of C5a we noted a steady decline in C5a levels with increasing postnatal age in both hSOD1^{G93A} and WT mice, however by disease end-stage, the levels of C5a were significantly lower in hSOD1^{G93A} mice when compared to WT mice (n=6, * P<0.05; **Figure 3.5B**).

Next, we immuno-stained lumbar spinal cords from hSOD1^{G93A} and WT mice for C5 with specific cellular markers for motor neurons (anti-ChAT), astrocytes (anti-GFAP), and microglia (anti-CD11b). C5 was clearly present in ChAT-positive lumbar motor neurons from end-stage hSOD1^{G93A} and WT mice (white arrows in **Figure 3.5C** and **3.5D**), but not in GFAP-positive astrocytes (**Figure 3.5E** and **3.5F**). For microglia, we did not see any C5 in CD11b-positive microglia in WT spinal cords (**Figure 3.5G**), but we did see some activated microglia (enlarged cell shape with thickening of proximal processes and decrease in ramification of distal branches (Raivich, 2005)) expressing low amounts of C5 in the spinal cords from end-stage hSOD1^{G93A} mice (white arrows; **Figure 3.5H**).

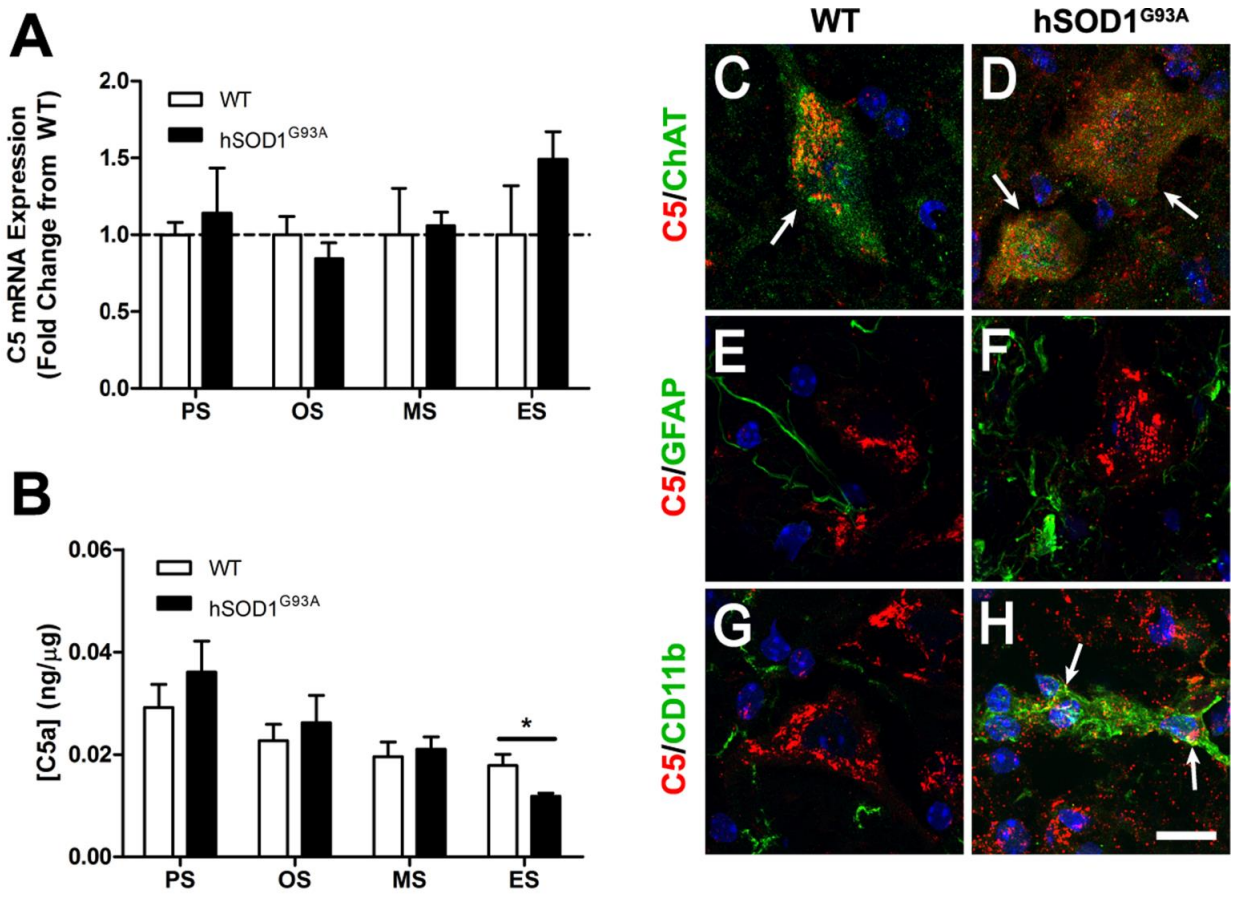

Figure 3.5: Expression and localisation of C5 and C5a in hSOD1^{G93A} and wild-type mice during disease progression. ***(A)*** *mRNA expression profile of C5 in lumbar spinal cord of hSOD1^{G93A} mice relative to wild-type (**WT**) mice. Dashed line, baseline expression in WT controls at each time point.* ***(B)*** *Protein expression of C5a in the lumbar spinal cord of hSOD1^{G93A} mice has decreased by end-stage when compared with WT mice. (**A**, **B**) Data expressed as mean ± SEM (n = 6 mice/group; *P <0.05, Student t test).* ***(C)*** *to* ***(H)*** *Double immunolabelling for C5 (red) with cellular makers (green) for motor neurons (ChAT; **C** and **D**, arrows), astrocytes (GFAP; **E** and **F**), and microglia (CD11b; **G** and **H**), in the ventral lumbar spinal cord region of hSOD1^{G93A} mice (**D**, **F** and **H**) and WT mice (**C**, **E** and **G**) at end-stage. Co-localisation of C5 with these cellular markers is seen as a merge of green and red (for example, white arrows in **C**, **D**, and **H**). PS, pre-symptomatic; OS, onset; MS, mid-symptomatic; ES, end-stage. Scale bar for C to H = 20μm*

3.2.4 CD88 is up-regulated during disease progression in hSOD1^{G93A} mice

Previous studies have shown increase in CD88 expression in multiple rodent models of ALS (Woodruff et al., 2008a, Humayun et al., 2009); hence this study aimed to investigate whether there were similar differences in expression of CD88 between hSOD1^{G93A} and WT mice during disease progression of ALS.

The mRNA expression levels for CD88 in the lumbar spinal cord of hSOD1^{G93A} mice were normalised and compared to WT mice during disease progression (30 days to 175 days) using quantitative real time PCR. CD88 expression was increased by 2.3 fold at mid-symptomatic, and by 8.6 fold at end stage of disease respectively, compared with WT mice (n = 6, * P < 0.05and *** P < 0.001; **Figure 3.6A**). The protein expression level of CD88 in the lumbar spinal cords of hSOD1^{G93A} and WT mice were measured using western blotting. A 45kDa CD88 immuno-reactive band was observed in all stages of hSOD1^{G93A} mice and their respective WT mice (**Figure 3.6B**, upper panel). Semi-quantitative densitometry analyses of these bands with respect to GAPDH loading controls (**Figure 3.6B**, lower panel), revealed increased CD88 protein in the lumbar spinal cord of hSOD1^{G93A} mice by 2.6 fold and 3.7 fold at mid-symptomatic and end stage respectively, when compared to WT mice (n=6, ** P < 0.01 and *** P < 0.001; **Figure 3.6C**).

3.2.5 CD88 is localised to motor neurons and activated microglia with minimal localisation to astrocytes in hSOD1^{G93A} mice

Next, we aimed to determine the cellular localisation of CD88 that has contributed to the increased expression seen in hSOD1^{G93A} mice. To achieve this, we performed immuno-labelling for CD88 on lumbar spinal cord sections from hSOD1^{G93A} mice and WT mice. These sections were immuno-stained for CD88 and with specific cellular markers to identify motor neurons (anti-ChAT), microglia (anti-Iba-1) and astrocytes (anti-GFAP).

In WT mice CD88 staining was observed on lumbar motor neurons (**Figure 3.6D**). CD88-stained cells were readily identified as motor neurons due to their large size, location and distinctive morphology (Banks et al., 2005). This was confirmed by double labelling with the motor neuron marker ChAT (white arrows in **Figure 3.6F**). CD88 immuno-staining was localized predominantly to the motor neurons' soma (**Figure 3.6D** to **3.6F**). To further support these immuno-histochemical findings, we confirmed that motor neurons expressed CD88 mRNA transcripts, by *in-situ* hybridization (**Figure 3.6D'**, inset). Next, we examined if CD88 immuno-staining was present on surrounding microglia and astrocytes in sections of WT lumbar spinal cords. We observed minimal

co-localization of CD88 to Iba-1 positive microglia (white arrows in **Figure 3.6G** and **3.6I**), but none to GFAP-positive astrocytes (**Figure 3.6J** to **3.6L**).

Following this demonstration of CD88 immuno-labelling in WT lumbar spinal cord, we then investigated CD88 cellular localisation in hSOD1^{G93A} mice. CD88 was also expressed on the few remaining motor neurons seen at the end-stage of disease (white arrow in **Figure 3.6O**). By contrast to WT lumbar spinal cords, we observed prominent CD88 immuno-staining on other cellular structures surrounding motor neurons in the lumbar spinal cords of hSOD1^{G93A} mice by the end-stage of disease (e.g. yellow arrows in **Figure 3.6M** and **3.6O**). This additional CD88 immuno-reactivity was investigated in hSOD1^{G93A} end-stage mice, using microglia marker Iba-1 and astrocyte marker GFAP. By contrast to Iba-1 positive microglia in WT mice, where these cells expressed little observable CD88 and appeared to be non-activated (i.e. small size with slender processes; (Raivich, 2005); **Figure 3.6H** and **3.6I**), Iba-1 positive microglia in hSOD1^{G93A} mice demonstrated an activated morphology with increased expression of CD88 (white arrows in **Figure 3.6P** and **3.6R**). As expected, astrocytes were seen to increase in numbers as disease progressed in hSOD1^{G93A} mice (Barbeito et al., 2004). This was noted by increased GFAP immuno-labelling in the lumbar spinal cord of hSOD1^{G93A} mice at end-stage compared to WT mice (**Figure 3.6K-L** for WT, and **Figure 3.6T-U** for hSOD1^{G93A} mice). Minimal CD88 co-localisation to GFAP-positive astrocytes was observed in WT (**Figure 3.6J-L**) and at the end-stage of disease in hSOD1^{G93A} mice (**Figure 3.6S-U**).

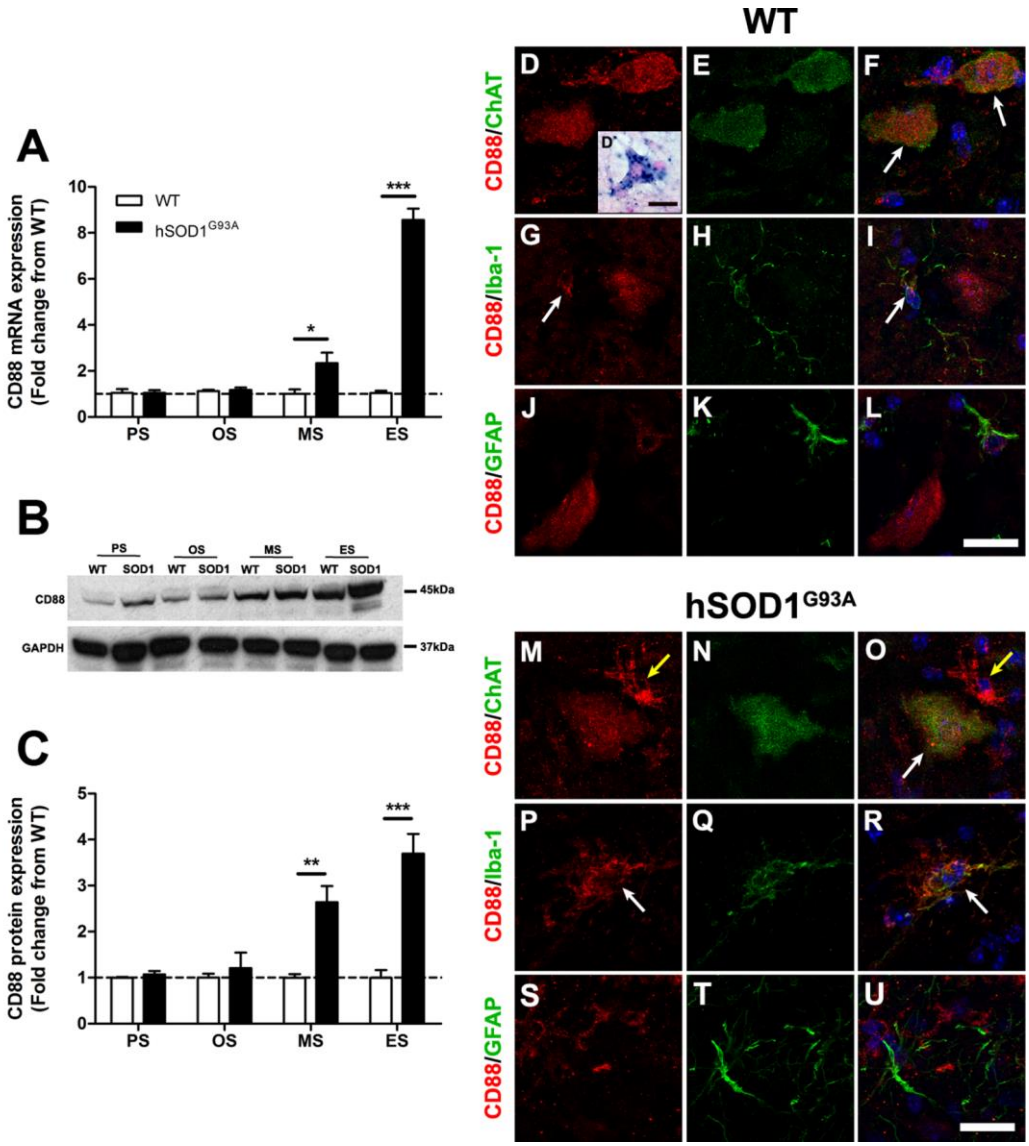

Figure 3.6: Expression and localisation of CD88 in hSOD1^{G93A} and wild-type mice at four different ages. *(**A**) mRNA expression of CD88 in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched wild-type (**WT**) mice at four different ages. (**B**) Representative western blot of CD88 with glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) in the lumbar spinal cord of hSOD1^{G93A} (SOD1) mice relative to age-matched WT mice at different ages. (**C**) Protein expression of CD88 determined by semi-quantitative densitometry in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched WT mice at four different ages. (**A**), (**C**) Dashed lines, baseline expression in WT controls at each time point; data expressed as mean ± SEM (n = 6 mice/group; *P <0.05, **P <0.01, ***P <0.001, Student t test). (**D**) to (**U**) Double immunolabelling of CD88 (red) with cellular markers (green) for motor neurons (ChAT; (**D**) to (**F**) WT mice, (**M**) to (**O**) hSOD1^{G93A} mice), microglia (Iba-1; (**G**) to (**I**) WT mice, (**P**) to (**R**) hSOD1^{G93A} mice), and astrocytes (GFAP; (**J**) to (**L**) WT mice, (**S**) to (**U**) for hSOD1^{G93A} mice) in the ventral lumbar spinal cord of WT and hSOD1^{G93A} mice at end-stage. CD88 was co-localised with ChAT-positive motor neurons (**F**, **O**, white arrows). (**D'**) CD88 mRNA transcript within lumbar motor neurons (determine by large cell size and location within the ventral horn). In hSOD1^{G93A} mice, immunolabelling of CD88 also evident on other cell types, indicated by lack of co-localisation with anti-ChAT (yellow arrows in **M** and **O**). (**G**), (**I**) White arrows, small amount of CD88 staining within nonactivated microglia in WT mice, with increased CD88 expression on activated microglia in hSOD1^{G93A} mice (**P** and **R**, white arrows). PS, pre-symptomatic; OS, onset; MS, mid-symptomatic; ES, end stage. Scale bars for all panels = 20 μM.*

3.3 DISCUSSION

While the pathogenesis of ALS is still unclear, there is persuasive evidence that complement factors are involved in promoting disease progression. Previous studies have demonstrated that C1q and C3 mRNA expression are significantly increased during ALS progression in hSOD1^{G93A} mice (Lee et al., 2012). In addition, up-regulation of CD88 has also been observed in numerous neurodegenerative diseases in rodents (Yasojima et al., 1999, Singhrao et al., 1999, Ager et al., 2010, Woodruff et al., 2006), including ALS (Woodruff et al., 2008a, Humayun et al., 2009), so it is plausible to propose that the complement system could be involved in the pathophysiology of ALS. The present study demonstrates that components of the classical and alternative complement pathways are up-regulated during the course of disease progression in hSOD1^{G93A} mice, and that C5a receptor CD88 expression level is also increased. In addition, we found a reduction in two major regulatory inhibitors of complement activation as the disease worsened, which is suggestive of a progressive dysregulation of complement in this model. Furthermore, we show that C5, the precursor of C5a, is expressed predominantly by motor neurons, suggesting that diseased motor neurons could be a major source of C5a generation during disease progression. This local complement "self-signalling" in the CNS might therefore contribute to motor neuron death in hSOD1^{G93A} mice as shown previously for cortical neurons (Pavlovski et al., 2012). Taken together, our results indicate that motor neurons may generate C5a under stress, and that this may promote self-damage under disease conditions that exist in ALS.

