THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Role of complement in ALS: regulating peripheral immune cells in skeletal muscle of hSOD1^{G93A} mouse model of ALS

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A thesis submitted for the degree of Doctor of Philosophy at
The University of Queensland in 2015

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Abstract

Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease. It is characterised by progressive loss of motor neurons and muscle atrophy. Recently, mounting evidence has suggested that complement, part of the innate immune system, is involved in the pathogenesis of ALS in both human patients and in animal models. Activation of complement in the central nervous system (CNS) has been well defined and has been proved to be critical to the death of motor neurons. However, less is known about the roles of complement in the skeletal muscle during the ALS disease progression.

The initial aim of this study was to examine the complement activation in skeletal muscle of hSOD1^{G93A} mice, a well-characterised ALS animal model. Expressions of major complement factors (C1qB, C3, factor B, C4, C5, C5aR1, and C3aR) and regulators (CD55, CD59) were determined, and shown to be significantly elevated in the skeletal muscle of hSOD1^{G93A} when compared to wide-type (WT) mice as disease progressed, suggesting that complement activation in the skeletal muscle of hSOD1^{G93A} mice is achieved through classical and possibly other complement cascades. In addition, expression levels of C5aR1 and C3aR, receptors for complement peptides C5a and C3a respectively, were also increased. Immunolocalisation studies shows that C5aR1 and C3aR are expressed on invading immune cells, CD11b^{+} macrophage and CD4^{+} helper T cells, in skeletal muscle of hSOD1^{G93A} mice.

The second aim of this study was to investigate the physiological roles of complement signalling in regulating immune cell migration in skeletal muscle of hSOD1^{G93A} mice. Massive invasions of macrophage and helper T cell were observed in tibialis anterior muscles of hSOD1^{G93A} mice when compared to age-matched wild-type mice. These infiltrations were remarkably attenuated in hSOD1^{G93A} mice lacking either C5aR1 or C3aR. By contrast, there was significantly less immune cell invasion into soleus muscles of hSOD1^{G93A} mice, but like for the tibialis anterior muscle, this invasion is significantly greater when compared to soleus muscles from age-matched wild-type mice, and attenuated in soleus muscle from hSOD1^{G93A} mice lacking either C5aR1 or C3aR. The soleus muscle, predominantly a slow-twitch muscle, is less vulnerable to denervation in hSOD1^{G93A} mice. Taken together, these results indicate that C5a-C5aR1 and C3a-C3aR signalling regulates the migration of immune cells into the skeletal muscle during ALS disease progression, and the extent of immune cell influx is related to physiological

function of skeletal muscle.

In summary, I have shown activation of the complement system in the skeletal muscle of hSOD1^{G93A} ALS mouse model, suggesting a role of complement C5a-C5aR1 and C3a-C3aR signalling in recruiting immune cells into skeletal muscle during disease progression. As skeletal muscle is the prime target for ALS, these findings may promote skeletal muscle as a therapeutic target for effects of complement factors in ALS treatment.

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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

Conference abstracts

1. **Wang, H**, Woodruff, TM, and Noakes PG (2012). The role of complement in the progression of naturally occurring motoneuron cell death. *Brisbane Cell and Developmental Biology Meeting, Brisbane, Australia, Oct 2012*

2. **Wang, H**, Lee, JD, Woodruff, TM, and Noakes PG (2014). Absence of C5a-C5aR1 signalling diminishes infiltration of peripheral immune cells in skeletal muscle of hSOD1^{G93A} mice. *25th International Symposium on ALS/MND, Brussels, Belgium, Dec 2014*

Publications included in the thesis

None.

Contributions by others to the thesis

I would like to thank Dr John D. Lee for his assistance with animal tissue collection and qPCR, WB and ELISA experiments (Chapter 3, 4, and 5). I would also like to acknowledge Dr Peter G. Noakes and Dr Trent M. Woodruff for their contributions in editing this thesis.

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

None.

Acknowledgements

First and foremost I would like to thank my supervisors A/Prof. Peter G. Noakes and A/Prof. Trent M. Woodruff for providing me the opportunity to study at the University of Queensland and pursue my dream of becoming a neuroscientist. I would like to thank Peter for the financial support to make this project possible, and his excellent guidance, caring, and patience throughout my training as a PhD student. I would also like to thank my supervisor Trent for his expert advice and encouragement throughout this difficult project.

I wish to express my appreciation to Dr. John D. Lee for his assistant with the project development and instruction on crucial laboratory techniques. I would also like to thank Dr. Shyuan T. Ngo for sharing her experience of becoming a successful neuroscientist with me, and helping me improving my presentation skills.

I would also like to acknowledge my committee members, Dr. Stephen Taylor and Dr. Nicolas Lavidis for their suggestions in general. My gratitude is also extended to all the members of the Noakes and Woodruff group, especially technicians Mary White and Maryam Shayegh, for their technical and moral support.

I would like to acknowledge the University of Queensland for providing me with the financial support (UQ International Scholarship).

Lastly, I would like to express gratitude to my immediate family. I would like to thank my mother and father for all of their love and encouragement through the years of my postgraduate studies overseas.

Keywords

complement, SOD, C5aR1, C3aR, macrophage, T cell, skeletal muscle, amyotrophic lateral sclerosis, motor neuron disease

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 110905 Peripheral Nervous System, 60%

ANZSRC code: 110707 Innate Immunity, 20%

ANZSRC code: 110704 Cellular Immunology, 20%

Fields of Research (FoR) Classification

FoR code: 1109 Neurosciences, 60%

FoR code: 1107 Immunology, 40%

Table of contents

List of Figures

List of Tables

List of Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
APC	antigen-presenting cell
BBB	blood-brain barrier
BNB	blood-nerve barrier
C1q	complement 1 subcomponent q
C1-INH	C1 inhibitor
C4BP	C4b-binding protein
Cdc42	cell division cycle 42
CNS	central nervous system
CR1	complement receptor type 1
CSF	cerebrospinal fluid
DAF	decay-accelerating factor
DAG	diacylglycerol
DAPI	4,6-diamidino-2-phenylindole
DC	dendritic cell
ERK	extracellular-signal regulated kinase
fALS	familial ALS
FUS	fused in sarcoma
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate
GRK	G-protein coupled receptor kinase
IGF	insulin-like growth factor
IL	interleukin
INF	interferon
IP3	inositol 1,4,5-trisphosphate
iTreg	inducible regulatory T cells
LPS	lipopolysaccharide
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase

MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MCP	membrane cofactor protein
MCT-1	monocarboxylate transporter 1
MEK	MAPK/ERK kinase
MG	myasthenia gravis
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MND	motor neuron disease
MPS	mononuclear phagocytic system
NK	natural killer
NMJ	neuromuscular junction
nTreg	natural regulatory T cell
PCD	programmed cell death
PKC	phosphokinase C
PLC	phospholipase C
PLD	phospholipase D
PMN	polymorphonuclaer cell
PNS	peripheral nerve system
PTX	pertussis toxin
RAG2	recombination activating gene 2
ROS	reactive oxygen species
sALS	sporadic ALS
SC	Schwann cell
SLE	systemic lupus erythematous
SOD1	superoxide dismutase 1
SOL	soleus
TA	tibialis anterior
TBST	tris-buffered saline tween
TCR	T cell receptor
TDP-43	TAR DNA-binding protein
TGF	transforming growth factor
TNF	tumour necrosis factor
Treg	regulatory T cell

UBQLN2	ubiquilin2
VCP	valosin-containing protein
WASP	Wiskot-Aldrich syndrome protein
WT	wild-type

Chapter 1

Introduction

1.1 Amyotrophic lateral sclerosis / Motor neuron disease

Amyotrophic lateral sclerosis (ALS), also known as Motor neuron disease (MND), is an idiopathic, fatal neurodegenerative disease of the human motor system (Kiernan et al., 2011). It was formally defined and identified for the first time by Jean-Martin Charcot in 1869 where degeneration and death of upper and lower motor neurons were observed (Cozzolino et al., 2008).