3.3.1 Classical and alternate complement pathways are activated in ALS progression in hSOD1^{G93A} mice.

The present study provided evidence for the activation of classical (C1qB and C4) and alternate (factor B) pathways of the complement system in the lumbar spinal cord of hSOD1^{G93A} mice during ALS disease progression. This is consistent with numerous studies in mouse models of ALS and human patients where increased levels of C1q, C3 and C4 have been found (Lee et al., 2012, Chiu et al., 2009). Furthermore this study also extended up-regulation of C1qB and C3 mRNA expression in previous studies to protein levels and localisation where C1q and C3b immuno-labelling was increased in hSOD1^{G93A} mice and expressed on motor neurons and microglia compared to WT. This may suggest that up-regulation of these components could assist in the removal of dying motor neurons via opsonisation, during disease progression in hSOD1^{G93A} mice.

It is possible that cell fragments or protein aggregates from dying motor neurons could lead to complement activation in the degenerating spinal cord (Woodruff et al., 2010). In our study, complement activation was seen at disease onset (Postnatal (**P**) 70 days) and was restricted to the

areas of motor neuron death in the spinal cord of hSOD1^{G93A} mice. Other studies have demonstrated that complement components C1q and C3b are located at the neuromuscular junction during the early stages of disease (P47) in hSOD1^{G93A} mice (Heurich et al., 2011). These findings are consistent with the idea that C1q and C3b may contribute to the cellular destruction of motor nerve terminals in these mice (Dupuis and Loeffler, 2009, Cappello et al., 2012, Schafer et al., 2012). Taken together, these findings and our own are consistent with the hypothesis that the early loss of motor neuron terminals is followed by the subsequent death of motor neurons within the spinal cord. However, there is current debate about the initiating site of degeneration in the cortical-motor system (upper motor neurons; (Vucic et al., 2008)) versus peripheral (neuromuscular junction; (Dupuis and Loeffler, 2009)). Future studies could contrast complement activation temporally at these different sites to determine the initiating site of complement-mediated neurodegeneration.

We also showed decreased mRNA expression levels of complement regulators CD55 and CD59a at later stages of the disease, which suggests that the homeostasis of the complement system is perturbed, which may lead to dysregulation and over-activation of the complement system. This supports other studies, which have shown that deficiency in CD55 and CD59a exacerbates neuronal degeneration (Wang et al., 2010, Britschgi et al., 2012, Stahel et al., 2009). Our findings are also consistent with those of Heurich et al., 2011, where decreased (but non-significant) levels of CD55 mRNA were observed during the later stages of disease in hSOD1^{G93A} mice. In the present study, we also confirmed the mRNA changes in CD55 at the protein level, which similarly showed decreased CD55 levels at later stages of disease. Interestingly, we also observed an initial increase in the mRNA expression levels of CD55 and CD59a during early stages of disease. This may indicate an early negative feedback mechanism to delay the activation of complement in host cells, however this needs further investigation. Collectively, our data adds further support to the notion that complement activation may play an important role in accelerating motor neuron loss and ultimately in progression of ALS.

3.3.2 C5 is expressed by wild-type and hSOD1^{G93A} motor neurons during disease progression.

C5, the precursor of C5a, is expressed by motor neurons in both wild type and hSOD1^{G93A} mice. This suggests that motor neurons are a major source of C5 generation in this tissue. We recently showed murine cortical neurons also expressed endogenous C5, and generated C5a in response to ischemia, which contributed to neuronal cell death (Pavlovski et al., 2012). It is plausible the same phenomenon is occurring in the hSOD1^{G93A} mouse, where stressed and dying motor neurons generate their own C5a, to act in an autocrine fashion by binding to CD88, present on these

neurons, to promote their death. Indeed, C5a has been suggested to directly cause neuronal cell death in a separate model of ALS (Humayun et al., 2009). C5a protein level in the lumbar spinal cord only appeared to be significantly decreased by end stage in hSOD1^{G93A} mice when compared to wild type mice. This could be due to fewer motor neurons (a source of C5/C5a), and the increased CD88 receptor levels by surrounding activated microglia as disease progressed. Increased CD88 on these cells would act to internalise and degrade C5a post activation of its receptor (Oppermann and Gotze, 1994), which could account for the decline in C5a levels over time. The consequence of C5a-CD88 signalling in wild type motor neurons is not yet fully understood and will need further investigation.

3.3.3 CD88 is up-regulated during disease progression in hSOD1^{G93A} mice

The present study provided evidence for a pathophysiological role of CD88 in hSOD1^{G93A} mice. Specifically, a significant increase in CD88 protein was observed at mid-symptomatic and end stage in hSOD1^{G93A} mice. This is in agreement with our previous studies conducted in hSOD1^{G93A} rats (Woodruff et al., 2008a). The increase in CD88 protein in hSOD1^{G93A} mice also parallels observations in other models of neurodegenerative diseases, such as Huntington's disease and Alzheimer's disease (Singhrao et al., 1999, Ager et al., 2010). Taken together, this suggests that complement activation is a generalised response to neuronal injury in neurodegenerative diseases. Furthermore, the ability of CD88 antagonists to attenuate both neurodegeneration and disease progression in rat models of ALS and Huntington's disease, and in mouse models of Alzheimer's disease, further suggests that increased CD88 activation actively contributes to neurodegeneration (Fonseca et al., 2009, Woodruff et al., 2006, Woodruff et al., 2008a).

In addition to an increase in CD88 in hSOD1^{G93A} mice, the present study observed CD88 on motor neurons in WT mice, as well as in hSOD1^{G93A} mice. The fact that CD88 was found on WT motor neurons suggests that it may play a non-inflammatory role in motor neuron function. Indeed, studies including our own, have shown CD88 is also present on other neurons within the brains of WT adult mice, including pyramidal neurons in the CA subfields of the hippocampus and neocortex, and Purkinje cells in the cerebellum (O'Barr et al., 2001, Crane et al., 2009); and CD88 has also been documented to be expressed on human motor neurons (Humayun et al., 2009). Hence the physiological significance of C5a receptor presence in motor neurons awaits further study.

Previous studies have shown that motor neuron death in animal models of ALS is exacerbated by toxic signals emanating from non-neuronal neighbouring cells (astrocytes and microglia), via an inflammatory response that accelerates disease progression (Kreutzberg, 1996, Nagai et al., 2007).

The present study also showed up regulation of CD88 on activated microglia, but minimal expression on astrocytes in hSOD1^{G93A} mice. The latter is in contrast to our previous study conducted in hSOD1^{G93A} rats, where CD88 was expressed primarily on proliferating astrocytes (Woodruff et al., 2008a), but is in support of other studies, which show CD88 on microglia in other neurodegenerative diseases (Griffin et al., 2007, Ager et al., 2010). The differential expression of CD88 on these proliferating glial cells between hSOD1^{G93A} rat and mouse models, may suggest a differential role for CD88 in these two species.

The strong co-localisation of CD88 with activated microglia in hSOD1^{G93A} mice suggests that CD88-activated microglia contribute to the propagation of disease as opposed to the aetiology of the disease. This is supported by previous studies where transplanted wild type microglia produced no delay of disease onset but survival was greatly extended through slowing of disease progression in hSOD1^{G93A} mice (Beers et al., 2006). The exact mechanism by which C5a-CD88 signalling in microglia plays a role in neuro-degeneration is still unknown, but may involve the release of reactive oxygen species through NADPH oxidase, or pro-inflammatory cytokine, which have been shown to be up-regulated in ALS (Wu et al., 2006).

3.4 CONCLUSION

In summary, the present study has demonstrated the up-regulation of classical and alternative pathway complement components, together with decreased levels of complement regulators, suggesting that complement activation and/or its dysregulation may play an important role in motor neuron loss and ultimately in progression of ALS. Expression of C5a receptor, CD88 was up-regulated in hSOD1^{G93A} mice, and the increased expression of CD88 in end-stage hSOD1^{G93A} mice appears to be due to increased microglial CD88 expression. Taken together, these results indicate that CD88 may play important role in the pathophysiology of ALS. These results pave the way for preliminary pharmacological experiments using specific downstream complement inhibitors in hSOD1^{G93A} mice, such as C5 inhibitors or antagonists to C5a receptor that could conceivably be extended to humans with positive therapeutic outcomes.

CHAPTER 4

"Complement factor C5a and its receptor CD88 contributes to the pathology in a mouse model of Amyotrophic lateral sclerosis"

*** Part of this chapter was published in the Proceeding of the National Academy of Sciences of the United States of America** - Woodruff, Trent M., **Lee, John D**. and Noakes, Peter G. (2014) Role for terminal complement activation in amyotrophic lateral sclerosis disease progression. *Proceeding of the National Academy of Sciences of the United States of America, 111* 1: E3-E4

4.1 INTRODUCTION

As discussed in previous chapters, complement activation has long been implicated in the pathogenesis of Amyotrophic lateral sclerosis (**ALS**), with numerous clinical and animal studies demonstrating strong up-regulation of activation fragments of complement components C1q, C3 and C4 in the serum, cerebrospinal fluid and neurological tissue (including spinal cord and motor cortex) of ALS patients and animal models of ALS (Lee et al., 2012). Intriguingly, recent studies have shown deletion of C1q, C3 and C4 in hSOD1 transgenic mouse models did not show any beneficial effects on the disease progression of ALS (Chiu et al., 2009, Lobsiger et al., 2013). Hence, these studies indicate that blocking upstream activation pathways of complement does not alter the disease course in hSOD1 transgenic mice. In contrast to these studies, our laboratories have previously shown that chronic administration of a specific C5a receptor CD88 antagonist PMX205 in $hSOD1^{G93A}$ transgenic rats markedly delayed the onset of motor symptoms and increased survival, compared to untreated animals (Woodruff et al., 2008a). Further, data presented in Chapter 3 demonstrated up-regulation of CD88 in the lumbar spinal cord of $hSOD1^{G93A}$ transgenic mice as disease progressed, suggesting that C5a-CD88 signalling may have a pathogenic role in the disease progression of ALS (Lee et al., 2013).

In the present study, we examined the effect of selective CD88 antagonist PMX205 on the disease progression of ALS in $hSOD1^{G93A}$ mice. Chronic oral administration of PMX205 to $hSOD1^{G93A}$ mice significantly improved hind-limb grip strength, slowed disease progression and extended survival. Additionally, we have also confirmed that $hSOD1^{G93A}$ mice lacking CD88 (**$hSOD1^{G93A}$ x $CD88^{-/-}$**) have a similarly extended survival. $hSOD1^{G93A}$ x $CD88^{-/-}$ mice also showed reductions in microglia and cytokine mRNA levels when compared to $hSOD1^{G93A}$ mice. These results indicate that downstream (terminal pathway) complement activation, leading to increased expression of CD88 in the $hSOD1^{G93A}$ mice has an important role in motor neuron death and contributes to the pathology in the $hSOD1^{G93A}$ mice.

4.2 RESULTS

4.2.1 Treatment with CD88 antagonist extends survival, improves hind-limb grip strength and slows disease progression in hSOD1^{G93A} mice in a concentration dependent manner.

Previous studies have shown increase in CD88 expression in multiple rodent models of ALS (Woodruff et al., 2008a, Humayun et al., 2009, Lee et al., 2013); hence this study aimed to elucidate whether the up-regulation of CD88 in the lumbar spinal cord of hSOD1^{G93A} mice were contributing to disease progression. hSOD1^{G93A} mice were orally dosed with the selective CD88 antagonist PMX205 beginning from 35 days of age with two different concentrations (3mg/kg/day and 9mg/kg/day). These concentrations were chosen based on previous reports of efficacy in a mouse transgenic model of Alzheimer's disease (3mg/kg; (Fonseca et al., 2009)), and inflammatory bowel disease (9 mg/kg; (Jain et al., 2013)). The end-stage of disease (survival time) in these animals was determined as complete hind-limb paralysis and an inability to right itself once placed on its back (Ludolph et al., 2007).

We first performed preliminary experiments in a small cohort of mice treated with PMX205 at 3mg/kg/day. PMX205 treatment at this lower dose did not affect survival time when compared with vehicle treated hSOD1^{G93A} mice (mean end stage of disease, vehicle = 170.6 ± 2.2 days and PMX205 = 174.5 ± 3.4 days, n = 5 – 6, P = 0.8897; **Figure 4.1A**). Decline in neuromotor performance was also assessed in these animals using body weight and hind-limb grip strength as it is a more sensitive measure of detecting motor deficit symptoms in hSOD1^{G93A} mice compared to rota-rod performance (Lee et al., 2013). There was no significant difference in body weight alterations between vehicle and PMX205 treated hSOD1^{G93A} mice (n = 5 – 6, P > 0.05; **Figure 4.1B**). Next we measured the maximal hind-limb grip strength as a measure of motor deficit symptoms in these animals. PMX205 treated hSOD1^{G93A} mice did not show any difference in maximal hind-limb grip strength when compared to vehicle treated hSOD1^{G93A} mice (n = 5 – 6, P > 0.05; **Figure 4.1C**). The overall disease progression was also determined by age at which the maximal grip strength declined by 25, 50, 75 and 100%. Vehicle treated hSOD1^{G93A} mice showed similar ages for decline in grip strength when compared to PMX205 treated hSOD1^{G93A} mice (n = 5 – 6, P = 0.5461; **Figure 4.1D**). Intriguingly, these preliminary experiments suggested that PMX205 treatment at 3mg/kg/day did not improve ALS pathology in hSOD1^{G93A} mice, despite being used at a similar dose to that previously reported in a transgenic mouse model of Alzheimer's disease. However, a separate study recently reported that an increased dose of 200µg/day (~8mg/kg/day) PMX205 is required to achieve efficacy in a mouse model of inflammatory bowel disease (Jain et al., 2013). Thus, for the next series of experiments, we decided to repeat our study with a higher dose of PMX205, choosing to dose at a 3-fold higher concentration of 9mg/kg/day.

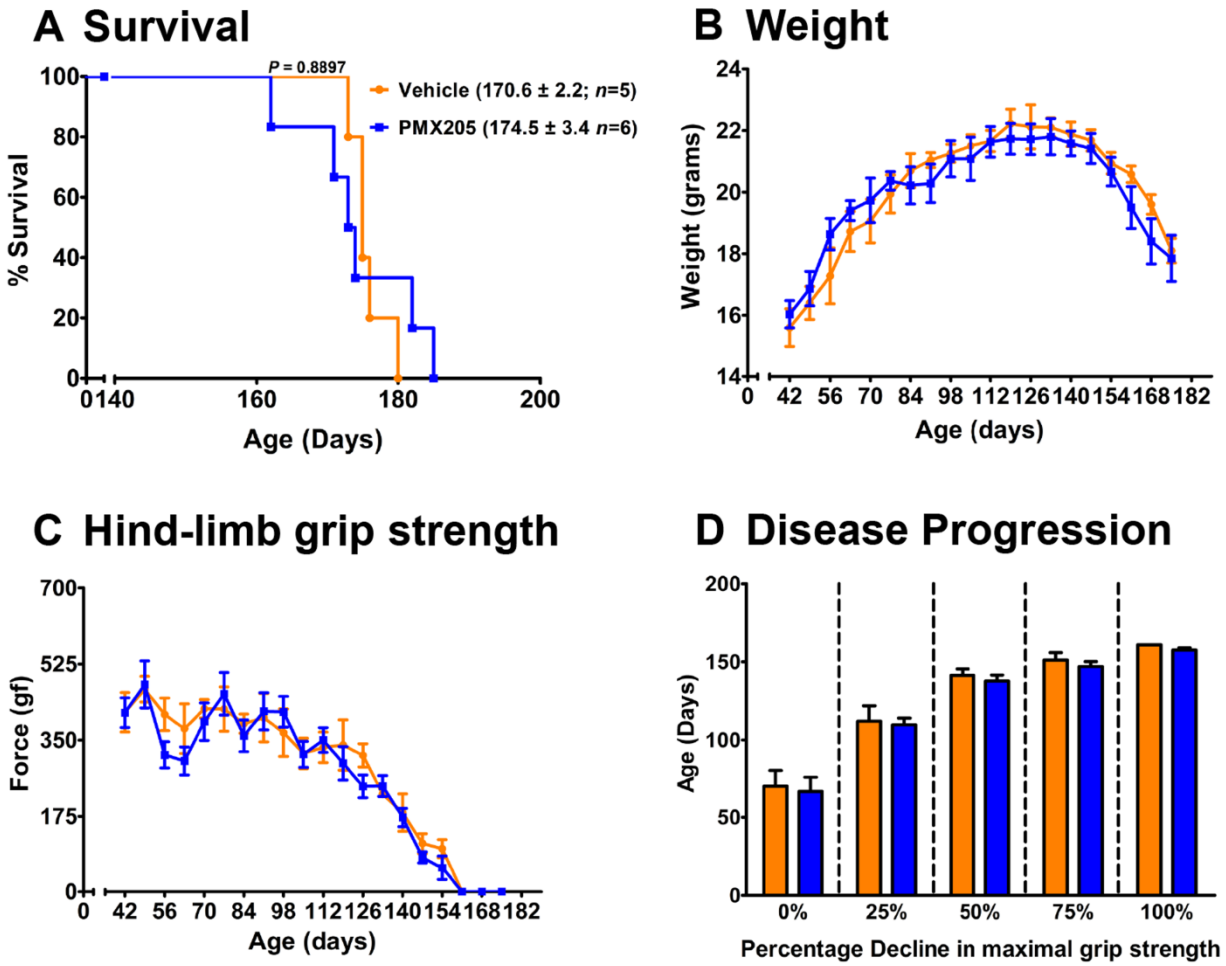

Figure 4.1: "Pre-Onset" PMX205 treatment at 3mg/kg/day does not affect survival, weight, hind-limb grip strength and disease progression in hSOD1^{G93A} transgenic mice. *hSOD1^{G93A} mice were orally dosed with the selective CD88 antagonist PMX205 at 35 days of age (3mg/kg/day).* ***A*** *shows a Kaplan-Meier plot of ages (in days) in which hSOD1^{G93A} mice were treated with dH_2O (vehicle; orange line) or PMX205 (blue line) reached end-stage of disease (complete hind-limb paralysis and an inability to right itself once placed on its back; n = 5 -6, P = 0.8897, log-rank test). PMX205 treatment at 35 days of age (3mg/kg/day) did not show alterations in survival of these animals.* ***B*** *and* ***C*** *shows no difference in weight and hind-limb grip strength between vehicle (orange line) and PMX205 (blue line) treated hSOD1^{G93A} mice (n = 5 – 6, P > 0.05, student t-test).* ***D*** *shows no difference in disease progression (determined by age at which maximal grip strength decline at 25, 50, 75 and 100%) between hSOD1^{G93A} mice treated with vehicle (orange bar) and PMX205 (blue bar) (n = 5 -6, P = 0.5461, student t-test). Data expressed as mean ± SEM.*

PMX205 treatment of hSOD1^{G93A} mice at 9mg/kg/day resulted in a significant extension in survival time when compared with vehicle treated hSOD1^{G93A} mice (mean end stage of disease, vehicle = 162.8 ± 3.2 days and PMX205 = 173.5 ± 3.2 days, n = 12 – 13, $P < 0.05$; **Figure 4.2A**). The weight of vehicle and PMX205 treated hSOD1^{G93A} mice both reached the maximum at 126 days of age, however there was no difference in body weight between vehicle and PMX205 treated hSOD1^{G93A} mice (n = 12 – 13, $P > 0.05$; **Figure 4.2B**). Although there was no difference in body weight, PMX205 treatment improved the hind-limb grip strength at 105 days of age (n = 12 – 13, * $P < 0.05$, + $P < 0.01$; **Figure 4.2C**). PMX205 treatment also significantly slowed the disease progression as the age at which it reached 25, 50, 75 and 100% decline in maximal grip strength was delayed (n = 12 – 13, * $P < 0.05$, ** $P < 0.01$, **Figure 4.2D**).

Finally, to confirm that CD88 is a valid therapeutic target in ALS, we also generated hSOD1^{G93A} mice lacking CD88, by crossing hSOD1^{G93A} mice with CD88$^{-/-}$ mice (**hSOD1^{G93A} x CD88$^{-/-}$**). These hSOD1^{G93A} x CD88$^{-/-}$ showed a similarly extended survival to PMX205 treated hSOD1^{G93A} mice when compared to hSOD1^{G93A} mice (mean end stage of disease, hSOD1^{G93A} = 168.2 ± 1.6 days and hSOD1^{G93A} x CD88$^{-/-}$ = 176.1 ± 2.1 days, n = 24, $P < 0.01$; **Figure 4.3A**).

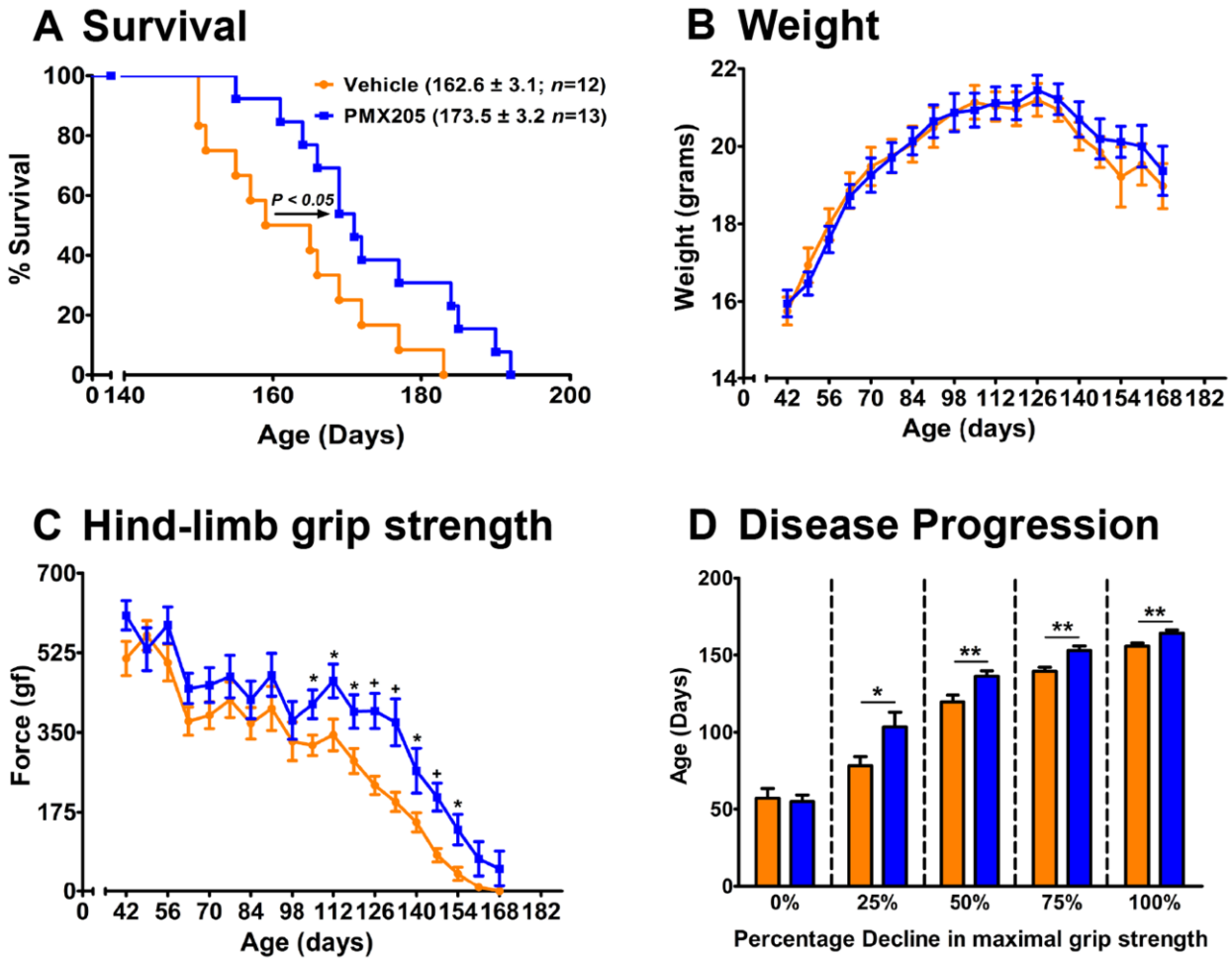

Figure 4.2: "Pre-Onset" PMX205 treatment at 9mg/kg/day extends survival, improves hind-limb grip strength and slows disease progression in hSOD1^{G93A} transgenic mice. *hSOD1^{G93A} mice were orally dosed with the selective CD88 antagonist PMX205 at 35 days of age (9mg/kg/day).* ***A*** *shows a Kaplan-Meier plot of ages (in days) in which hSOD1^{G93A} mice were treated with dH_2O (vehicle; orange line) or PMX205 (blue line) reached end-stage of disease (complete hind-limb paralysis and an inability to right itself once placed on its back; n = 12 – 13, P < 0.05, log-rank test). PMX205 treatment at 35 days of age (9mg/kg/day) resulted in significant extension in survival time compared with vehicle treatment.* ***B*** *shows no difference in weight between vehicle (orange line) and PMX205 (blue line) treated hSOD1^{G93A} mice (n = 12 – 13, P > 0.05, student t-test).* ***C*** *shows significant improvement in hind-limb grip strength for PMX205 treated versus vehicle treated hSOD1^{G93A} mice at 105 days of age (n = 12 – 13, * P<0.05, + P<0.01, student t-test).* ***D*** *shows slowed disease progression (determined by age at which maximal grip strength decline at 25, 50, 75 and 100%) in hSOD1^{G93A} mice treated with PMX205(blue bar) when compared with vehicle treated hSOD1^{G93A} mice (orange bar) at 25, 50, 75 and 100% decline in maximal grip strength (n = 12 – 13, * P < 0.05, ** P < 0.01, student t-test). hSOD1^{G93A} mice lacking C5a signaling via CD88 shows a significant extension in survival, improved hind-limb grip strength and slowed disease progression, demonstrating a pathogenic role for C5a in this mutant ALS mouse model. Data expressed as mean ± SEM.*

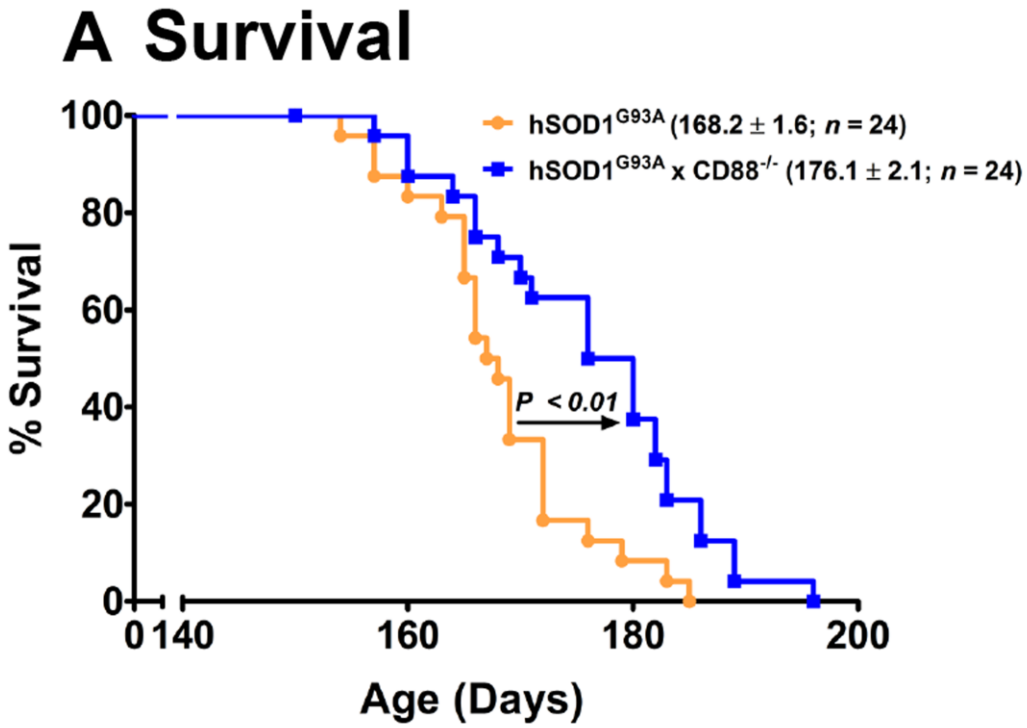

Figure 4.3: The C5a receptor CD88 contributes to disease progression in the hSOD1^{G93A} genetic model of ALS. A *shows a Kaplan-Meier plot of ages (in days) in which hSOD1^{G93A} mice with normal (CD88$^{+/+}$, orange line) or fully deleted (CD88$^{-/-}$, blue line) CD88 reached end-stage of disease (complete hind-limb paralysis and an inability to right itself once placed on its back; n = 24, P < 0.01, log-rank test). hSOD1^{G93A} mice lacking CD88 showed a similarly extended survival to PMX205 treated hSOD1^{G93A} mice. Data expressed as mean ± SEM.*

4.2.2 Post-Onset treatment with CD88 antagonist does not affect motor performance but extends survival and slows disease progression in hSOD1^{G93A} mice.

We next aimed to determine if CD88 inhibition at a later stage of disease could still reduce ALS pathology in hSOD1^{G93A} mice. This is important, as ALS patients generally present in the clinic with manifest disease, where motor neuron loss has already progressed. Thus, this experiment provides a scenario which would more closely model a therapeutic intervention for human ALS patients. hSOD1^{G93A} mice were therefore orally dosed with PMX205 at 91 days of age with the efficacious dose of 9mg/kg/day. As shown in Chapter 3, 91 days is the age at which there is already considerable motor decline, and motor neuron loss. PMX205 treatment in hSOD1^{G93A} mice from this post-onset disease age showed an increase in survival time when compared to vehicle treated hSOD1^{G93A} mice (mean end stage of disease, vehicle = 169.2 ± 2.8 days and PMX205 = 179.7 ± 1.8 days, $n = 9$, $P < 0.01$; **Figure 4.4A**). However post-onset PMX205 treatment did not affect the body weight and hind-limb grip strength of hSOD1^{G93A} mice when compared to vehicle treatment ($n = 9$, $P > 0.05$; **Figure 4.4B** and **4.4C**). Importantly though, PMX205 treatment significantly slowed disease progression at the later stages of disease as the age at which it reached 75 and 100% decline in maximal grip strength was delayed ($n = 9$, * $P < 0.05$, **Figure 4.4D**). This demonstrates that treatment of ALS mice with PMX205, well into their disease progression, can still have therapeutic benefits.