ALS is characterised by progressive muscular paralysis reflecting degeneration of motor neurones in the primary motor cortex, brainstem and spinal cord (Wijesekera and Leigh, 2009). “Amyotrophic” refers to muscle atrophy, weakness and visible fasciculation that reflect the degeneration of the corresponding lower motor neurons. “Lateral sclerosis” refers to hardening of the anterior and lateral corticospinal tracts where motor neurons are degenerating and replaced by gliosis (Rowland and Shneider, 2001, Wijesekera and Leigh, 2009). The clinical course of ALS is progressive. About 50% of patients die within 30 months and 15-20% survive between 5-10 years of symptom onset (Talbot, 2009). The most common cause of death among ALS patients is respiratory failure that results from progressive weakening of the respiratory muscles (Kiernan et al., 2011).

The incidence of ALS in Europe is estimated to be 2-3 cases per 100,000 person-years, and the overall lifetime risk of ALS is 1:350 for men and 1:400 for woman (Uenal et al., 2014, Johnston et al., 2006). Even though ALS affects people worldwide, an exact incidence of this disease remains unknown. The age of onset for ALS varies between 45-65 years with a median age of onset of 50 years (Cozzolino et al., 2008, Wijesekera and Leigh, 2009). Only 5% of patients have an onset before the age of 30 years, although

cases of juvenile sporadic onset have been increasingly noted (Haverkamp et al., 1995, Gouveia and De Carvalho, 2007).

Several factors contributing to the pathogenesis of the ALS have been proposed, including excitotoxicity, oxidative stress, mitochondrial dysfunction, defective axonal transport, neurofilament aggregation, abnormal protein aggregation, altered neuron hyperexcitability and genetic factors (Shaw and Ince, 1997, Wood et al., 2003, Manfredi and Xu, 2005, Lin and Schlaepfer, 2006, Pieri et al., 2009, Barber and Shaw, 2010, Bilsland et al., 2010, Chen et al., 2013). The vast majority (90-95%) of ALS cases are sporadic (sporadic ALS, sALS), while the remaining ALS cases are inherited (familial ALS, fALS).

To date, more than 20 genes including superoxide dismutase 1 (*SOD1*), TAR DNA-binding protein (*TDP-43*), fused in sarcoma (*FUS*), Ubiquilin2 (*UBQLN2*), *C9ORF72*, and Valosin-contaning protein (*VCP*) are found associated with fALS (Rosen et al., 1993, Sreedharan et al., 2008, Vance et al., 2009, Johnson et al., 2010, DeJesus-Hernandez et al., 2011, Deng et al., 2011). Among them, mutations in SOD1 account for 20% of familial ALS and 5% of sALS (Rosen et al., 1993). More than 130 different SOD1 mutations have been reported in ALS patients (Andersen, 2006).

1.1.1 SOD1 mutations

The discovery of disease-associated mutations in the SOD1 gene in 1993 was one of the most important breakthroughs in ALS research (Rosen et al., 1993). SOD1 is a homodimer of a ubiquitous 153-amino-acid cytosolic metalloenzyme with a catalytic copper ion and a stabilizing zinc ion in each subunit. It is expressed in all cells and widely distributed in the cytoplasm, nucleus, lysosomes and intermembrane space of mitochondria (Liu et al., 2004, Pasinelli et al., 2004). The SOD1 gene is composed of five exons separated by four introns. The main known function of SOD1 is to provide defence against oxygen toxicity by catalysing the reduction of dangerous superoxide radicals to O_2 and H_2O_2 (Chen et al., 2013).

In the central nervous system (CNS), SOD1 accounts for about 1% of total brain protein. In addition, motor neurons generally possess a much higher content of SOD1 protein compared to other cells in the nervous system (Shaw and Eggett, 2000). The mechanism underlying the nature of the mutation in the SOD1 gene still remains unclear. Increasing

evidence indicates that the motor neuron damaging effects from SOD1 is induced through several pathways, including protein misfolding and aggregation, oxidative stress and cytoskeletal abnormalities. Protein misfolding and aggregation are prominent features of ALS. SOD1 inclusions have been demonstrated in lower motor neurons of fALS patients, mutant SOD1 mouse models, and in cultured COS cells or motor neurons expressing mutant SOD1. Mitochondria abnormalities caused by mutant SOD1 inclusion formation are also an early event pathogenesis in $SOD1^{G93A}$ transgenic mice. The abnormal accumulation of intermediate filaments (IF) proteins in the axons of motor neurons is one of the universal pathological features of ALS. In particular, the aggregation of abnormal neurofilaments (NF), the major type of IFs in adult motor neurons, is a common pathological hallmark in fALS patients, due to SOD1 mutations and in mutant SOD1 mouse models, suggesting that NFs may act as toxic intermediates in the disease (Shaw, 2005). Given the complexity of SOD1-related ALS, it is hard to determine the primary or secondary causes, but it is likely that pathogenesis results from a complex interplay between protein misfolding and cellular stress.

Besides motor neurons, expression of mutant SOD1 in non-neuronal cells also contributes ALS disease and motor neuron death. For instance, astrocytes, the major type of glia in the CNS, expressing mutant SOD1 can actively decrease motor neuron survival by secreting neurotoxic factors (Nagai et al., 2007, Ferraiuolo et al., 2011, Haidet-Phillips et al., 2011, Phatnani et al., 2013). Meanwhile, astrocytes lose some of their neuro-supportive functions during the disease progression, and ultimately undergo degeneration themselves (Rossi et al., 2008, Martorana et al., 2012). Expression of mutant SOD1 on microglia, the surveillance immune cells in CNS, also accelerates the disease progression (Boillee et al., 2006). In addition, oligodendroglia, also affects motor neuron survival. Oligodendroglia expresses monocarboxylate transporter 1 (MCT1), which is crucial in providing motor neurons with metabolic support. Reduced expression level of MCT1 was found in mutant SOD1 transgenic mice, suggesting that impaired oligodendroglia metabolic support to neurons contributes to ALS pathogenesis (Lee et al., 2012b, Philips et al., 2013). Taken together, these findings implicate that glial cells carrying mutant SOD1 are involved in the pathogenesis of ALS by triggering motor neuron degeneration.

ALS is classically regarded as a “neurocentric” disease that causes the progressive loss of upper and lower motor neurons followed by axonal degeneration and muscle atrophy

(Strong and Rosenfeld, 2003). In recent years, the “dying-back” hypothesis has drawn much attention in which pathological changes in motor axons and nerve terminals appear to precede motor neuron degeneration and the onset of clinical symptoms (Fischer et al., 2004, Xie et al., 2005, Parkhouse et al., 2008, Sotelo-Silveira et al., 2009, Carrasco et al., 2010). In the $SOD1^{G93A}$ mouse, quantitative analysis demonstrated denervation at neuromuscular junction (NMJ) by 47 days of age, followed by severe loss of motor axons from the ventral root between days 47 and 80 days, and loss of motor neuron cell bodies from the lumbar spinal cord after day 80. This pattern suggests that motor neuron disease in the $SOD1^{G93A}$ mouse is actually a “dying-back” motor neuropathy where distal axonal degeneration occurs early during the disease, before neuronal degeneration, and onset of symptoms (Fischer et al., 2004). Neuropathological analysis of a 58-year-old patient with sporadic ALS who died unexpectedly also showed a “dying-back” phenomenon where denervation and innervation changes at the muscle were observed while pathological changes in the motor neuron itself were not detected (Fischer et al., 2004). In addition, selective loss of motor units in most forceful fast-twitch muscle fibres was observed prior to the onset of symptoms in the $SOD1^{G93A}$ mouse, implicating that motor neurons innervating the slower muscle fibres are more resistant than those innervating the faster ones (Frey et al., 2000, Schaefer et al., 2005, Hegedus et al., 2007, Hegedus et al., 2008). These findings support the idea that neuromuscular denervation and symptom appearance in ALS can occur regardless of motor neuron survival, suggesting that alternations elsewhere, for instance in skeletal muscle, muscle satellite cells or terminal Schwann cells were able to influence the integrity of axons, thus challenging the “neurocentric” view of ALS (Pansarasa et al., 2014).