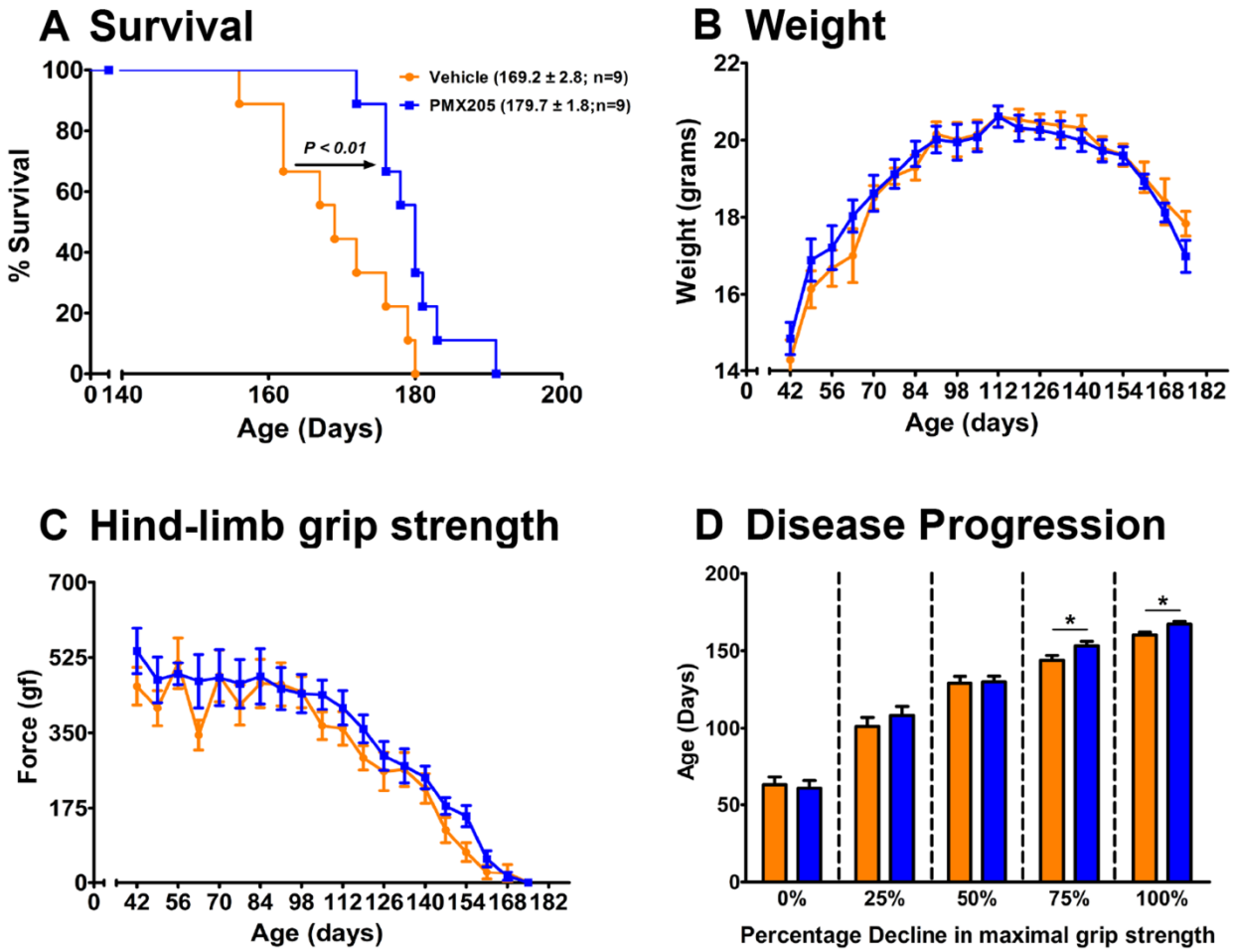

Figure 4.4: "Post-Onset" PMX205 treatment at 9mg/kg/day does not affect motor performance but extends survival and slows disease progression at later stages in hSOD1^{G93A} transgenic mice. *hSOD1^{G93A} mice were orally dosed with the selective CD88 antagonist PMX205 at 91 days of age (9mg/kg/day).* ***A*** *shows a Kaplan-Meier plot of ages (in days) in which hSOD1^{G93A} mice treated with dH_2O (vehicle; orange line) or PMX205 (blue line) reached end-stage of disease (complete hind-limb paralysis and an inability to right itself once placed on its back; n = 9, P < 0.01, log-rank test). PMX205 treatment at 91 days of age (9mg/kg/day) resulted in significant extension in survival time compared with vehicle treatment.* ***B*** *and* ***C*** *shows no difference in weight and hind-limb grip strength between vehicle (orange line) and PMX205 (blue line) treated hSOD1^{G93A} mice (n = 9, P > 0.05, student t-test).* ***D*** *shows slowed disease progression (determined by age at which maximal grip strength decline at 25, 50, 75 and 100%) in hSOD1^{G93A} mice treated with PMX205 (blue bar) when compared to vehicle treated hSOD1^{G93A} mice (orange bar) at 75% and 100% decline in maximal grip strength (n = 9, * P < 0.05, student t-test). Data expressed as mean ± SEM.*

4.2.3 hSOD1^{G93A} mice lacking CD88 have reduced microglia and monocyte mRNA levels when compared to hSOD1^{G93A} mice at end-stage of disease progression.

We next aimed to identify some of the underlying mechanisms for protection from pathology in mice treated with PMX205, or deficient in CD88. Since the studies described above used PMX205-treated mice which were taken to end-stage of disease, we decided to use hSOD1^{G93A} x CD88$^{-/-}$ for these experiments. This allowed us to collect tissue from animals at different ages throughout disease progression. Importantly, inhibition of CD88 using PMX205 (pharmacological approach) or hSOD1^{G93A} mice lacking CD88 (genetic approach) showed similarly extended survival when compared to vehicle and hSOD1^{G93A} mice, validating this approach. Given our previous experiments demonstrating marked CD88 accumulation in activated microglia in hSOD1^{G93A} mice (Chapter 3), we focussed our attention on glial activation markers, and their downstream activation products. CD88 activation is known to have generally pro-inflammatory functions on microglia (Woodruff et al., 2010) and microglia activation is known to contribute to ALS pathology (Chiu et al., 2013).

We first investigated whether the absence of CD88 in hSOD1^{G93A} mice had any effect on astrocytosis and microgliosis in the lumbar spinal cord. mRNA expression levels of GFAP (marker of astrocytes), Iba-1 (marker of both resident microglia and infiltrating monocyte/macrophages), Entpd1 (CD39; predominant marker of resident microglia) and Ly6C (predominant marker of early infiltrating monocyte/macrophages) were measured in the lumbar spinal cord of hSOD1^{G93A} mice and hSOD1^{G93A} x CD88$^{-/-}$ mice at mid-symptomatic and end-stage of disease progression using quantitative real time PCR. GFAP transcripts were unaltered in hSOD1^{G93A} x CD88$^{-/-}$ mice at mid-symptomatic and end-stage of disease when compared to hSOD1^{G93A} mice (n = 6, $P > 0.05$; **Figure 4.5A**), in line with the lack of expression of CD88 on astrocytes (Chapter 3). Iba-1 transcripts however, were significantly reduced in hSOD1^{G93A} x CD88$^{-/-}$ mice by 0.37 fold at end-stage of disease when compared to hSOD1^{G93A} mice respectively (n = 6, ** $P < 0.01$; **Figure 4.5B**). Entpd1 mRNA expression was initially increased in hSOD1^{G93A} x CD88$^{-/-}$ mice by 0.6 fold at mid-symptomatic, and decreased at end-stage of disease by 0.34 fold when compared to hSOD1^{G93A} mice (n = 6, * $P < 0.05$, *** $P < 0.001$; **Figure 4.5C**). Ly6C mRNA was also decreased at end-stage by 0.24 fold when compared to hSOD1^{G93A} mice (n = 6, * $P < 0.05$; **Figure 4.5D**).

4.2.4 hSOD1^{G93A} mice lacking CD88 have reduced cytokine tumour necrosis factor α and interleukin-1β levels when compared to hSOD1^{G93A} mice at end-stage of disease progression.

Activation of CD88 also induces synthesis of cytokines to modulate inflammatory processes, and has been shown to induce cytokine expression in microglia (Woodruff et al., 2010). Importantly,

pro-inflammatory cytokine such as tumour necrosis factor α (**TNFα**) and interleukin-1β (**IL-1β**) are thought to propagate disease progression in ALS (Elliott, 2001, Nguyen et al., 2001, Hensley et al., 2003). Hence we investigated whether the absence of CD88 in hSOD1^{G93A} mice had any effect on the cytokine TNFα and IL-1β levels in the lumbar spinal cord. mRNA expression of TNFα and IL-1β were measured in the lumbar spinal cord of hSOD1^{G93A} mice and hSOD1^{G93A} x CD88$^{-/-}$ mice at mid-symptomatic and end-stage of disease progression using quantitative real time PCR. TNFα transcripts were significantly reduced in hSOD1^{G93A} x CD88$^{-/-}$ mice by 0.38 fold at end-stage of disease when compared to hSOD1^{G93A} mice (n = 6, *** P < 0.001; **Figure 4.6A**). IL-1β mRNA expression was also decreased in hSOD1^{G93A} x CD88$^{-/-}$ mice by 0.52 fold at end-stage of disease when compared to hSOD1^{G93A} mice (n = 6, *** P < 0.001; **Figure 4.6B**).

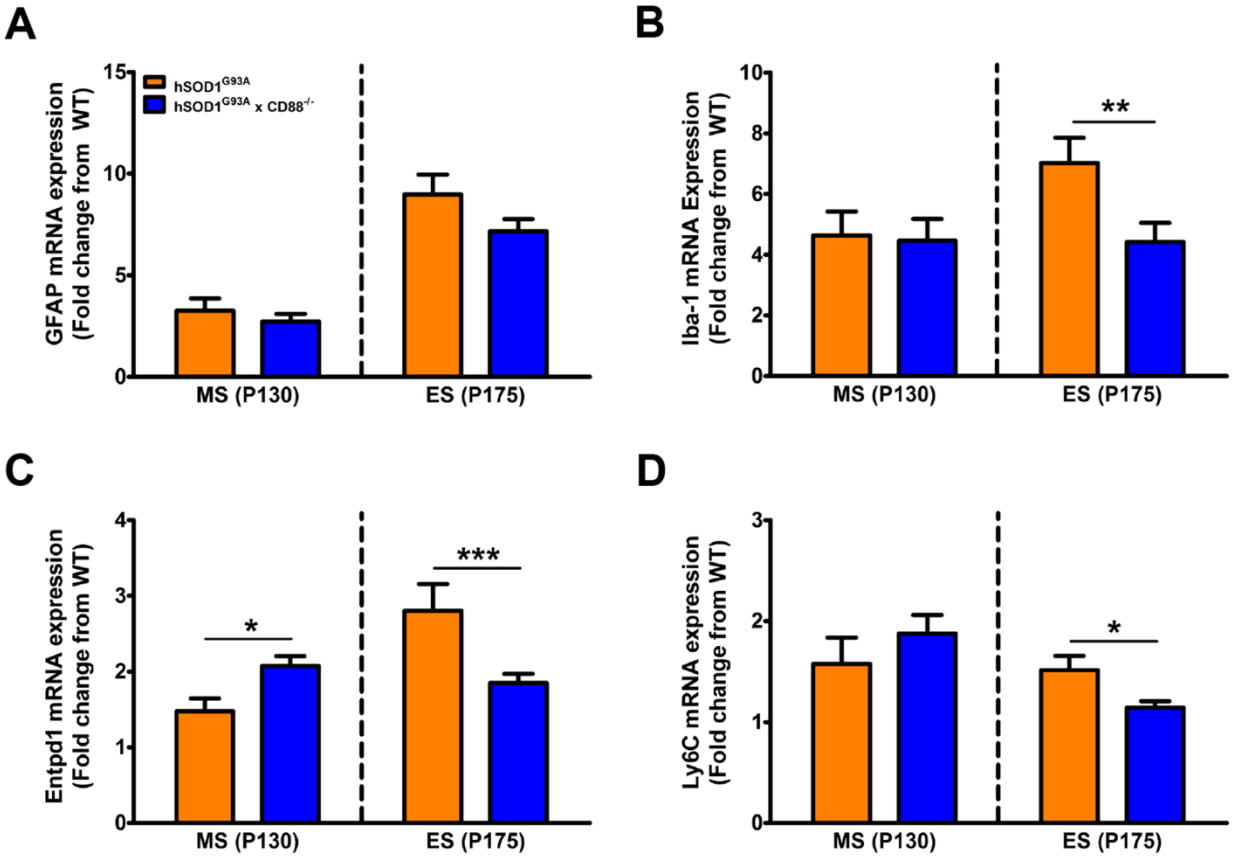

Figure 4.5: hSOD1^{G93A} mice lacking CD88 have reduced microglia and monocyte mRNA levels when compared with hSOD1^{G93A} mice at end-stage of disease progression. (A) to (D) mRNA expressions of GFAP (astrocytes, A), Iba-1 (microglia/monocyte, B), Entpd1 (CD39; microglia, C) and Ly6C (monocyte, D) in lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched hSOD1^{G93A} mice lacking CD88 (hSOD1^{G93A} x CD88$^{-/-}$) at mid-symptomatic and end-stage of disease progression. Data expressed as mean ± SEM (n = 6 mice/group; *P <0.05, **P <0.01, ***P <0.001, Student t test). MS, mid-symptomatic = 130 days postnatal (P130); ES, end-stage = 175 days postnatal (P175).

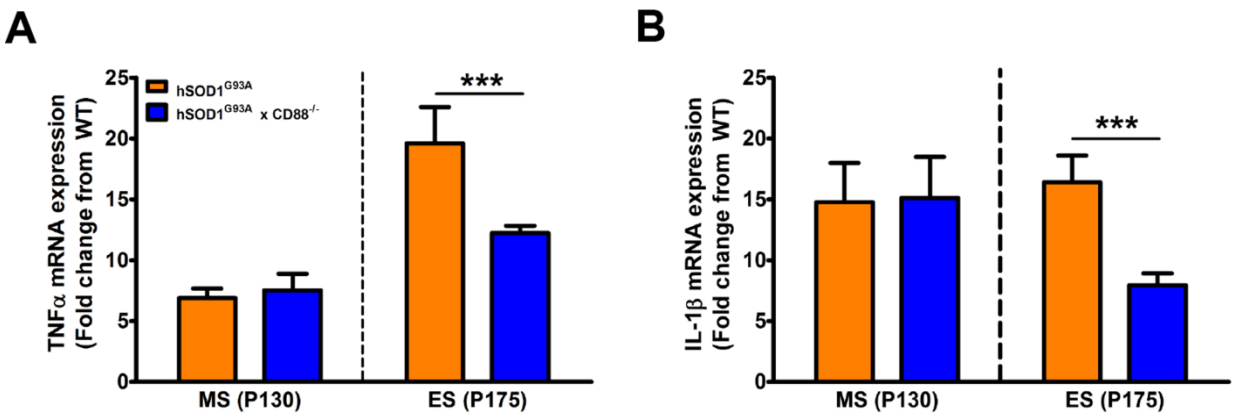

Figure 4.6: hSOD1^{G93A} mice lacking CD88 have reduced cytokine tumour necrosis factor α and interleukin-1β mRNA levels when compared with hSOD1^{G93A} mice at end-stage of disease progression. ***(A)*** *mRNA expression of TNFα in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched hSOD1^{G93A} mice lacking CD88 (**hSOD1^{G93A} x CD88$^{-/-}$**) at mid-symptomatic and end-stage of disease progression.* ***(B)*** *mRNA expression of IL-1β in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched hSOD1^{G93A} x CD88$^{-/-}$ at mid-symptomatic and end-stage of disease progression. Data expressed as mean ± SEM (n = 6 mice/group; ***P <0.001, Student t test). MS, mid-symptomatic = 130 days postnatal (**P130**); ES, end-stage = 175 days postnatal (**P175**).*

4.3 DISCUSSION

Currently there is no effective treatment for ALS. Although the pathogenesis of ALS is still unclear, there is convincing evidence that complement factors are involved in promoting disease progression. Previous studies have demonstrated that C1q, C3 and C4 mRNA expression are significantly increased during ALS progression in hSOD1^{G93A} mice. However, given that deletion of C1q, C3 and C4 in hSOD1 transgenic mouse models did not show any beneficial effects on the disease progression of ALS, it indicates that blocking upstream complement activation pathways does not contribute to ALS mice neurotoxicity. Hence, it is possible instead, that downstream complement factors, potentially generated by the fourth "extrinsic pathway", may play a role in the disease progression of ALS. In support of this finding, we have shown that chronic administration of a specific CD88 antagonist PMX205 in hSOD1^{G93A} transgenic rats have markedly delayed the onset of motor symptoms and increased survival, compared to untreated animals (Woodruff et al., 2008a). In addition, up-regulation of CD88 has also been observed in numerous neurodegenerative diseases in rodents (Yasojima et al., 1999, Singhrao et al., 1999, Ager et al., 2010, Woodruff et al., 2006), including ALS (Woodruff et al., 2008a, Humayun et al., 2009), so it is plausible to propose that downstream complement C5a-CD88 signalling may have a pathogenic role in the disease progression of ALS (Lee et al., 2013).

The present study demonstrates that inhibition of CD88 using oral administration of a specific CD88 antagonist significantly improved hind-limb grip strength, slowed disease progression and extended survival in the most commonly used model of ALS (hSOD1^{G93A} mice). Additionally, inhibition of CD88 using hSOD1^{G93A} x CD88$^{-/-}$ mice also showed similarly extended survival. Furthermore, I showed that hSOD1^{G93A} x CD88$^{-/-}$ mice also showed reductions in microglia, monocytes and cytokine mRNA levels when compared to hSOD1^{G93A} mice. Taken together these results indicate that CD88 activation in hSOD1^{G93A} mice actively contributes to the pathogenesis of ALS, potentially through over-activation of reactive microglia.