Many strains of transgenic murine ALS models with human SOD1 mutations have been generated, particularly the $SOD1^{G93A}$ mutation in which amino acid glycine in position 93 is substituted by alanine, to investigate the disease pathogenesis (Gurney et al., 1994). The $SOD1^{G93A}$ mouse model displays an onset of clinical symptoms with the development of tremor and hindlimb weakness detected by locomotor deficits at ~90 days, progressing to paralysis and premature death at ~120-140 days (Gurney et al., 1994).

One of the earliest pathological events is the degeneration of neuromuscular junctions at ~47 days of age. This degeneration appears to be selective for neuromuscular junctions supplied by fast-fatigable motor neurons (Fischer et al., 2004, Pun et al., 2006). These

neurons are large motor neurons, which have a higher metabolic load as they are more excitable (e.g. capable for generating high frequency action potentials), and thus are more vulnerable to cellular stress (e.g. greater levels of reactive oxygen species [ROS]), when compared to smaller motor neurons that are less excitable and which innervate slow-twitch muscle fibres (Frey et al., 2000, Pun et al., 2006, Hegedus et al., 2007). This might explain why denervation of fast-twitch muscles occurs before denervation of slow-twitch muscle fibres. By 80 days postnatal, proximal motor axon loss is prominent, coinciding with motor impairment, and followed by a drastic 50% loss of lower motor neurons at 100 days (Fischer et al., 2004). Pathological features such as vacuolization of mitochondria, fragmentation of the Golgi apparatus, endoplasmic reticulum stress, and astrogliosis and microgliosis in the spinal cord are observed at early postnatal ages. As the transgenic $SOD1^{G93A}$ mice develop a disease strikingly similar to ALS, including selective loss of upper and lower motor neurons, paralysis and significant loss of muscle mass, they are extensively used as models to probe ALS (Mourelatos et al., 1996, Hall et al., 1998, Bendotti et al., 2001, Saxena et al., 2009) (Figure 1).

The standard $hSOD1^{G93A}$ transgenic mouse model harbours 25 copies of the mutant human SOD1 gene (Gurney, 1997). The onset of symptoms and survival of $SOD1^{G93A}$ mice are directly related to the copy number of the mutant transgene. Lower copy number of the $hSOD1^{G93A}$ transgene results in a prolonged asymptomatic phase and protracted survival in these mice (Henriques et al., 2010, Acevedo-Arozena et al., 2011, Deitch et al., 2014). As the development of disease is delayed, $SOD1^{G93A}$ low-copy transgenic mice may more closely mimic human pathophysiology, making it a more appropriate model for studying early-stage pathogenesis of human ALS and benefits the development of early-stage preventive strategies.

1.2 Complement System

The innate immune system, also known as the non-specific immune system, plays a vital role in the inflammatory response to infection through the activity of receptors that are capable of recognizing defined molecular patterns present in a variety of microorganisms. The complement system is an essential part of the innate immune system and is able to discriminate and eliminate invading pathogens (Friec and Kemper, 2009).

The Belgium immunologist Jules Bordet described complement for the first time in the 1890s as a heat-labile substance that 'complements' the heat-stable fraction of normal blood serum to realise the anti-bacterial function of an antibody (Lachmann, 2006). We now know that the complement system consists of more than 40 soluble factors, cellular receptors, and regulatory molecules present in blood plasma and on cell surfaces (Walport, 2001). The main biological function of complement is to recognise and eliminate the 'foreign' microorganisms. Complement proteins collaborate as a cascade to opsonise pathogens and trigger a series of inflammatory responses modulating the activity of T- and B- cells to fight infection and maintain homeostasis (Merle et al., 2015). The complement system also bridges the innate and adaptive immune system, destroys host components such as apoptotic and necrotic cells and disrupts protein assemblies (Morgan et al., 2005). Four distinct pathways, including classical, lectin, alternative and extrinsic, can initiate the complement system, depending on the context. Each of the pathways leads to a common terminal pathway. These pathways are reviewed in the following sections, along with the major complement factors and regulators.

1.3 Complement activation pathways

Activation of complement is known to occur through three pathways – the classical, alternative and lectin pathways. All three pathways result in the cleavage of C3, the most abundant complement protein, followed by generation of complement peptide C3a and C5a, and the C5b-9 membrane-attack complex (MAC), which creates a pore that disrupts cell homeostasis and eventually leads to lysis (Ramaglia and Baas, 2009)(Figure 2). Recently, a new complement pathway – the extrinsic protease pathway was discovered. Unlike the three pathways previously described whose activation relies on the generation of C3, the extrinsic protease pathway generates C5a in the absence of C3 (Huber-Lang et al., 2006)(Figure 2).

Complement factors can opsonise bacteria for enhanced phagocytosis. They can recruit and activate various cells including polymorphonuclear cells (PMNs) and macrophages. Complement is also involved in regulation of antibody responses and it can aid in the clearance of immune complexes and apoptotic cells. Furthermore, complement can have a detrimental effect for the host, it can contribute to inflammation and tissue damage and can trigger anaphylaxis (Walport, 2001).

1.3.1 Classical pathway

The classical pathway, or antibody dependent pathway, was the first complement pathway discovered. It is initiated by binding of the C1 complex (composed of C1q, C1r and C1s) to the complement-fixing antibodies (IgG1 and IgM) attached to the antigen on the target surface. Upon the binding of C1q to antigen-antibody complexes, C1r and C1s are subsequently activated which in turn cleave C4 and C2. C4 is cleaved into two fragments, C4a and C4b. The larger C4b molecule contains an exposed active thioester bond allowing it to attach to a variety of target surfaces and act as an opsonin. Activated C1 cleaves C2 into C2a and C2b, and C2b binds to C4b on the cell surface, leading to the formation of the C3 convertase C4b2b (Ricklin et al., 2010, Wallis et al., 2010)(Figure 2).

The generation of the C3 convertase, which cleaves C3 into the anaphylatoxin C3a and the opsonin C3b, is the crucial point in complement activation where all complement pathways converge. The opsonic C3 fragment, C3b, either binds covalently to pathogenic surfaces, or to C4b in the C4b2b complex producing the C5 convertase (C4b2b3b), which cleaves C5 into C5b and the anaphylatoxin C5a. Once released, C5b interacts with C6, C7, C8 and multiple copies of C9 molecules ranging from 1 to 18 to form the MAC complex, resulting in ion flux and eventually lysis of target cells (Xiong et al., 2003, Carroll and Sim, 2011)(Figure 2).

1.3.2 Lectin pathway

The lectin pathway of complement is initiated by the binding of mannose-binding lectin (MBL) or ficolins to carbohydrate groups on bacterial cells surfaces. MBL belongs to the collectin protein family as it contains a collagen-like domain while ficolins consist of a collagen-like stem structure (Holmskov et al., 2003). Both MBL and ficolins are pathogen-recognizing proteins, which form complexes with the MBL-associated serine proteases (MASPs). MASPs are regarded as homologous to C1r and C1s molecules of the classical complement pathway Activated MASP-1 and MASP-2 cleave C4 and C2, leading to the formation of the lectin pathway C3 and C5 convertases, C4b2b and C4b2bC3b, respectively (Thiel et al., 2000).

1.3.3 Alternative pathway

Activation of the alternative pathway can be triggered by almost any foreign substances, including lipopolysaccharide (LPS), yeast, virus, and necrotic cells (Guo and Ward, 2005).

Additionally, in contrast to the classical and lectin pathways, the alternative pathway is capable of auto-activation through the spontaneous conversion of C3 to C3b termed 'tick-over' (Thurman and Holers, 2006). Spontaneous hydrolysis of the unstable thioester bond in C3 continually generates the C3b-like protein C3(H_2O). This configuration change in C3 allows the binding of factor B to C3(H_2O), followed by the cleavage of factor B by factor D into Ba and Bb. When the Ba fragment is released, the active serine protease Bb remains associated with the C3(H_2O) complex, forming the alternative pathway fluid-phase C3 convertase C3(H_2O)Bb. C3(H_2O)Bb further cleaves plasma C3 into C3a and C3b (Pangburn and Muller-Eberhard, 1983, Thurman and Holers, 2006, Bexborn et al., 2008).