4.3.1 Treatment with CD88 antagonist improves motor function, slows disease progression and extends survival time

The present study provided evidence for a pathophysiological role of CD88 in hSOD1^{G93A} transgenic mice. Specifically, we inhibited CD88 activation with the CD88 selective antagonist PMX205. This orally active compound crosses the blood-brain barrier and has been shown to have a protective effect in multiple models of neurodegenerative diseases such as Huntington's disease and Alzheimer's disease (Woodruff et al., 2006, Fonseca et al., 2009). In our preliminary experiments, treatment with PMX205 at concentration of 3mg/kg/day did not extend survival time,

improve motor performance or slow disease progression in hSOD1^{G93A} mice in contrast to its protective effect in other neurodegenerative diseases, suggesting that the concentration of PMX205 used was not optimal in hSOD1^{G93A} mice. Indeed, a recent study by our colleagues demonstrated that an 8mg/kg/day oral dose was required to achieve efficacy in a mouse model of inflammatory bowel disease; a 4mg/kg/day dose was without effect (Jain et al., 2013). Hence I also dosed ALS mice with PMX205 at a higher concentration of 9mg/kg/day and our results were striking. PMX205 treatment of hSOD1^{G93A} mice at 9mg/kg/day resulted in a significant extension in survival time, improved hind-limb grip strength and slowed disease progression. This is in agreement with our previous studies conducted in hSOD1^{G93A} transgenic rats where it was shown PMX205 significantly extended survival time and delayed the onset of motor dysfunction. Our result was also confirmed with hSOD1^{G93A} x CD88$^{-/-}$ mice as these mice showed similarly extended survival when compared to hSOD1^{G93A} mice. Taken together this suggests that increased CD88 activation in hSOD1^{G93A} mice actively contributes to the pathogenesis seen in this model of ALS. These findings also demonstrate that optimal selection of PMX205 dosing is required to achieve efficacy. There are previous reports of a lack of efficacy with PMX205 in a hSOD1 mouse model of ALS (Klos et al., 2013), and based on our current findings, this may be due to an inadequate dose of PMX205 used (*P. Monk personal communication*). The reasons why a higher dose of PMX205 was required in our ALS studies, whereas a lower (~2mg/kg/day) dose was required in a transgenic mouse model of Alzheimer's disease is unknown (Fonseca et al., 2009). However, importantly, it should be noted that these 2 models use distinctly different strains of mice, which might explain these differences. We are currently performing pharmacokinetic studies to quantify the concentrations of PMX205 in the brain after oral dosing, and to correlate this with efficacy, in order to optimise therapeutic dosing of PMX205 in future studies.

Our drug treated experiments included hSOD1^{G93A} mice dosed from very early age (Day 35) to determine the maximum effect of CD88 inhibition, as well as at a later point after the onset of motor deficits (Day 91). Our results showed that there was no significant difference in survival time extension between the two dosing groups, suggesting that the pathogenic involvement of C5a occurs subsequent to day 91. This is in agreement with our previous experiments showing up-regulation of CD88 at 130 and 175 days in hSOD1^{G93A} mice, indicating that C5a exacerbates disease pathology but does not initiate the onset of disease (Lee et al., 2013). Although there was no difference in survival time extension between the two dosing groups, hSOD1^{G93A} mice treated post onset of motor deficit did not show improved hind-limb grip strength but delayed age reaching 75% and 100% decline in their maximal grip strength (i.e. slowed disease progression). The onset of motor deficit measured by hind-limb grip strength is 70 days in hSOD1^{G93A} mice which also

correlates with the decline in motor neuron numbers (Lee et al., 2013). Hence treating these animals at Day 91 when there is significant decline in motor neuron numbers would be expected to only show minimal improvement in motor functions.

4.3.2 Reduced microglia/monocytes and cytokine levels in hSOD1^{G93A} mice lacking CD88

Many studies have shown that motor neuron death in ALS animal models is exacerbated by toxic signals emanating from non-neuronal neighbouring cells (astrocytes and microglia); via an inflammatory response that accelerates disease progression (Kreutzberg, 1996, Nagai et al., 2007). Our previous study showed strong co-localisation of CD88 to microglia and minimal co-localisation of CD88 to astrocytes in hSOD1^{G93A} transgenic mice. Hence to elucidate the mechanism of C5a-CD88 signalling in microglia in the disease progression of ALS, we investigated whether the absence of CD88 in hSOD1^{G93A} mice had any effect on astrocytosis and microgliosis. We showed that there was no difference in astrocyte mRNA levels between hSOD1^{G93A} and hSOD1^{G93A} x CD88$^{-/-}$ mice while there was a significant reduction in microglia mRNA levels (Iba-1 and CD39) in hSOD1^{G93A} x CD88$^{-/-}$ mice. This suggests that at least some of the therapeutic benefits seen with CD88 antagonism could be due to the reduction of C5a-mediated activation of microglia. In addition to microglia, we have also demonstrated that hSOD1^{G93A} x CD88$^{-/-}$ mice has decreased levels of inflammatory monocytes. This supports recent studies where they have shown that using anti-Ly6C mAb in hSOD1^{G93A} mice slowed disease progression and attenuated neuronal damage by decreasing infiltration of Ly6C-positive monocytes to the spinal cord (Butovsky et al., 2012). This suggests that C5a-CD88 signalling may play a role in recruiting inflammatory monocytes into the lumbar spinal cord that contributes to the pathogenesis of ALS.

Microglia are a well-known contributor to the pathology observed in hSOD1 mouse models of ALS (Henkel et al., 2009). Microglia derived from hSOD1-transgenic mice release reactive oxygen species and cytokine that induce motor neuron cell death (Wu et al., 2006, Tonges et al., 2014, Beers et al., 2008, Hensley et al., 2002, Alexianu et al., 2001). Several studies have shown that expression of pro-inflammatory cytokines such as TNFα and IL-1β is an early event in hSOD1 mouse models of ALS and promotes disease progression (Elliott, 2001, Nguyen et al., 2001, Hensley et al., 2003). We show here that there is a reduction in both TNFα and IL-1β in the lumbar spinal cord of hSOD1^{G93A} x CD88$^{-/-}$ mice when compared to hSOD1^{G93A} mice. This suggests that lack of C5a-CD88 signalling in the microglia reduces cytokine levels, ultimately leading to reduced inflammation which attenuates disease pathogenesis in this model of ALS. Our findings are also consistent with those of Meissner et al., 2010, where they showed extended survival and slowed disease progression in hSOD1^{G93A} mice lacking IL-1β (Meissner et al., 2010). Unexpectedly, there

is a conflicting literature on the neurotoxic effect of TNFα in hSOD1^{G93A} mice as they reported that disruption of TNFα in hSOD1^{G93A} mice failed to influence survival or progression of disease (Gowing et al., 2006). However, an increase in transcripts encoding for IL-1β was observed in these animals. Hence suggesting that up-regulation of IL-1β may be part of a compensatory process (Gowing et al., 2006). Importantly, C5a-CD88 signalling can inhibit the release of both pro-inflammatory cytokine TNFα and IL-1β, which would likely contribute to the disease progression of ALS.

4.4 CONCLUSION

In summary, the present study has demonstrated that inhibition of CD88 using oral administration of a specific CD88 antagonist significantly improved hind-limb grip strength, slowed disease progression and extended survival. Additionally, inhibition of CD88 using hSOD1^{G93A} x CD88$^{-/-}$ mice also showed similarly extended survival, suggesting that C5a-CD88 signalling in these animals contributes to motor neuron death and ultimately disease progression of ALS. Expression of microglia, monocytes and cytokine (TNFα and IL-1β) transcripts was also reduced in hSOD1^{G93A} x CD88$^{-/-}$ mice. Taken together these results indicate that inhibition of CD88 significantly attenuates disease progression potentially by reducing levels of microglia, monocytes and cytokines which all have an important role in motor neuron death and contributes to the pathology in the hSOD1^{G93A} mice. Specifically, our studies suggest that inhibiting the pro-inflammatory C5 activation fragment, C5a, which is central to, and generated by, all complement pathways, may be an important therapeutic strategy to treat ALS.

CHAPTER 5

"Absence of C3a-C3aR signalling worsens disease pathology in a mouse model of Amyotrophic lateral sclerosis"

5.1 INTRODUCTION

Many studies including our own have shown that complement activation is involved in the pathogenesis of Amyotrophic lateral sclerosis (**ALS**) (Chapters 3, 4). However, recent studies have shown that inhibition of upstream complement components C1q, C3 and C4 in hSOD1 transgenic mouse models did not show any beneficial effects on the disease progression of ALS (Chiu et al., 2009, Lobsiger et al., 2013). Hence, these combined studies indicate that blocking upstream complement activation pathways does not alter the disease course in hSOD1 transgenic mice. However, it introduces the new possibility that downstream complement factors, potentially generated by the fourth "extrinsic pathway", may play a role in the disease progression of ALS (Woodruff et al., 2014).

Complement activation triggers the generation of cleavage products, including the complement peptides C3a and C5a (Klos et al., 2013). C5a-CD88 signalling has been studied extensively to show a pathogenic role in the disease progression of ALS by inducing chemotaxis and activation of microglia, with subsequent release of pro-inflammatory cytokine tumour necrosis factor α (**TNFα**) and interleukin-1β (**IL-1β**) as mentioned in chapter 4 (Woodruff et al., 2014). By contrast to C5a, the biological action of C3a in the disease progression of ALS has never been studied. Although less work has been done with C3a, several studies have suggested that C3a-C3aR signalling could have several protective roles in the central nervous system diseases as mentioned in chapter 1 (Yanamadala and Friedlander, 2010).

In the present study, we examined the expression and cellular location of central component C3 and its receptor C3aR during disease progression. We also examined the effect of C3aR deletion on the disease progression of ALS using $hSOD1^{G93A}$ mice lacking C3aR (**$hSOD1^{G93A}$ x $C3aR^{-/-}$**). $hSOD1^{G93A}$ x $C3aR^{-/-}$ mice had significantly reduced hind-limb grip strength and body weight. These mice also had accelerated disease progression and shortened survival time. $hSOD1^{G93A}$ x $C3aR^{-/-}$ mice also showed up-regulation of astrocytes and microglia mRNA levels but down-regulation of monocyte mRNA level. $hSOD1^{G93A}$ x $C3aR^{-/-}$ mice also showed no change in cytokine levels when compared to $hSOD1^{G93A}$ mice. These results suggest a novel neuroprotective role for

C3aR in hSOD1^{G93A} mice and identify C3aR up-regulation or activation as a novel target to slow down the progression of disease progression in ALS patients.

5.2 RESULTS

5.2.1 C3 and C3aR are up-regulated during disease progression in hSOD1^{G93A} mice

The mRNA expression levels for C3 and C3aR in the lumbar spinal cord of hSOD1^{G93A} mice were normalised and compared to wild-type (**WT**) mice during disease progression (30 days to 175 days) using quantitative real time PCR. C3 mRNA expression was increased by 1.2 fold at pre-symptomatic, 1.6 fold at onset, 1.6 fold at mid-symptomatic and 12.9 fold at end stage of disease, when compared with WT mice (n = 9, * P < 0.05, ** P < 0.01, *** P < 0.001; **Figure 5.1A**). The mRNA expression level of C3aR in the lumbar spinal cords of hSOD1^{G93A} mice also increased by 1.4 fold, 5.2 fold and 34.0 fold at onset, mid-symptomatic and end stage respectively, when compared to WT mice (n=9, * P < 0.05 and *** P < 0.001; **Figure 5.1B**).

Next we immuno-stained the lumbar spinal cords from hSOD1^{G93A} and WT mice for C3 and its activation product C3b with specific cellular markers for motor neurons (anti-ChAT), microglia (anti-Iba-1) and astrocytes (anti-GFAP). C3/C3b appeared primarily on motor neurons and astrocytes in hSOD1^{G93A} mice (white arrows in **Figure 5.2L** and **5.2R**), compared to WT where there was little to no C3/C3b staining (**Figure. 5.2A-C** and **Figure. 5.2G -I**). Furthermore we did observe minimal C3/C3b staining on Iba-1 labelled microglia in hSOD^{G39A} mice but not in WT mice (**Figure. 5.2D-F** for WT mice and **Figure 5.2M-O** for hSOD1^{G93A} mice).

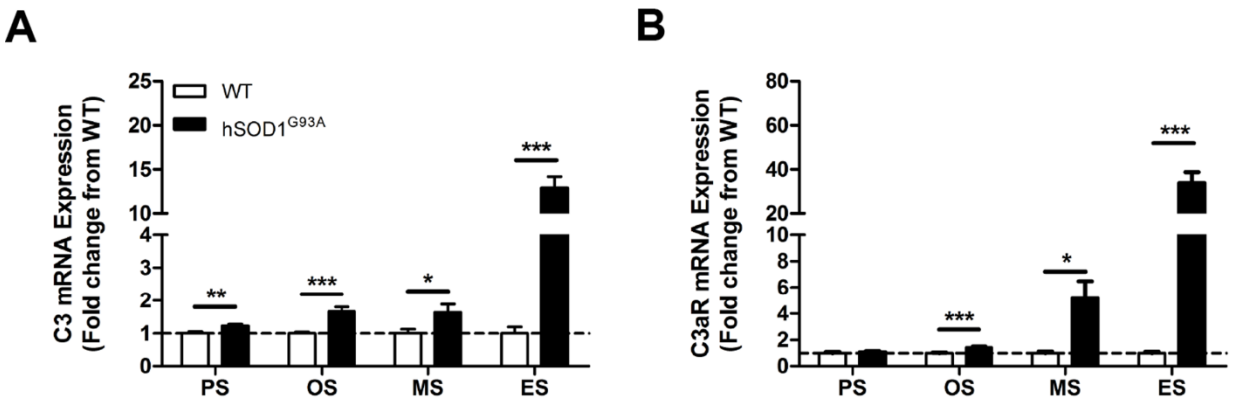

Figure 5.1: Increased expression of C3 and C3aR in hSOD1^{G93A} mice relative to control wild-type mice during disease progression. *(**A**) mRNA expression of C3 in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched wild-type (**WT**) mice at four different ages. (**B**) mRNA expression profile of C3aR in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched WT mice at four different ages. Dashed lines, baseline expression in WT controls at each time point. Data expressed as mean ± SEM (n = 9 mice/group; *P <0.05, ** P < 0.01, ***P <0.001, Student t test). PS, pre-symptomatic; OS, onset; MS, mid-symptomatic; ES, end stage.*

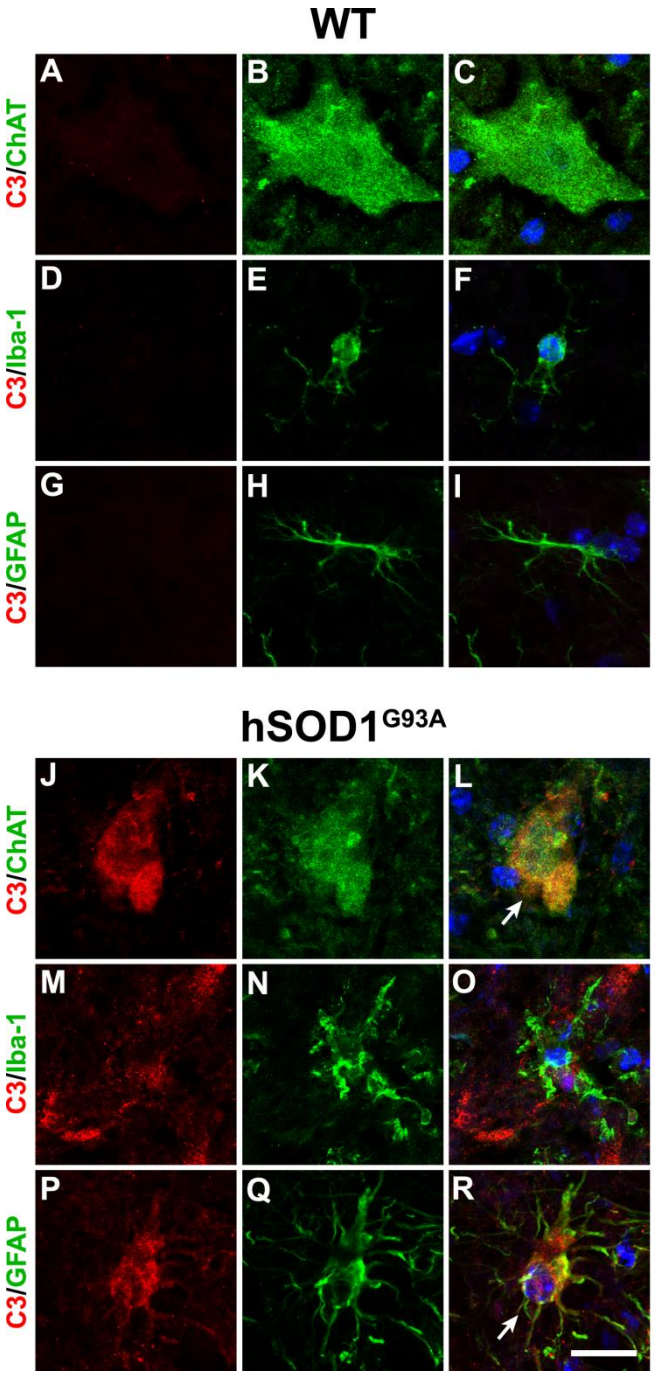

Figure 5.2: Expression of C3/C3b in wild-type and hSOD1^{G93A} mice at end-stage of disease. *(**A**) to (**R**) Double immunolabelling of C3/C3b (red) with cellular markers (green) for motor neurons (ChAT; (**A**) to (**C**) wild-type (WT) mice, (**J**) to (**L**) hSOD1^{G93A} mice), microglia (Iba-1; (**D**) to (**F**) WT mice, (**M**) to (**O**) hSOD1^{G93A} mice), and astrocytes (GFAP; (**G**) to (**I**) WT mice, (**P**) to (**R**) for hSOD1^{G93A} mice) in the ventral lumbar spinal cord of WT and hSOD1^{G93A} mice at end-stage of disease. There was minimal expression of C3/C3b in WT (**A**, **D** and **G**) with marked increase in hSOD1^{G93A} mice (**J**, **M** and **P**). In hSOD1^{G93A} mice, C3/C3b was co-localised with ChAT-positive motor neurons (white arrows in **L**). There was little to no co-localisation of C3/C3b with Iba-1 positive microglia (**O**), and strong co-localisation with GFAP-labelled astrocytes (white arrows in **R**). Scale bars for all panels = 20 μM.*

5.2.2 C3aR is localised to motor neurons and astrocytes with minimal localisation to microglia in hSOD1^{G93A} mice

We also aimed to determine the cellular localisation of C3aR that has contributed to the increased expression seen in hSOD1^{G93A} mice. To achieve this, we performed immuno-labelling for C3aR on lumbar spinal cord sections from hSOD1^{G93A} and WT mice. These sections were immuno-stained for C3aR with specific cellular markers to identify motor neurons (anti-ChAT), microglia (anti-Iba-1) and astrocytes (anti-GFAP).