The initiating component of the alternative pathway is the C3b deposited on cell surfaces. C3b generated from the spontaneous cleavage of C3 randomly binds to protein and carbohydrates expressed on cell surfaces, forming a C3bB complex upon interacting with factor B in an Mg^{2+}-dependent manner. Factor D then cleaves the bound factor B in the surface-associated C3bB complex, generating the alternative pathway surface-bound C3 convertase C3bBb. C3bBb is an unstable enzyme complex homologous to the classical pathway convertase C4b2b and decays spontaneously. The serum protein properdin, also known as factor P, binds to C3bBb and stabilises it through slowing down the dissociation of Bb from the enzyme complex (Hourcade, 2006, Bexborn et al., 2008).

Formation of C3 convertase allows for the cleavage of more C3, resulting in additional C3b production. In addition, activation of the classical and alternative pathways can provide C3b for the formation of the alterative pathway C3 convertase, which in turn amplifies further cleavage of C3 into C3a and C3b (Thurman and Holers, 2006). Amplification of C3b production results in an additional covalent binding of C3b to C3bBbP, forming the alternative pathway C5 convertase C3bBb3b (Ricklin et al., 2010).

1.3.4 Extrinsic protease pathway

Aside from the three established pathways, a fourth pathway of complement activation, the extrinsic pathway was identified resulting from the link between complement and coagulation pathways. The extrinsic pathway of coagulation was discovered in the 1980's (Ploplis et al., 1987). Proteolytic enzymes such as thrombin, plasmin, and kallikrein have long been known to be able to cleave and activate complement component C3 (Thoman et al., 1984, Markiewski et al., 2007). Thrombin was able to activate C5 in the C3 knockout

mouse in which C5 convertases cannot be produced (Huber-Lang et al., 2006). This route can bypass the traditional C3-dependent upstream pathways to initiate downstream pathway activation (Woodruff et al., 2010). *In vitro* studies have also shown that individual cells are capable of generating bioactive complement fragments. For instance, cultured cortical neurons can produce their own C5a, which exacerbates neuronal death under metabolic stress, suggesting that similar pathways may be active in dysfunctional motor neurons in ALS (Pavlovski et al., 2012).

In a recent study, Lobsiger et al. claimed that global complement activation does not affect disease progression in $SOD1^{G93A}$ mice lacking in C1q or C3 (Lobsiger et al., 2013). One thing this study overlooked was that the "extrinsic pathway" of complement activation could bypass the traditional C3-dependent pathways and consequently alter the disease progression. In support of this theory, our lab has demonstrated that a selective C5aR1 receptor antagonist extended survival of the $SOD1^{G93A}$ rat, and a similar extended survival has been observed in $SOD1^{G93A}$ mice lacking C5aR1 (Woodruff et al., 2008a, Woodruff et al., 2014). These findings suggest that extrinsic pathway, particularly at the downstream step of C5, may contribute to ALS pathogenesis.

1.5 Key effectors of complement

The primary function of complement is to recognize invading microorganisms and promote their elimination through opsonisation of pathogens with C1q and C3b, followed by lysis via the membrane attack complex (MAC), C5b-9, the final product of the five terminal proteins C5, C6, C7, C8 and C9 (Carroll and Sim, 2011).

1.5.1 The initiator of the classical pathway

C1q is the recognition component of the classical pathway, belonging to a family of soluble proteins categorised as defence collagens (Bohlson et al., 2007). It is a 460-kDa glycoprotein composed of six identical peripheral globular regions, each of which contains three distinct polypeptide chains (Reid, 1983). The non-collagen-like domain of C1q mediates the recognition of activators of C1, converting C1r and C1s into active serine proteases which in turn triggers the initiation of classical complement cascade (Tenner, 1998). C1q is synthesised by monocyte/macrophage, microglia and dendritic cells (DCs) (Korotzer et al., 1995, Kaul and Loos, 2001, Cortes-Hernandez et al., 2004, Reis et al., 2007). Deficiency of C1q is associated with excessive inflammation and autoimmunity,

leading to the development of systemic lupus erythematous (SLE) and glomerulonephritis (Botto et al., 1998, Botto and Walport, 2002). There is mounting evidence suggesting that C1q serves as a bridging molecule to facilitate the physiologic clearance of apoptotic cells by phagocytes (Ogden et al., 2001, Vandivier et al., 2002, Fraser et al., 2010). In addition, C1q functions as opsonin to promote the uptake of apoptotoic cells by phagocytes through interacting with complement receptors on the cell surface (Francis et al., 2003). Interestingly, recent studies demonstrate that C1q also plays a role in mediating synaptic pruning in CNS during development. In the developing brain, astrocytes induce the generation of C1q in neurons. Neuron and microglia-derived C1q tags weak or inappropriate synapses for removal via the activation of classical complement cascade, resulting in C3 cleavage and synaptic C3b deposition. Those complement-tagged synapses are eliminated through phagocytosis by microglia (Stevens et al., 2007, Schafer and Stevens, 2010).

1.5.2 The complement peptides

As complement activation products opsonise or lyse cells, complement can defend against pathogenic materials but also damage healthy host cells and tissues. The complement exerts its detrimental roles through the formation of anaphylatoxins, especially C3a and C5a (Guo and Ward, 2005).

Complement component C3a is a 77-amino-acid protein. It is cleaved from C3 by C3 convertase upon activation of the complement pathways. C3a mediates assorted immunoregulatory functions, including chemotaxis, smooth muscle contraction and increased vascular permeability (Stimler et al., 1983, Williams, 1983, Daffern et al., 1995, Legler et al., 1996). It performs these functions through binding to its C3aR, a seven transmembrane G protein-coupled receptor (Tornetta et al., 1997).

C5a, a serum protein of 74-amino-acid residues, is regarded as one of the most potent inflammatory peptides known. It carries out a broad spectrum of biological activities on different cells, including phagocytosis, degranulation, H_2O_2 production, granule enzyme release, delay or enhancement of apoptosis, chemokine and cytokine productions and chemotaxis (Guo and Ward, 2005, Lee et al., 2008). The C-terminal arginine on C5a is removed by the plasma enzyme carboxypeptidases, forming C5a-des-Arg that has a 10-1000 times reduced potency compared with C5a (Lee et al., 2008). C5a exerts its effects

through two high affinity receptors, the C5aR1 (also known as CD88) and the C5aR2 (also known as C5L2/GRP77) (Huber-Lang et al., 2002).

C3a and C5a are powerful inflammatory molecules, especially C5a. These two anaphylatoxins are chemically and biologically similar. They share a striking 36% sequence similarity and both possess highly conserved C-terminal pentapeptide sequences that are essential for activation of their receptors (Klos et al., 2009). C3a contains four α-helical cationic regions stabilised by three disulphide bonds while C5a contains an additional fifth helix at the C terminus (Nettesheim et al., 1988). These structural features make C3a, but not C5a, a highly potent antibacterial peptide as it induces breaks in bacterial membranes (Nordahl et al., 2004).

As potent inflammatory mediators, C3a and C5a can trigger contraction of smooth muscle, increase the permeability of small blood vessels, and induce vasodilation (Julia et al., 1998). C3a and C5a also promote oxidative bursts in macrophages, neutrophils, and eosinophils while inducing histamine release from basophils and mast cells (Kretzschmar et al., 1993, Murakami et al., 1993, el-Lati et al., 1994, Elsner et al., 1994a, Elsner et al., 1994b). In addition, C3a induces serotonin release from platelets and modulate synthesis of IL6 and TNF-α by B cells and monocytes while C5a regulates the production of eosinophil cationic protein and the adhesion to endothelial cells by eosinophils (Fukuoka and Hugli, 1988, Rivier et al., 1994, Fischer and Hugli, 1997, DiScipio et al., 1999, Fischer et al., 1999, Jagels et al., 2000). Both anaphylatoxins are chemoattractive for a wide range of immune cells, and this is particularly for C5a. C5a is a strong chemoattractant for basophils, macrophages, neutrophils, mast cells, and activated B and T cells (Lett-Brown and Leonard, 1977, Aksamit et al., 1981, Ehrengruber et al., 1994, Ottonello et al., 1999, Nataf et al., 1999). C3a on the other hand predominantly chemoattracts mast cells and eosinophils (Hartmann et al., 1997).