In WT mice C3aR staining was observed on lumbar motor neurons (**Figure 5.3A**). C3aR-stained cells were readily identified as motor neurons due to their large size, location and distinctive morphology (Banks et al., 2005). This was confirmed by double labelling with the motor neuron marker ChAT (white arrow in **Figure 5.3C**). Next, we examined if C3aR immuno-staining was present on surrounding microglia and astrocytes in sections of WT lumbar spinal cords. We observed no co-localization of C3aR to Iba-1 positive microglia (**Figure 5.3D** and **5.3F**) and GFAP-positive astrocytes (**Figure 5.3G** to 5.**3I**) in WT mice. Following this demonstration of C3aR immuno-labelling in WT lumbar spinal cord, we then investigated C3aR cellular localisation in hSOD1^{G93A} mice. C3aR was also expressed on the few remaining motor neurons seen at the end-stage of disease (white arrow in **Figure 5.3L**). In hSOD1^{G93A} mice, C3aR appeared primarily on GFAP-positive astrocytes (white arrow in **Figure 5.3R**) with no observable C3aR staining on Iba-1 positive microglia in hSOD^{G39A} mice (**Figure 5.3M-O**).

5.2.3 hSOD1^{G93A} mice lacking C3aR have shortened survival, poorer motor performance and faster disease progression when compared to hSOD1^{G93A} mice

Given our findings of strong up-regulation of C3aR, and its ligand precursor C3, in hSOD1^{G93A} mice, we next sought to determine the role of this potential increased C3aR-activation in these mice by generating hSOD1^{G93A} mice lacking C3aR. Interestingly, in stark contrast to hSOD1^{G93A} mice lacking CD88, an absence of C3aR in hSOD1^{G93A} mice (**hSOD1^{G93A} x C3aR$^{-/-}$**) showed shortened survival when compared to hSOD1^{G93A} mice (mean end stage of disease, hSOD1^{G93A} = 167.2 ± 2.2 days and hSOD1^{G93A} x C3aR$^{-/-}$ = 158.2 ± 1.7 days, n = 24, $P < 0.01$; **Figure 5.4A**). Concomitant with this, there was a decrease in weight of hSOD1^{G93A} x C3aR$^{-/-}$ mice when compared to hSOD1^{G93A} mice at 56 days of age (n = 12, * $P < 0.05$, + $P < 0.01$, # $P < 0.001$, **Figure 5.4B**). At 77 days hSOD1^{G93A} x C3aR$^{-/-}$ mice also showed a significant reduction in hind-limb grip strength when compared to hSOD1^{G93A} mice (n = 12, * $P < 0.05$, + $P < 0.01$, # $P < 0.001$, **Figure 5.4C**). Absence of C3aR in hSOD1^{G93A} mice also significantly accelerated the disease progression as the

age at which it reached 50 and 100% decline in maximal grip strength was faster in hSOD1^{G93A} x C3aR$^{-/-}$ when compared to hSOD1^{G93A} mice (n = 12, * P < 0.05, *** P < 0.001, **Figure 5.4D**).

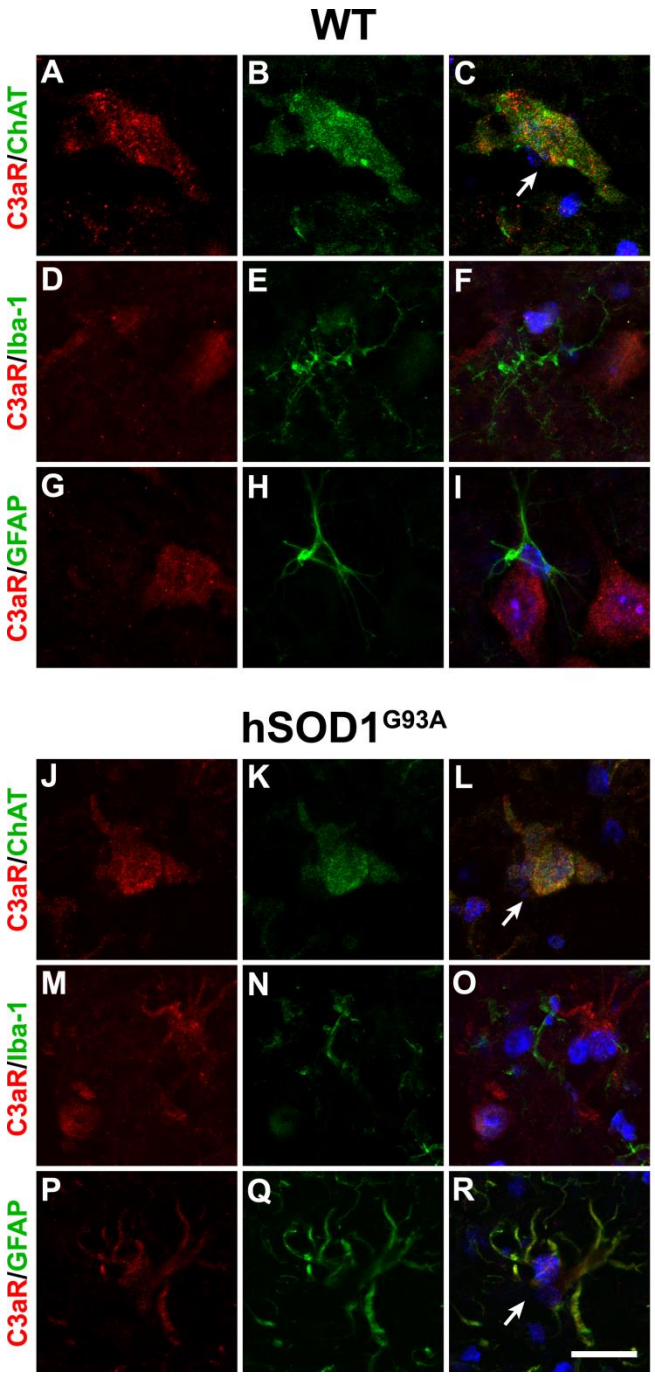

Figure 5.3: Expression of C3aR in wild-type and hSOD1^{G93A} mice at end-stage of disease. (A) *to* ***(R)*** *Double immunolabelling of C3aR (red) with cellular markers (green) for motor neurons (ChAT; (**A**) to (**C**) wild-type (**WT**) mice, (**J**) to (**L**) hSOD1^{G93A} mice), microglia (Iba-1; (**D**) to (**F**) WT mice, (**M**) to (**O**) hSOD1^{G93A} mice), and astrocytes (GFAP; (**G**) to (**I**) WT mice, (**P**) to (**R**) for hSOD1^{G93A} mice) in the ventral lumbar spinal cord of WT and hSOD1^{G93A} mice at end-stage of disease. C3aR was mainly co-localised with ChAT-positive motor neurons in the WT mice (**C**, white arrow) with minimal co-localisation with Iba-1 labelled microglia and GFAP positive astrocytes (**F** and **I**). In hSOD1^{G93A} mice, C3aR immunolabelling was evident on ChAT positive motor neurons and GFAP positive astrocytes (**L** and **R**, white arrows). Scale bars for all panels = 20 μM.*

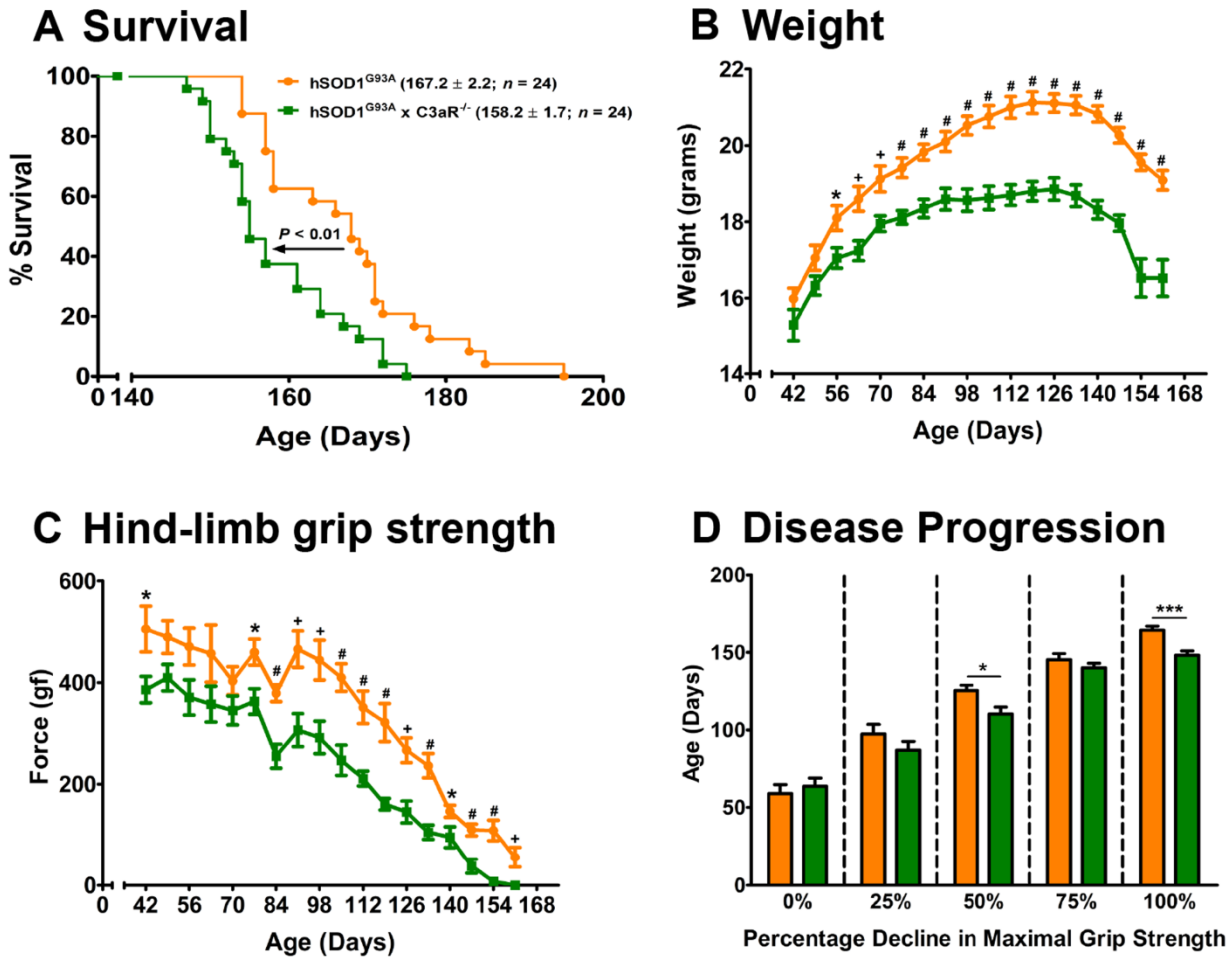

Figure 5.4: hSOD1^{G93A} mice lacking C3aR have shortened survival, poorer motor performance and faster disease progression when compared to hSOD1^{G93A} mice. ***A*** *shows a Kaplan-Meier plot of ages (in days) in which hSOD1^{G93A} mice with normal (C3aR$^{+/+}$, orange line) or fully deleted (C3aR$^{-/-}$, green line) C3aR reached end-stage of disease (complete hind-limb paralysis and an inability to right itself once placed on its back; n = 24, P < 0.01, log-rank test). hSOD1^{G93A} mice lacking C3aR (**hSOD1^{G93A} x C3aR$^{-/-}$**) showed significant reduction in survival time relative to hSOD1^{G93A} mice.* ***B*** *shows significant reduction in weight of hSOD1^{G93A} x C3aR$^{-/-}$ mice when compared to hSOD1^{G93A} mice at 56 days of age (arrows, n = 12, * P<0.05, + P<0.01, # P<0.001, Student t-test).* ***C*** *shows significant decrease in hind-limb grip strength for hSOD1^{G93A} x C3aR$^{-/-}$ mice versus hSOD1^{G93A} mice at 42 days of age initially and then from 77 days of age onwards (n = 12, * P<0.05, + P<0.01, # P<0.001, Student t-test).* ***D*** *shows faster disease progression (determined by age at which maximal grip strength decline at 25, 50, 75 and 100%) in hSOD1^{G93A} x C3aR$^{-/-}$ mice (green bar) when compared with hSOD1^{G93A} mice (orange bar) at 50 and 100% decline in maximal grip strength (n = 12, * P < 0.05, *** P < 0.001, Student t-test). hSOD1^{G93A} mice lacking C3a signaling via C3aR shows a significantly shortened in survival, reduction in weight, worsened hind-limb grip strength and faster disease progression, demonstrating a neuroprotective role for C3a in this mutant ALS mouse model. Data expressed as mean ± SEM.*

5.2.4 hSOD1^{G93A} mice lacking C3aR have increased astrocytes and microglia mRNA levels but decreased monocyte mRNA levels, when compared to hSOD1^{G93A} mice throughout disease progression.

One of the known actions of C3aR activation is to induce pro-inflammatory functions such as chemotaxis and activation of glial cells. Therefore to elucidate the mechanism of C3aR in the disease progression of ALS, we investigated whether the absence of C3aR in hSOD1^{G93A} mice had any effect on astrocytosis and microgliosis in the lumbar spinal cord. mRNA expression levels of GFAP (marker of astrocytes), Iba-1 (marker of both resident microglia and infiltrating monocyte/macrophages), Entpd1 (CD39; predominant marker of resident microglia) and Ly6C (predominant marker of early infiltrating monocyte/macrophages) were measured in the lumbar spinal cord of hSOD1^{G93A} mice and hSOD1^{G93A} x C3aR$^{-/-}$ mice at mid-symptomatic and end-stage of disease progression using quantitative real time PCR.

GFAP transcripts were significantly increased in hSOD1^{G93A} x C3aR$^{-/-}$ by 1.3 fold at end-stage of disease when compared to hSOD1^{G93A} mice (*n = 6, * P < 0.05*; **Figure 5.5A**). Iba-1 transcripts were not changed in hSOD1^{G93A} x C3aR$^{-/-}$ mice at mid-symptomatic and end-stage of disease when compared to hSOD1^{G93A} mice (n = 6, $P > 0.05$; **Figure 5.5B**). Entpd1 mRNA expression significantly increased in hSOD1^{G93A} x C3aR$^{-/-}$ mice by 2.2 fold at mid-symptomatic stage of disease when compared to hSOD1^{G93A} mice (n = 6, *** $P < 0.001$; **Figure 5.5C**). However Ly6C mRNA was decreased at mid-symptomatic stage 0.64 fold when compared to hSOD1^{G93A} mice (n = 6, ** $P < 0.01$; **Figure 5.5D**).