1.5.3 The anaphylatoxin receptors

The anaphylatoxin receptors, which belong to the superfamily of G-protein coupled receptors, include the C3a receptor (C3aR) and C5a receptors, C5aR1 as well as C5aR2. Although these receptors share high sequence homology, they differ in ligand specificity, signal transduction capacity and function (Lee et al., 2001).

1.5.3.1 C3aR

The C3aR is a G-protein coupled, seven-transmembrane segment protein. Compared to C5aR, it is unique with a reduced N-terminal domain and a remarkable large second extracellular loop of 172 amino acids between the fourth and fifth transmembrane domains, required for ligand binding (Gao et al., 2003) (Figure 3).

C3aR is found in a wide range of tissues, including lung, liver, kidney, brain, heart, muscle and testis (Hsu et al., 1997). It is predominantly expressed on the surface of myeloid-derived cells, such as neutrophils, eosinophils, basophils, mast cells, dendritic cells, microglia, and monocytes/macrophages (Klos et al., 1992, Daffern et al., 1995, Hartmann et al., 1997, Zwirner et al., 1998a, Gutzmer et al., 2004). In addition, non-myeloid cells including astrocytes, endothelial cells, epithelial cells and smooth muscle cells from asthma patients (Gasque et al., 1998, Ischenko et al., 1998, Monsinjon et al., 2003, Fregonese et al., 2005). C3aR is also expressed on B cells and activated T cells (Martin et al., 1997, Zwirner et al., 1999, Werfel et al., 2000). Moreover, recent studies have demonstrated up-regulated expression of C3aR on murine $CD4^+$ cells upon dendritic cells stimulation (Strainic et al., 2008).

C3aR mediates chemotaxis of eosinophils, mast cells, dendritic cells and monocytes, but not neutrophils (Daffern et al., 1995, Nilsson et al., 1996, Gutzmer et al., 2004). It also induces an oxidative burst in macrophages, neutrophils and eosinophils (Burg et al., 1996). Besides this, basophils and mast cells undergo degranulation and release histamine upon C3a-C3aR signalling (Bischoff et al., 1990, Venkatesha et al., 2005).

Upon C3a binding to its receptor C3aR, intracellular signal transduction is promoted via heterotrimeric guanosine triphosphate (GTP)-binding proteins (G proteins). C3aR mediates its effect on immune cells through coupling to the pertussis toxin (PTX)-sensitive and –insensitive G proteins, $G_{\alpha i}$ and $G_{\alpha 16}$, respectively (Norgauer et al., 1993, Zwirner et al., 1997, Yang et al., 2001). In endothelial cells, C3aR also couples to insensitive G proteins $G_{\alpha 12}$ and $G_{\alpha 13}$ (Schraufstatter et al., 2002). The downstream pathway activates phosphoinositol-3-kinase gamma (PI3K-γ), which in turn activates phospholipase C (PLC)-β and PLC-γ. PLC is a multidomain phosphodiesterase that generates the second messenger inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which further leads to Ca^{2+} mobilization and phosphokinase C (PKC) activation, respectively. PI3K can also

activate the mitogen-activated protein kinases (MAPK)/extracellular-signal regulated kinase (ERK) 1/2 (Langkabel et al., 1999, Sayah et al., 2003, Venkatesha et al., 2005)

1.5.3.2 C5aR1

The classic C5aR1 receptor is a 42-kDa membrane glycoprotein, belonging to the superfamily of G protein-coupled receptors with seven transmembrane segments (Leslie and Hansen, 2001). Murine C5aR1 displays an overall 65% sequence identity to its human counterpart (Gerard et al., 1992). For the interaction between C5a and C5aR1, a “two-site binding” model has been proposed. The first binding site is at the aspartate-rich acidic N terminus of C5aR1, which interacts with the basic core of C5a. The second binding site is between the agonistic C terminus of C5a and the transmembrane domains and charged residues at the base of the C5aR1 extracellular loops. Unlike the N-terminal binding site, the second binding site is indispensible for receptor activation (Mery and Boulay, 1993, DeMartino et al., 1994, Gerber et al., 2001). Current data suggests that at least three different discontinuous regions of C5a are involved in its interaction with C5aR1 (Huber-Lang et al., 2003). In spite of the fact that C5a was first considered as an anaphylatoxin and later as a leukocyte chemoattractant, the wide spread expression of C5aR1 implicated more general functionality (Figure 4).

Similar to C3aR, C5aR1 is expressed on various myeloid originated cells such as neutrophils, eosinophils, basophils, mast cells, dendritic cells and monocytes, as well as on non-myeloid cells, including epithelial cells, Kupffer cells, stellate cells, astrocytes and microglial cell (Chenoweth and Hugli, 1978, Chenoweth and Goodman, 1983, Gerard et al., 1989, Morelli et al., 1996, Werfel et al., 1997, Drouin et al., 2001, Koleva et al., 2002, Schlaf et al., 2004, Gasque et al., 1997).

C5aR1 is a potent chemoattractant for monocytes, neutrophils, eosinophils, basophils, mast cells, B cells and T cells (Pieters et al., 1995, Webster et al., 1980, DiScipio et al., 1999, Lett-Brown and Leonard, 1977, Hartmann et al., 1997, Kupp et al., 1991, Nataf et al., 1999). It is also responsible for other biological events such as mast cell degranulation, oxidative burst in granulocytes, and secretion of proinflammatory mediators from monocytes, eosinophils and mast cells (Subramanian et al., 2011, Guo et al., 2003, Takafuji et al., 1994, Hartmann et al., 1997). Recent studies have shown up-regulation of C5aR1 in the CNS of murine hSOD1^{G93A} transgenic models, suggesting a pathogenic role

for C5a-C5aR1 signalling in ALS (Lee et al., 2013, Woodruff et al., 2008b, Woodruff et al., 2008a).

Signal transduction of C5aR1 depends on heterotrimeric G-proteins. C5aR1 mainly couples to the PTX-sensitive G protein $G_{\alpha i2}$ in cells such as neutrophils and mast cells or, less frequently, to PTX-insensitive G protein $G_{\alpha16}/G_{\alpha15}$ in cells of the hematopoietic lineage (Skokowa et al., 2005, Amatruda et al., 1993, Monk and Partridge, 1993, Davignon et al., 2000). C5a-C5aR1 interaction causes calcium fluxes from both intercellular compartments and extracellular medium. Phosphorylation of C5aR1 leads to association with β-arrestins 1 and 2 and subsequently targeting to clathrin-coated pits for internalization (Braun et al., 2003). Binding of arrestins depends on phosphorylation of the C terminus of C5aR1 by G-protein coupled receptor kinases (GRKs). Apart from their function as kinase, GRKs can also interact with other elements of intracellular signalling including Akt, MAPK/ERK kinase (MEK) and PI3K-γ (Ribas et al., 2007). It has been demonstrated that C5a-C5aR1 interaction leads to downstream activation of several components of different signalling pathways, such as PI3K-γ, phospholipase C β2 (PLC-β2), phospholipase D (PLD) and MEK-1 (Perianayagam et al., 2002, la Sala et al., 2005, Mullmann et al., 1990, Buhl et al., 1994). Another binding partner of the C terminus of activated C5aR1 is Wiskot-Aldrich syndrome protein (WASP). This interaction is strongly potentiated in the presence of active cell division cycle 42 (cdc42), a GTP binding protein that induces a conformational change of WASP to its active state. WASP is a multifunctional protein that regulates actin dynamics and therefore could be involved in the chemotactic response to C5a (Tardif et al., 2003).

1.5.3.3 C5aR2

The second receptor for C5a, C5aR2, is a recently discovered seven-transmembrane segment protein expressed on granulocyte and immature dendritic cells (Ohno et al., 2000). Like C5aR1, C5aR2 contains the classical G-protein coupled receptor structure but is not coupled to intracellular G protein signalling pathways (Okinaga et al., 2003). C5aR2 shares 58% sequence identity with C5aR1 and 55% with C3aR in its conserved transmembrane regions (Lee et al., 2001). C5aR2 is expressed in various myeloid derived and non-myeloid cells. It seems to frequently co-express with C5aR1 in most cells and tissues (Gavrilyuk et al., 2005, Lee et al., 2001, Okinaga et al., 2003, Gao et al., 2005).

By contrast to C5aR1, C5aR2 appears to be a non-functional decoy receptor as no mobilization of intracellular calcium, extracellular signal-related kinase phosphorylation or receptor internalization was shown when it was treated with C5a (Okinaga et al., 2003). However, this view has been challenged where recent evidence showed that C5aR2 functions as an intracellular receptor triggered by C5aR1 activation, which acts to regulate C5aR1 signalling (Bamberg et al., 2010).

1.6 Regulation of complement activation

Complement is important for the elimination of invading pathogens, but it can also attack host cells when over-activated. Therefore, activation of the complement system must be tightly controlled. This delicate balance between activation and inhibition is achieved by complement regulators and inhibitors.

Complement regulators are divided into two forms, cell-surface regulators and fluid-phase regulators. In humans, there are four well-characterized cell-surface complement regulatory proteins, which protect human cells against autologous complement attack. Three of the regulators, decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and complement receptor type 1 (CR1, CD35) act as inhibitors of C3/C5 convertases while protectin (CD59) functions as an inhibitor of membrane attack complex (MAC). DAF inhibits the activation of C3 and C5 by binding to and dissociating C3/C5 convertase enzymes of both the classical and the alternative pathway (Lublin and Atkinson, 1989). MCP regulates C3 activation by acting as a cofactor protein for factor I-mediated cleavage of C3b (Liszewski et al., 1991). CR1 possesses both DAF and MCP functions. It has decay-accelerating activity against both the classical and the alternative pathway C3/C5 convertase. It also acts as a cofactor for factor I-mediated cleavage of C3b and C4b, as well as for the cleavage of iC3b to C3c and C3dg. Additionally, CR1 is a major immune adherence receptor and plays a role in immune complex processing and clearance (Ahearn and Fearon, 1989). Lastly, CD59 prevents the assembly of the MAC at the final step of the complement activation cascade. By binding to C5b-8 complex, CD59 limits C9 incorporation and polymerization in the MAC (Miwa and Song, 2001).

Due to the rapid and self-amplifying activation tendencies, the complement system needs to be well controlled in the fluid-phase. This is achieved by a set of fluid-phase regulators such as C1 inhibitor (C1-INH), C4b-binding protein (C4BP), factor H, clusterin and

vitronectin. C1-INH prevents spontaneous activation of the complement system by inhibiting the C1r and C1s serine protease of the classical pathway, as well as MBL-associated serine protease (MASP) -1 and -2 of the lectin pathway (Davis et al., 2008). C4BP acts as an inhibitor of the classical pathway by preventing the formation of the classical C3 convertase. It also acts as a factor I cofactor for the cleavage of C4b, consequently inhibits the alternative pathway to some extent as well (Blom et al., 2004). Factor H binds to C3b, accelerates the disassociation of the alternative pathway C3-convertase (C3bBb) and acts as a cofactor for the factor I-mediated proteolytic inactivation of C3b (Józsi and Zipfel, 2008). Clusterin and vitronectin bind to the terminal complement complexses and prevents their incorporation into cell membranes (Schwarz et al., 2008, Preissner and Seiffert, 1998).

1.7 Complement activation within the CNS

The CNS tissue is separated from plasma by the blood-brain barrier (BBB), a blood vessel network that forms tightly binding endothelial cells and perivascular astrocytes (Morgan and Gasque, 1996, Gasque et al., 2000). The BBB serves as a molecular sieve, which prevents infiltration of circulating immune cells such as B- and T- lymphocytes and restricts passage of plasma proteins into the brain parenchyma and cerebrospinal fluid (Abbott et al., 2010). The liver is the primary site of plasma complement protein synthesis. Though most of the complement proteins are unlikely to penetrate the intact BBB, local synthesis of complement components was found on astrocytes, microglia, neurons and oligodendrocytes (Barnum, 1995). The complement proteins produced by systemic immune cells may not reach the cerebral tissue when the BBB is intact; however, they could be found during CNS inflammation and neurodegenerative diseases (Morgan and Gasque, 1996, Gasque et al., 2000).

The roles of complement activation in the brain remain controversial. It can lead to cytolytic death of neurons and enhance a pro-inflammatory reaction contributing to the pathogenesis and progression of neurodegenerative disease (Shen et al., 1995). On the other hand, complement could also trigger brain tissue remodelling and repair by clearing toxic deposits, such as amyloid fibrils present in neurotic plaques in Alzheimer's disease (AD) or by enhancing the expression of growth factors involved in the early processes of regeneration (Stevens et al., 2007). Given the multiple functions of complement in CNS, it is suggested that complement may play a role in CNS homeostasis (Brennan et al., 2012).

1.8 Complement activation in ALS

Up-regulation of complement components was found in a number of neurodegenerative diseases, including ALS, AD and glaucoma (Rosen et al., 1993, Alexander et al., 2008). Transgenic hSOD1^{G93A} rats with end-stage disease showed a remarkable deposition of complement factor C3/C3b and significant up-regulation of the C5aR1 and C5L2 in the lumbar spinal cord (Woodruff et al., 2008a). In the mutant hSOD1^{G93A} mice, elevated levels of complement factors (C1qB, C4, factor B, C3/C3b, C5 and C5aR1) and down-regulation of complement regulators (CD55 and CD59a) were found during disease progression. Interestingly, increased microglial C5aR1 expression surrounding the regions of motor neuron death were shown in end-stage hSOD1^{G93A} mice (Lee et al., 2013). With chronic oral application of PMX205, a selective C5aR1 antagonist, transgenic SOD1^{G93A} rats displayed reduced astroglial proliferation in the regions of motor neuron degeneration, resulting in enhanced survival times (Woodruff et al., 2008a). Moreover, C5aR1-deleted hSOD1^{G93A} mice showed a similarly significant extension in survival compared with SOD1^{G93A} (Woodruff et al., 2014). Taken together, these findings suggest that complement system is over-activated in animal models of ALS and C5a-C5aR1 signalling contributes to the disease.

1.9 Immune cells of peripheral nervous system

The peripheral nervous system (PNS) is part of the nervous system, which contains the nerves and ganglia that reside outside of the spinal cord and brain. The primary function of the PNS is to facilitate the complex movements and behaviours by connecting the central nervous system to the organs, limbs, and skin. The PNS can be divided into two parts, the somatic nervous system and the autonomic nervous system. The somatic nervous system contains motor axons that innervate skeletal muscles, while the autonomic nervous system consists of cells and axons that connect to smooth muscle, cardiac muscle and glands.

The peripheral nervous system has long been regarded as an immunologically privileged site. It is separated from the external environment by the blood-nerve barrier (BNB), a highly specialised unit that limits the entry of immune cells and soluble mediators (Kieseier et al., 2006). However, the restriction is not complete as cells or soluble factors can easily access the PNS at the root entry and exit zones and at the nerve terminals where no barrier exists. Activated T and B-lymphocytes constantly patrol throughout the PNS.

Antigen-presenting cells (APC), like macrophages, and Schwann cells also contribute to the local immune networks (Wekerle et al., 1986).