5.2.5 No change in cytokine tumour necrosis factor α and interleukin-1β mRNA levels in hSOD1^{G93A} mice lacking C3aR when compared to hSOD1^{G93A} mice throughout disease progression.

Activation of C3aR is also known to induce synthesis of cytokines to modulate inflammatory processes in some cell types (Takabayashi et al., 1996, Takabayashi et al., 1998). Hence we investigated whether the absence of C3aR in hSOD1^{G93A} mice had any effect on the levels of the keystone cytokine TNFα and IL-1β in the lumbar spinal cord. mRNA expression of TNFα and IL-1β were measured in the lumbar spinal cord of hSOD1^{G93A} mice and hSOD1^{G93A} x C3aR$^{-/-}$ mice at mid-symptomatic and end-stage of disease progression using quantitative real time PCR. TNFα and IL-1β transcripts were not significantly different in hSOD1^{G93A} x C3aR$^{-/-}$ mice when compared to hSOD1^{G93A} mice at mid-symptomatic and end-stage of disease (n = 6, $P > 0.05$; **Figure 5.6A and 5.6B**).

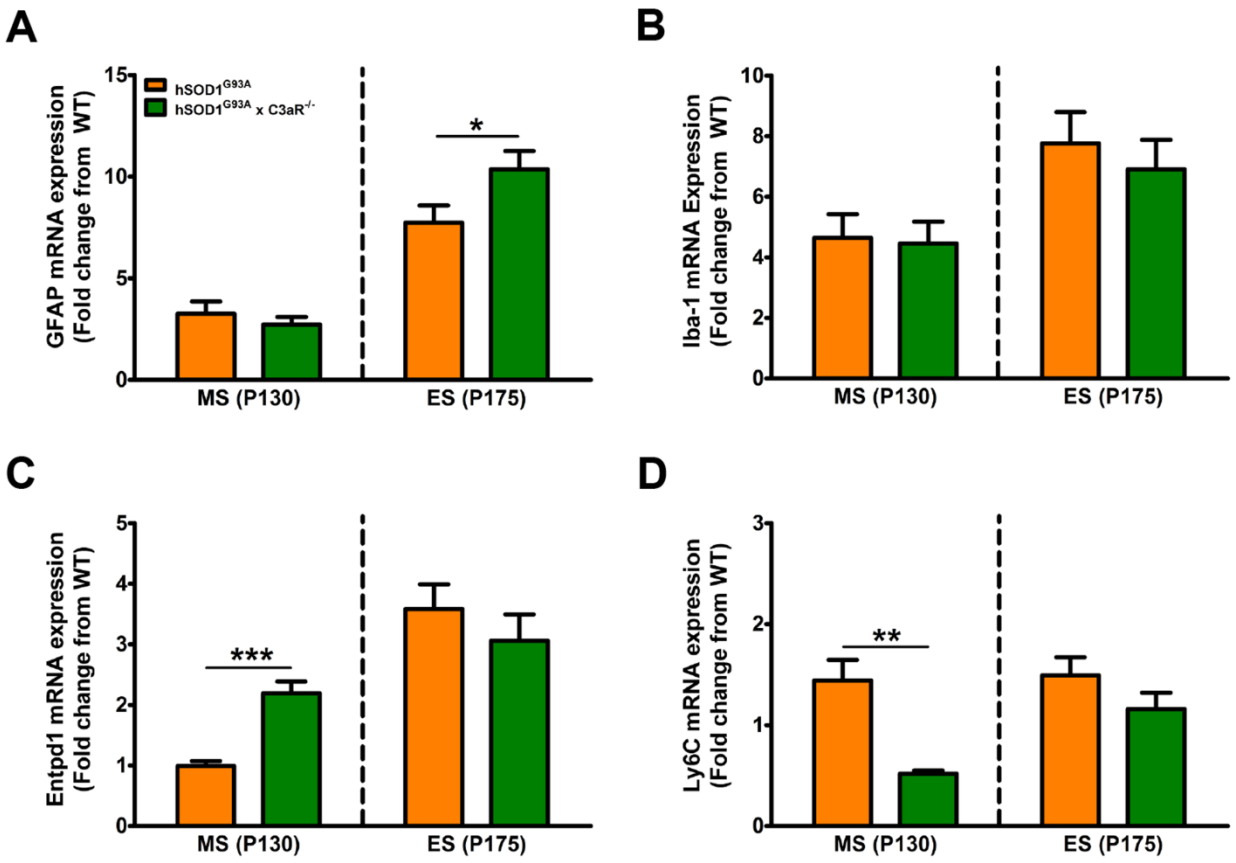

Figure 5.5: hSOD1^{G93A} mice lacking C3aR have increased astrocytes and microglia mRNA levels while decreased monocytes mRNA levels when compared with hSOD1^{G93A} mice. *(**A**) to (**D**) mRNA expressions of GFAP (astrocytes, **A**), Iba-1 (microglia/monocyte, **B**), Entpd1 (CD39; microglia, **C**) and Ly6C (monocyte, **D**) in lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched hSOD1^{G93A} mice lacking C3aR (**hSOD1^{G93A} x C3aR$^{-/-}$**) at mid-symptomatic and end-stage of disease progression. Data expressed as mean ± SEM (n = 6 mice/group; *P <0.05, **P <0.01, ***P <0.001, Student t test). MS (**P130**), mid-symptomatic - 130 days postnatal; ES (**P175**), end-stage - 175 days postnatal.*

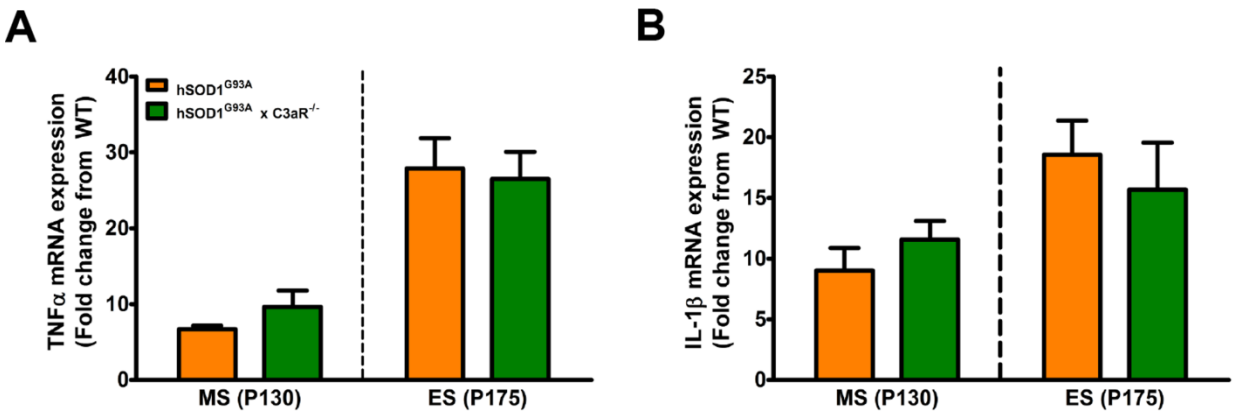

Figure 5.6: There is no change in cytokine tumour necrosis factor α and interleukin-1β mRNA levels in hSOD1^{G93A} mice lacking C3aR when compared with hSOD1^{G93A} mice. *(**A**) mRNA expression of tumour necrosis factor α (**TNFα**) in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched hSOD1^{G93A} mice lacking C3aR (**hSOD1^{G93A} x C3aR$^{-/-}$**) at mid-symptomatic and end-stage of disease progression. (**B**) mRNA expression of interleukin-1β (**IL-1β**) in the lumbar spinal cord of hSOD1^{G93A} mice relative to age-matched hSOD1^{G93A} x C3aR$^{-/-}$ mice at mid-symptomatic and end-stage of disease progression. Data expressed as mean ± SEM (n = 6 mice/group; P > 0.05, Student t test). MS (**P130**), mid-symptomatic - 130 days postnatal; ES (**P175**), end-stage - 175 days postnatal.*

5.3 DISCUSSION

We have previously shown that dysregulation and over-activation of complement system may be involved in promoting disease progression in ALS. However, deletion of C1q, C3 and C4 in hSOD1 transgenic mouse models did not show any beneficial effects on the disease progression of ALS, which indicates that blocking upstream complement activation pathways does not contribute to ALS mice neurotoxicity (Chiu et al., 2009, Lobsiger et al., 2013). This introduces the possibility that downstream complement factors (C3 and C5) potentially generated by the fourth "extrinsic pathway", may play a role in the disease progression of ALS. While C5a-CD88 signalling has clearly shown to play a pathogenic role in the disease progression of ALS in multiple rodent models as shown in chapter 4 (Woodruff et al., 2008a, Woodruff et al., 2014), the role of complement peptide C3a has never been examined in the disease progression of ALS due to a lack of selective and CNS permeable C3aR inhibitors (Mathieu et al., 2005, Proctor et al., 2004). Given this, we utilised hSOD1^{G93A} x C3aR$^{-/-}$ mice to elucidate the function of C3aR in the disease progression of ALS.

The present study demonstrates that the central component of complement system C3 and its receptor C3aR are markedly up-regulated during the course of disease progression in hSOD1^{G93A} mice and is expressed on motor neurons and astrocytes, but surprisingly not microglia. Inhibition of C3aR-signalling using hSOD1^{G93A} x C3aR$^{-/-}$ mice resulted in shortened survival, reduced body weight, worsened hind-limb grip strength and ultimately accelerated disease progression. Furthermore, we showed that hSOD1^{G93A} x C3aR$^{-/-}$ mice also had up-regulation of astrocytes and resident microglia mRNA levels, while reduction in monocyte mRNA levels when compared to hSOD1^{G93A} mice. Taken together these results indicate that C3aR activation in hSOD1^{G93A} mice may play a neuroprotective role in the disease progression of ALS.

5.3.1 C3 is expressed by wild-type and hSOD1^{G93A} motor neurons and is increased during disease progression

C3, the central component of complement system and the precursor of C3a and C3b is expressed by motor neurons in both the WT and hSOD1^{G93A} mice. This suggests that motor neurons are a major source of complement activation in this tissue. Previously we have shown that C3b is increased in hSOD1^{G93A} mice and expressed on motor neurons when compared to WT (Lee et al., 2013). This may suggest that stressed and dying motor neurons generate their own C3b where it could assist in the removal of dying motor neurons via opsonisation during disease progression in hSOD1^{G93A} mice, similar to synaptic pruning in nervous system development (Stevens et al., 2007). We also showed that C5, the downstream complement factor of C3 is generated in the motor neurons of

hSOD1^{G93A} mice. Additionally, our previous studies showed that cortical neurons can generate their own C5a to act in an autocrine fashion by binding to CD88 present on these neurons (Pavlovski et al., 2012). This suggests that motor neurons could also be capable of generating their own complement peptides, which can directly cause neuronal cell death in this mouse model of ALS. Like C5a-CD88 signalling in motor neurons, the consequence of C3a-C3aR signalling in WT motor neurons is not yet fully understood and will need further investigation.

5.3.2 C3aR is expressed on motor neurons and up-regulated during disease progression in hSOD1^{G93A} mice

The present study provided evidence for pathophysiological role of C3aR in hSOD1^{G93A} mice. Specifically, a significant up-regulation in C3aR mRNA level was observed at onset, mid-symptomatic and end-stage of disease in hSOD1^{G93A} mice. Unlike CD88, where it has been extensively studied to show that CD88 activation could actively contribute to neurodegeneration in various neurodegenerative diseases, the function of C3aR has received limited attention due to its complex, multifarious and context - dependent role of C3a (Klos et al., 2009). Interestingly, recent studies have proposed two contradicting functions of C3a-C3aR signalling in neurodegeneration where some showed pro-inflammatory and anti-inflammatory functions (Jacob et al., 2010, Yanamadala and Friedlander, 2010).

In addition to an increase in C3aR in hSOD1^{G93A} mice, the current study observed localization of C3aR on motor neurons in both the WT and hSOD1^{G93A} mice. The fact that C3aR was found on WT motor neurons suggests that C3aR may play a non-inflammatory role in the homeostasis of motor neuronal viability. Indeed, other studies have shown C3aR is also expressed in other neurons within the brains of the WT adult mice including hippocampal and cortical neurons (Davoust et al., 1999). Local expression of C3aR in motor neurons could be explained in terms of its involvement in increasing neuronal motility and migration during neurogenesis (Benard et al., 2008). Further evidence to support this notion is a study which utilised C3 and C3aR deficient mice, where these mice developed impairment of neurogenesis indicating that C3a – C3aR signalling is essential in neuroblast migration (Rahpeymai et al., 2006). It was also shown that C3a – C3aR signalling can induce neural progenitor cells in the CNS to differentiate, mature and migrate (Shinjyo et al., 2009).

Although there is substantial evidence for the role of C3a – C3aR signalling in the early development of cerebellar neurons there is no evidence to date reported in adult motor neurons. Hence, future studies could be developed using cultured motor neuron cells to elucidate the physiological significance of C3a receptor presence in motor neurons. Specifically motor neurons

could be exposed to stress and treated with C3a to assess the role that C3a – C3aR signalling may play in maintaining motor neuronal survival. However, based on the current evidence mentioned above, it is postulated that the expression of C3aR on motor neurons serves to provide homeostasis for motor neuronal viability, neurogenesis and migration, as a protective mechanism under normal physiology condition or a repair mechanism after CNS insult.

5.3.3 C3aR is expressed on proliferating astrocytes in hSOD1^{G93A} mice

It has become increasingly clear that non-neuronal neighbouring cells (astrocytes and microglia) can release toxic signals that can exacerbate motor neuron death in animal models of ALS (Kreutzberg, 1996, Nagai et al., 2007). The present study showed up regulation of C3aR on proliferating astrocytes, but minimal expression on activated microglia in hSOD1^{G93A} mice. The strong co-localisation of C3aR with proliferating astrocytes during disease progression of ALS suggests that the presence of C3aR on astrocytes is triggered by injury/insult from the disease. This is postulated to be a protective mechanism for the survival of adjacent motor neurons, as it has been previously found that C3a exerts beneficial effects against N-methyl-D-aspartate (**NMDA**)-induced neuronal death in mixed cultures of neurons and astrocytes but not in pure neuronal cultures (van Beek et al., 2001). Since the neuroprotective effect could only be seen in the presence of astrocytes, it suggests that C3a-C3aR signalling in astrocytes could be involved in modulating neuronal glutamate signalling and ultimately providing beneficial effects against NMDA toxicity. This also provides evidence of C3a-C3aR signalling on proliferating astrocytes in hSOD1^{G93A} mice protecting motor neurons against glutamate excitoxicity, as NMDA receptors are known to regulate Ca^{2+} influx into neurons and thus mediate glutamate excitoxicity (Yanamadala and Friedlander, 2010).

Despite the above evidence for C3a-C3aR signalling being neuroprotective, other studies have also shown that C3a-C3aR signalling in astrocytes could have a pathogenic role in the disease progression of ALS. It was found that co-stimulation of rat primary astrocytes with C3a/IL-1β resulted in a synergistic effect where nerve growth factor (**NGF**) production was increased (Jauneau et al., 2006). Although NGF is a neurotrophic factor which plays an essential role in maintaining the function and survival of neurons (Hetman and Xia, 2000, Yuen and Mobley, 1996), a study using hSOD1^{G93A} mice and cultured reactive astrocytes showed that NGF induced motor neuron death due to the activation of p75^{NTR} receptor pathway in response to pathological conditions (Pehar et al., 2004, Domeniconi et al., 2007).

Collectively, these studies suggest that C3a-C3aR signalling in hSOD1^{G93A} mice could have both pro- and anti-inflammatory functions, indicating that the equilibrium between these two functions will be important factor when looking at the role of C3aR in disease progression of ALS.

5.3.4 Absence of C3a-C3aR signalling in hSOD1^{G93A} mice worsens hind-limb grip strength, lowers body weight, accelerates disease progression and shortens survival time.

In the present study we used hSOD1^{G93A} x C3aR$^{-/-}$ mice to elucidate the role of C3a-C3aR signalling in the disease progression of ALS. We report for the first time that hSOD1^{G93A} x C3aR$^{-/-}$ mice have shortened survival time, worsened hind-limb grip strength, reduced body weight and accelerated disease progression when compared to hSOD1^{G93A} mice, suggesting that C3aR plays a neuroprotective role in these mice. This is in agreement with previous studies where they showed that C3a might provide a protective role in the central nervous system (**CNS**). Transgenic mice overexpressing C3a in astrocytes were significantly resistant to mortality induced by endotoxic shock compared with WT and C3aR deficient mice (Boos et al., 2005). Another study using mouse model of Alzheimer's disease showed that knocking out amyloid precursor protein (the protein that forms the plaque in the disease) resulted in neuroprotection, with elevated levels of C3a in the cerebrospinal fluid (Zhou et al., 2008). Taken together these results suggest that C3a-C3aR signalling in the context of CNS provides neuroprotective role, hence neuroprotection in the disease progression of ALS.

5.3.5 Increased astrocytes/microglia mRNA levels while reduced monocyte mRNA levels in hSOD1^{G93A} mice lacking C3aR

As mentioned previously, there is increasing evidence that motor neuron death in ALS animal models is exacerbated by toxic signals emanating from non-neuronal neighbouring cells (astrocytes and microglia) via an inflammatory response that accelerates disease progression (Nagai et al., 2007, Kreutzberg, 1996). We showed strong co-localisation of C3aR to astrocytes and minimal co-localisation of C3aR to microglia in hSOD1^{G93A} transgenic mice. Hence to elucidate the mechanism of C3a-C3aR signalling in astrocytes in the disease progression of ALS, we investigated whether the absence of C3aR in hSOD1^{G93A} mice had any effect on astrocytosis and microgliosis. We showed that there was a significant increase in astrocytes and microglia mRNA levels (GFAP, Entpd1) in hSOD1^{G93A} x C3aR$^{-/-}$ mice when compared to hSOD1^{G93A} mice at end-stage and mid-symptomatic stage of disease respectively, while there was a significant reduction in monocyte mRNA levels (Ly6C) in hSOD1^{G93A} x C3aR$^{-/-}$ mice at mid-symptomatic stage of disease. This suggests that at least some of the detrimental effects seen with C3aR inhibition could be due to the lack of C3a-mediated reduction of astrocytes and microglia in this mouse model of ALS. This

supports many studies where they have shown non-neuronal neighbouring cells (astrocytes and microglia) release toxic factors that are detrimental to motor neuron survival (Julien, 2007, Henkel et al., 2009, Fritz et al., 2013, Rojas et al., 2014). Although we showed increased microglia mRNA levels (Entpd1) in hSOD1^{G93A} x C3aR$^{-/-}$ mice when compared to hSOD1^{G93A} mice at mid-symptomatic stage, we also showed a decrease in monocyte mRNA level (Ly6C) in hSOD1^{G93A} x C3aR$^{-/-}$ mice at the same age. This was an interesting result as it contradicts recent studies where they showed decreasing infiltration of Ly6C-positive monocytes to the spinal cord attenuated motor neuron death and slowed disease progression in hSOD1^{G93A} mice (Butovsky et al., 2012). However, this supports other studies where they showed that resident microglia but not peripheral monocytes are major participants in ALS spinal cord innate immune activation (Chiu et al., 2013).

The activation states of microglia and astrocytes with respect to protection versus toxicity cannot be inferred solely from their morphology markers (GFAP, Iba-1, Entpd1 and Ly6C) (Beers et al., 2008). Hence we looked at mRNA expression of pro-inflammatory cytokine such as TNFα and IL-1β, which is known to be functional markers of activation (Beers et al., 2008). Interestingly, we showed that there was no difference between hSOD1^{G93A} x C3aR$^{-/-}$ mice and hSOD1^{G93A} mice for TNFα and IL-1β. This suggests that absence of C3a-C3aR signalling in astrocytes of hSOD1^{G93A} mice is worsening the disease outcome through other mechanism rather than increasing inflammation. For example, astrocytes are well known to respond to inflammation and injury of the CNS through the release of neutrophic factors such as glial cell line-derived neurotrophic factor (**GDNF**) (Llado et al., 2004, Appel et al., 1997), brain-derived nerurotrophic factor (**BDNF**) (Dougherty et al., 2000) and neurotrophin-3 (**NT-3**) (Dougherty et al., 2000). These factors are known to support motor neuronal survival (Koliatsos et al., 1993, Rakowicz et al., 2002, Whitehead et al., 2005, Gibbons et al., 2005). In the case for GDNF, it is released from astrocytes when such cells are exposed to inflammatory cytokines TNF-α, lipopolysaccharides and interferon-γ (Appel et al., 1997, Llado et al., 2004). Not only does GDNF promote motor neuron survival (Zhao et al., 2004), but can provide protection from apoptosis by inhibiting caspase-3 (Yu et al., 2007).

As mentioned, astrocytes have also been shown to increase its release of BDNF and NT-3 upon stimulus of spinal cord injury (Dougherty et al., 2000). BDNF prevents motor neuronal degeneration and induces axonal growth (Kishino et al., 1997). While the role of NT-3 in protecting motor neurons has not been investigated, other studies have found that it does play a protective role in sensory neurons (Tessarollo et al., 1994). In support of these ideas, we have recently shown that C3a can stimulate up-regulation of BDNF and NT-3 mRNA levels from astrocytes (*unpublished data*). Hence it is plausible that C3a-C3aR signalling in astrocytes in hSOD1^{G93A} mice can release

GDNF, BDNF and NT-3 to dampen the detrimental effects of astrocytes and protect against motor neuronal death.

5.4 CONCLUSION

In summary, the present study has demonstrated the up-regulation of central component C3 and its receptor C3aR in $hSOD1^{G93A}$ mice, and the increased expression of C3 and C3aR in end-stage $hSOD1^{G93A}$ mice appears to be due to increased astroglial C3 and C3aR expression. We also showed for the first time that inhibition of C3aR using $hSOD1^{G93A}$ x $C3aR^{-/-}$ mice significantly reduced hind-limb grip strength and body weight. It also accelerated disease progression and shortened survival, suggesting that C3a-C3aR signalling in these animals plays a neuroprotective role and protects motor neurons from dying. Expression of astrocytes and microglia mRNA level was increased while monocyte mRNA level was decreased in $hSOD1^{G93A}$ x $C3aR^{-/-}$ mice. Interestingly cytokines (TNFα and IL-1β) transcripts were not changed between $hSOD1^{G93A}$ x $C3aR^{-/-}$ mice and $hSOD1^{G93A}$ mice. Taken together these results indicate that inhibition of C3aR significantly exacerbates disease progression by increasing levels of astrocytes and microglia but not via modulation of inflammatory cytokines. This also opens the new window of opportunity as lack of C3a-C3aR signalling in astrocytes worsens the disease outcome through other mechanisms rather than increasing inflammation. Specifically, whilst our previous studies suggest that inhibiting the pro-inflammatory C5 activation fragment, C5a, which is central to, and generated by, all complement pathways, may be a novel therapeutic strategy to treat ALS, our data shows C3aR agonism offers a novel alternate or complementary therapeutic strategy to treat ALS.

CHAPTER 6

"Conclusion and future direction"

Amyotrophic lateral sclerosis (**ALS**), also known as Lou Gehrig's disease, is the most common form of motor neuron disease. It is a debilitating, devastating late onset neurodegenerative disorder that is characterized by the progressive death of upper and lower α-motor neurons within the central nervous system (**CNS**) (Bruijn et al., 2004). Over 50% of ALS patients die within 3-5 years after diagnosis and there is no cure for this disease. Our limited understanding of the cellular and molecular mechanisms that leads to the motor neuron death restricts the development of treatments for the disease. While the pathogenesis of ALS is still unclear, there is persuasive evidence that complement factors are involved in promoting the progression of disease. Therefore the aim of this thesis was to investigate the role of complement system in the disease progression of ALS using $hSOD1^{G93A}$ mice as an animal model. The key findings of the present study are: 1) dysregulation of complement including up-regulation of CD88 and C3aR in $hSOD1^{G93A}$ mice; 2) confirmation that CD88 has a pathogenic role in $hSOD1^{G93A}$ mice and 3) identification of C3aR as playing a neuroprotective role in $hSOD1^{G93A}$ mice.

In chapter 3 I demonstrated up-regulation of components of classical pathway (C1qB and C4), alternative pathway (factor B) and the central component common to all pathways (C3), together with decreased levels of complement regulators (CD55 and CD59a). This suggests that complement activation and/or its dysregulation may play an important role in motor neuron loss and ultimately in progression of ALS. Additionally this study also showed increased C1q and C3b immuno-labelling in $hSOD1^{G39A}$ mice and expression on motor neurons and microglia compared to wild-type. This may suggest that up-regulation of these components could assist in the removal of dying motor neurons via opsonisation, during disease progression in $hSOD1^{G93A}$ mice, similar to synaptic pruning during development (Stevens et al., 2007). Collectively, our data from chapter 3 added further support to the notion that complement activation may play an important role in accelerating motor neuron loss and ultimately in progression of ALS.

Despite these findings, recent studies have shown that deletion of upstream complement component C1q, C3 and C4 in hSOD1 transgenic mouse models did not show any beneficial effects on the disease progression of ALS (Chiu et al., 2009, Lobsiger et al., 2013). This is a somewhat surprising result, given the findings presented in Chapter 3, and that of previous published work showing strong activation of complement in ALS. These recent complement knockout studies indicate that

blocking upstream complement activation pathways do not contribute to ALS mice neurotoxicity. In addition to these opsonins (C1q and C3/C3b), cleavage products of C3 and C5 (C3a and C5a) and their receptors (C3aR and CD88) are also critical for mediating and executing the majority of complement-mediated inflammatory response. Hence it also introduces the new possibility that downstream complement factors, potentially generated by the fourth "extrinsic pathway", may play a role in the disease progression of ALS

In support of this hypothesis, my research also showed a significant increase in CD88 protein in hSOD1^{G93A} mice (Chapter 3). This supports other studies where they have shown up-regulation of CD88 in numerous neurodegenerative diseases including ALS, which makes it plausible to propose that downstream complement C5a-CD88 signalling may have a direct pathogenic role in the disease progression of ALS (Lee et al., 2013). Furthermore I demonstrated that inhibition of CD88 using oral administration of a specific CD88 antagonist, PMX205, significantly improved hind-limb grip strength, slowed disease progression and extended survival. This was also confirmed using hSOD1^{G93A} mice lacking CD88 (**hSOD1^{G93A} x CD88$^{-/-}$**), suggesting that CD88 activation contributes to motor neuron death seen in this model of ALS. I also showed in Chapter 4 that hSOD1^{G93A} x CD88$^{-/-}$ mice had reduced expression of microglia and monocyte markers, and cytokine (TNFα and IL-1β) mRNA transcripts. This suggests that the increased CD88 expressed on microglia in hSOD1^{G93A} mice may have many pathogenic functions including chemotaxis/proliferation of microglia, recruitment of inflammatory monocytes, and ultimately pro-inflammatory cytokine induction that may propagate the disease progression of ALS. Taken together these results indicate that CD88 has a pathogenic (pro-inflammatory) role and inhibition of CD88 can significantly attenuate disease progression by reducing inflammation that may accelerate motor neuron death and contribute to the pathology in hSOD1^{G93A} mice. One caveat with this hypothesis is that this study to date has only looked at the mRNA level, and now needs to be extended to the protein levels for the markers microglia, monocyte and cytokine using western blotting, immunohistochemistry and ELISA.

Another interesting finding I obtained from Chapter 3, was that C5, the precursor of C5a, is expressed predominantly by motor neurons, suggesting that diseased motor neurons could be a major source of C5a generation during disease progression. This local complement "self-signalling" in the CNS might therefore contribute to motor neuron death in hSOD1^{G93A} mice directly as shown previously by our group for cortical neurons (Pavlovski et al., 2012). Alternatively, locally generated C5a by injured motor neurons could act indirectly by acting as a chemoattractant and mitogen for resident microglia, infiltrating monocytes, resulting in their activation to induce

cytokine release from these cells which will ultimately contribute to motor neuron death. To further understand the relative contribution of CD88 expression in the CNS and circulating white blood cells in relation to ALS progression, we are proposing to utilise the bone-marrow (BM) chimeric studies where we can generate a number of BM chimeric mice with CD88 deficiency in CNS or peripheral blood to elucidate whether the benefits of CD88 ablation in disease progression of ALS are partially or solely mediated via altered peripheral immune cell responses in hSOD1^{G93A} mice. This will help us to pin point the mechanism by which CD88 activation worsens disease pathology in ALS and assist in future therapeutic strategies targeting complement activation. These BM chimeric studies are currently underway in our laboratory.

By contrast to C5a-CD88 signalling where it has clearly been shown to play a pathogenic role in the disease progression of ALS in multiple rodent models (Woodruff et al., 2008a, Woodruff et al., 2014), the role of complement peptide C3a has never been examined in the disease progression of ALS, possibly due to a lack of selective and CNS permeable C3aR inhibitors (Mathieu et al., 2005, Proctor et al., 2004). Given the lack of data for a role of C3a-C3aR signalling in ALS, we utilised hSOD1^{G93A} mice lacking C3aR (**hSOD1^{G93A} x C3aR$^{-/-}$**) to elucidate the function of C3aR in the disease progression of ALS. In addition to an up-regulation of the central component of complement system C3, we also showed that its receptor C3aR is up-regulated during the course of disease progression in hSOD1^{G93A} mice and is expressed on motor neurons and astrocytes, but interestingly completely absent on microglia. Inhibition of C3a-C3aR signalling using hSOD1^{G93A} x C3aR$^{-/-}$ mice resulted in shortened survival, reduced body weight, worsened hind-limb grip strength and ultimately accelerated disease progression. Taken together these results indicate that C3aR activation in hSOD1^{G93A} mice may play a neuroprotective role in the disease progression of ALS.

Like C5a-CD88 signalling, the knowledge about the cellular and molecular mechanism of C3a-C3aR signalling in the disease progression of ALS is very limited. Hence in chapter 5, I tried to dissect out some of the mechanisms that C3a-C3aR signalling could contribute to its neuroprotective role in the disease progression of ALS. We showed that hSOD1^{G93A} x C3aR$^{-/-}$ mice had up-regulation of astrocytes and resident microglia markers, but reduction in infiltrating monocyte markers when compared to hSOD1^{G93A} mice. We also showed that there was no difference in cytokines (TNFα and IL-1β) levels between hSOD1^{G93A} x C3aR$^{-/-}$ mice and hSOD1^{G93A} mice. Taken together these results indicate that C3aR activation on astrocytes in hSOD1^{G93A} mice may play a role in slowing down proliferation of astrocyte and microglia which may reduce the toxic signals (other than cytokine) emanating from these cells and ultimately slowing down the disease progression of ALS (Re et al., 2014, Phatnani et al., 2013). However, as

mentioned above, this study needs to be extended to the protein levels for the markers microglia, monocyte and cytokine using western blotting, immunohistochemistry and ELISA. It is also possible that C3a could exert its beneficial effects by modulating neuronal glutamate signalling and providing protection against N-methyl-D-aspartate toxicity seen in hSOD1^{G93A} mice. Previous studies have demonstrated as similar scenario whereby C3a-induced production of cytokine such as interleukin-6 and growth factors such as nerve growth factor (**NGF**) by these glial cells were found to display a neuroprotective effect against glutamate-mediated neuronal cell death (Ali et al., 2000, Park et al., 2012, Carlson et al., 1999, El Idrissi and Trenkner, 1999, Kume et al., 2000). In support of this idea, we have recently shown that C3a can stimulate up-regulation of brain-derived neurotrophic factor (**BDNF**) and neurotrophin-3 (**NT-3**) mRNA levels from astrocytes (*unpublished data*). Hence it is plausible that C3a-C3aR signalling in astrocytes in hSOD1^{G93A} mice can release NGF, glial-derived neurotrophic factor, BDNF and NT-3 to dampen the glutamate excitoxicity and protect against motor neuronal death. These ideas are currently being pursued in our laboratory.

Taken together these results of this thesis indicate that the role of complement activation in the disease progression of ALS is complex and can be viewed as a double-edge sword (similar to inflammation in general), which is either detrimental or beneficial depending on which complement pathway (C5a-CD88 or C3a-C3aR signalling pathway) is dominant. This emphasises the importance of equilibrium within the complement system as the complement peptides C3a and C5a generated during complement activation and their receptors C3aR and CD88 have two opposing roles in the progression of ALS in the hSOD1^{G93A} mouse. Hence, my results propose that modulating complement activation in ALS should be targeted towards downstream C5a inhibition, in order to avoid blocking any endogenous protective effects of upstream factors such as C3a in ALS progression.

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