1.9.1 Schwann cell

Schwann cell (SC), the primary glial cell of peripheral nervous system, is derived from neural crest cells located in the dorsal neural tube (Jessen and Mirsky, 2005). SC has critical roles in development, differentiation, physiological homoeostasis, myelination and nerve regeneration (Bunge, 1993). During the development of PNS, SC interacts with both the extracellular matrix and axons to form the myelin sheath that insulates axons and dramatically increases nerve conduction velocity (Balice-Gordon et al., 1998). In addition to myelinating Schwann cells, they can be further divided into three classes: non-myelinating Schwann cell, the presynaptic Schwann cell and the satellite cell. By contrast to myelinating Schwann cells which envelops large-diameter axons, non-myelinating Schwann cells can surround the small-diameter axons (Griffin and Thompson, 2008). The presynaptic Schwann cells, also known as terminal Schwann cells, are found at neuromuscular junctions (NMJ). These play several active and essential roles in synaptic function, formation and maintenance of NMJs (Corfas et al., 2004). Following injury to mammalian peripheral nerves, presynaptic Schwann cells extend their processes guiding reinnervating axons to endplates (Son and Thompson, 1995). The satellite cells are associated with the neuronal cell bodies of ganglia (Hanani, 2005).

1.9.2 Macrophage

Macrophages are phagocytes that were firstly identified by zoologist Metchnikoff in the late 19th century. They form the mononuclear phagocytic system (MPS) along with monocytes and dendritic cells. The MPS, neutrophils and mast cells are considered as ‘professional’ phagocytic cells, which express a number of receptors on their surface detecting signals that are not normally presented in healthy tissues. For instance, scavenger receptors are responsible for binding apoptotic and necrotic cells, opsonized pathogen sand cell debris (Murray and Wynn, 2011).

Macrophages are located in all tissues throughout the body and perform important various immune surveillance roles, including phagocytosis, antigen presentation and immune responses. They can be categorized into subpopulations according to their anatomical location and functional phenotype, such as osteoclasts (bone), alveolar macorphages

(lung), histiocytes (interstitial connective tissue) and Kupffer cells (liver) (Murray and Wynn, 2011). Classification of macrophages depends on their distinct functions, including activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Mantovani et al., 2005). M1 macrophages activation is induced by interferon (IFN)-γ alone or in combination with Toll-like receptor ligands (e.g. lipopolysaccharide (LPS)) or cytokines (e.g. tumor necrosis factor (TNF) and Granulocyte-macrophage colony-stimulating factor (GM-CSF)), whereas interleukin (IL)-4 or IL-13 stimulates an alternative M2 fashion of macrophage activation (Mantovani et al., 2005).

In the peripheral nervous system, there are large amounts of resident macrophages located within the endoneurium of peripheral nerves (Griffin et al., 1993). When inflammation or injury occurs, a great number of hematogenous macrophages rapidly invade the nerve following PNS lesion. The recruitment of macrophages is mediated by chemokines, such as macrophage inflammatory protein (MIP)-1α and transforming growth factor (TGF)-β1 (Zou et al., 1999, Kiefer et al., 1996). In addition, complement components, specifically anaphylatoxin C5a, can act as chemotactic agents to attract macrophages (Don et al., 2007).

1.9.3 T cell lymphocytes

T lymphocytes, also known as T cells, are one of the major lymphocyte populations in the immune system. T cells originate from the pluripotent hematopoietic stem cell population produced by bone marrow and they mature in the thymus. The thymus is the primary organ for T-lymphocyte development where T cells differentiate into helper, cytotoxic or regulatory T cells. The major function of T cells is to work with B cells, another type of lymphocyte that develop in bone marrow, to provide adaptive immunity. Unlike B cells, T cells cannot detect pathogens by themselves. They need the assistance of antigen-presenting cells (APC) like dendritic cells or macrophages, which engulf and digest pathogens becoming antigen fragments. These fragments are then presented on Major Histocompatibility Complex (MHC) class II, a protein located on the surface of APCs, to activate the immune response of T cells (Janeway et al., 2011).

T cells are subdivided into several groups based on their lineage markers and functional activities. CD4 and CD8 are the major surface co-receptors that define two separate T cell lineages with different functions. $CD4^+$ cells recognize antigen in the context of MHC class

II molecules and produce cytokines as effector helper T cells. $CD8^+$ cells are activated by antigenic peptides presented by MHC class I molecules and generate cytotoxic T cells (Broere et al., 2011).

Helper T cells and cytotoxic T cells are the two major classes of T cells. Helper T cells are the most common T cells, which make up over 75% of the T cell population. They perform their immune functions mainly through secreting cytokines, which assist B cells producing antibodies and recruiting phagocytic cells such as natural killer (NK) cells and macrophages to the site of infection. By contrast to helper T cells that indirectly exert their functions by communicating with other cells, cytotoxic T cells attack and destroy pathogens directly. Then are able to trigger the apoptosis, known as programmed cell death (PCD), in the targeted cells by releasing specialised lytic granules upon recognition of antigens on the surface or their targets (Janeway et al., 2011).

The other major subset of T cells is regulatory T cells (Tregs), which play pivotal roles in the development and maintenance of peripheral tolerance, modulation of immune response and prevention of autoimmune disease. The majority of Tregs appears within the $CD4^+$ T cell population, and can be divided into naturally occurring (nTreg) and inducible (iTreg) subtypes. nTregs are derived from thymus and are characterized by constitutive expression of the α-chain of the IL-2 receptor (CD25) and the transcription factor Foxp3, while iTregs arise from the naïve $CD4^+$ T cells in the periphery (Broere et al., 2011).

1.10 Immune cells in ALS mouse models

A common feature of ALS and other neurodegenerative diseases is the occurrence of a neuroinflammatory reaction consisting of activated glial cells, mostly microglia and astrocytes. Microglia are considered as the resident macrophages of the CNS while astrocytes are the largest glial cell population within the brain (Kreutzberg, 1996, Dong and Benveniste, 2001). Activation of microglia and astrocytes were found in both patients and mouse models of ALS (McGeer and McGeer, 2002, Hall et al., 1998). In concert with CNS resident immune cells, the peripheral immune cells, and in particular T cells, play a pivotal role in ALS pathogenesis. Infiltrating T cells were found in spinal cord tissue of ALS patients and mouse models (Engelhardt et al., 1993, Kawamata et al., 1992, Alexianu et al., 2001). Recent studies have suggested a neuroprotective role of $CD4^+$ T lymphocytes in mutant SOD1 mice (Beers et al., 2008, Banerjee et al., 2008, Chiu et al., 2008).

Evidence shows impaired T cell immune function and diminished T cell proliferative capacity in hSOD1^{G93A} mice as well as increased number of necrotic lymphocytes in the spleen (Banerjee et al., 2008).

When hSOD1^{G93A} transgenic mice were bred with functional T lymphocytes deficient or CD4+ T lymphocytes deficient mice (for instance, mice lacking recombination activating gene 2 (RAG2), hSOD1^{G93A} x RAG2$^{-/-}$ mice; CD4 knockout, hSOD1^{G93A} x CD4$^{-/-}$ mice, and T cell receptor β chain (TCR) deficient, hSOD1^{G93A} x TCRβ$^{-/-}$ mice), their motor neuron disease progression was accelerated and the survival length was shortened, and these mutant SOD1 mice displayed attenuated expression of morphological markers of microglia activation and microglial neurotropic factors such as IGF (insulin-like growth factor)-1 (Beers et al., 2008, Chiu et al., 2008). Interestingly, the adoptive T lymphocytes transfer from WT donor mice proved to be protective in a mutant SOD1 transgenic mouse model (Beers et al., 2008). The combined results indicate that T lymphocytes play an endogenous neuroprotective role in ALS by modulating the trophic/cytotoxic balance of glia.

1.11 Muscle denervation in ALS mouse models

Motoneuron death is a prominent feature of ALS. However, rescuing motor neurons from death has only a limited impact on the progression of disease and lifespan. It does not slow down muscle denervation in either SOD1 mouse models or humans (Fischer et al., 2004, Gould, 2006, Kostic et al., 1997, Sagot et al., 1995).

The skeletal muscle is one of the three types of muscle in the body, along with smooth and cardiac muscle. It represents the majority of muscle tissue, which makes up about 55% of individual body mass in most mammals (Zierath and Hawley, 2004). Skeletal muscle is composed of various muscle fibre types, which are distinguished by the distribution of different myosin heavy chian (MHC) isoforms. Generally, mammalian skeletal muscles consist of a “slow” myosin isoform (MHCI), and three “fast” myosin isoforms (MHCIIa, IId/x and IIb). Muscle fibres contain only one myosin isoform are regarded as pure fibres (type I with MHCI, type IIA with MHCIIa, type IID/X with MHCIId/x, and type IIB with MHCIIb). Fibres containing two myosin isoforms are referred as hybrid fibres, which can be subgrouped into: type IC (MHCI>MHCIIa); type IIC (MHCIIa>MHCI); type IIAD (MHCIIa>MHCIId); type IIDA (MHCIId>MHCIIa); type IIDB (MHCIId>MHCIIb), and type

IIBD (MHCIIb>MHCIId) (Pette and Staron, 2000). Under normal conditions, human and other large mammals do not express MHCIIb. Interestingly, MHCIIx transcript are more abundant in type IIB fibres of human skeletal muscle (Smerdu et al., 1994).

In this project, tibialis anterior (TA) and soleus (SOL) muscles from hSOD1^{G93A} mice and age-matched wild-type mice were used. Previous studies have shown that in C57BL6J mice, the fast-twitch tibialis anterior muscle consisted predominantly type IIB (59.68 ± 9.95%) and type IIDB (33.83 ± 15.85%) fibres while type IIAD, IID, IIA, I, and IC/IIC fibres were also observed. In the soleus muscle, the majority of muscle fibres are type I (37.42 ± 8.20%) and type IIA (38.62 ± 6.81%) where type I, IIA, IIAD and IID fibres were also seen (Augusto et al., 2004). However, several studies have termed the soleus muscle as a slow-twitch muscle as it is primarily composed of slow fibres (type I 58% vs type II 42%). Therefore, I defined the soleus muscle as a slow-twitch muscle in the present study (Barclay et al., 1993, Atkin et al., 2005, Hegedus et al., 2007).

Muscle fibres are dynamic structures and their phenotypes change in response to various conditions, such as altered neuromuscular activity, mechanical loading or unloading, different hormonal profiles, and aging (Pette and Staron, 2000). Animal models of ALS also demonstrated skeletal muscle fibre-type shifting during disease progression. In hSOD1^{G93A} mice, tibialis anterior muscles showed a 65% decreased in the number of innervated IIB fibres and a 28% reduction in the number of IID/X fibres around the age of 50 days (P50) prior to disease onset, while the number of type IIA muscle fibres increased by nearly twofold. These alternations suggested a preferential loss of the most forceful motor units containing type IIB fibres in tibialis anterior muscles of hSOD1^{G93A} mouse, as well as activity-dependent fibre type transition from fast fatigable fibres (FF = IIB) and fast fatigue intermediate fibres (FI = IID/X) to slower fatigue resistant (FR) type IIA fibres (Hegedus et al., 2007, Hegedus et al., 2008). On the other hand, the soleus muscle that is primarily composed of slow fibres showed no denervation at 30 days of age (P30) whereas tibialis anterior muscle denervation occurs demonstrated a 40% of denervation at the same age (Vinsant et al., 2013). These results suggest that muscle denervation occurs in a fast fibre preferential manner, which is attributed to the selective vulnerability of large motor neurons innervating fast-twitch fibres in hSOD1^{G93A} mouse model of ALS (Frey et al., 2000, Pun et al., 2006, Hegedus et al., 2007). This selective vulnerability of fast-twitch muscle (tibialis anterior) in ALS has also been confirmed by a separated study using

SOD1^{G93Adl}, a low-copy mutant human *SOD1* mouse model that shows a slower course of disease (Acevedo-Arozena et al., 2011).

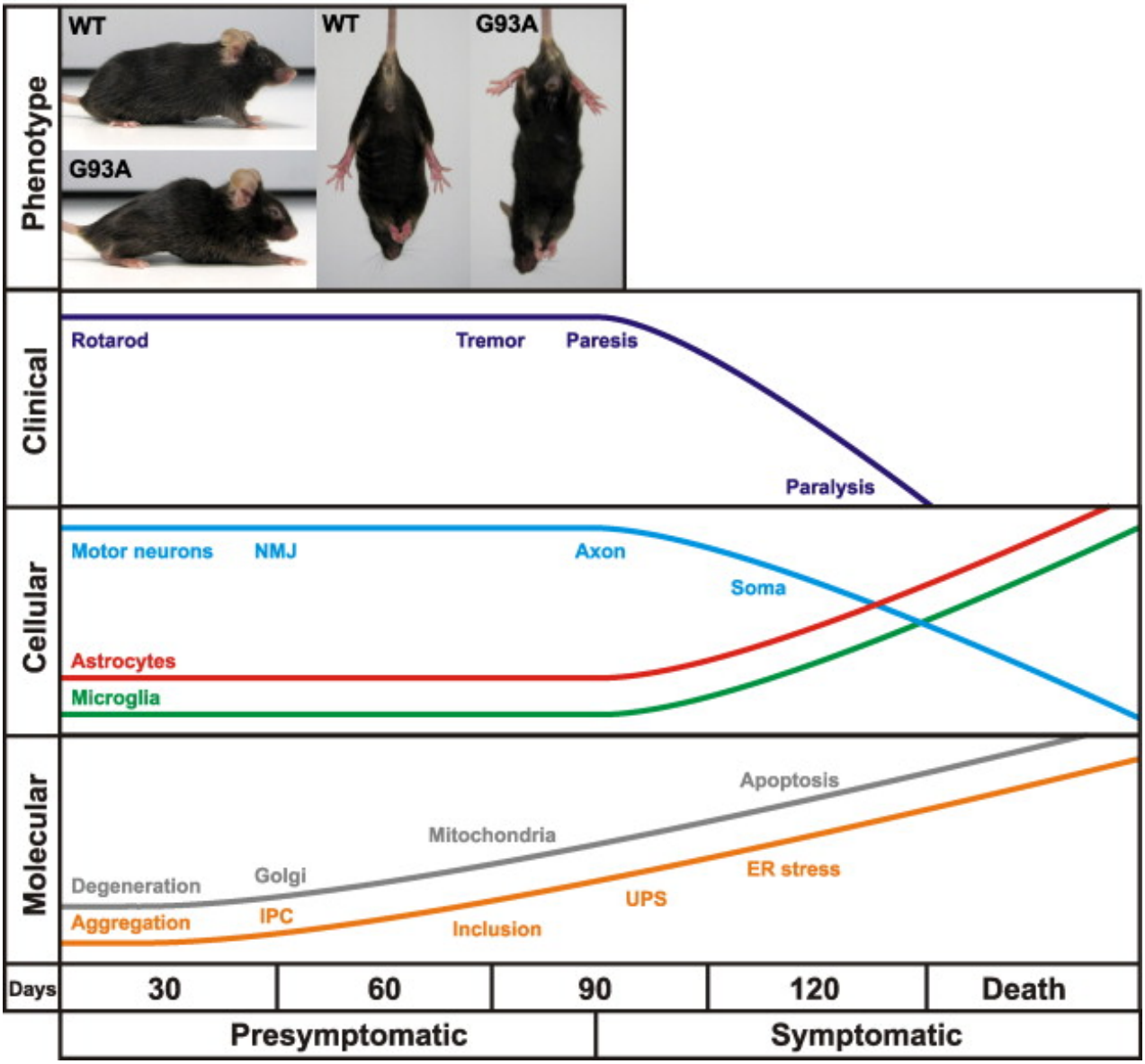

Figure 1. Time course of clinical and neuropathological events in high copy number transgenic hSOD1^{G93A} mice.

Mice develop hindlimb tremor, weakeness and locomotor deficits at about 90 days which is preceded by distal synaptic and axonal degeneration. These symptoms are followed by fatal paralysis about 1 month later concomitant with spinal motor neuron loss and reactive gliosis (Figure adpated from (Turner and Talbot, 2008).

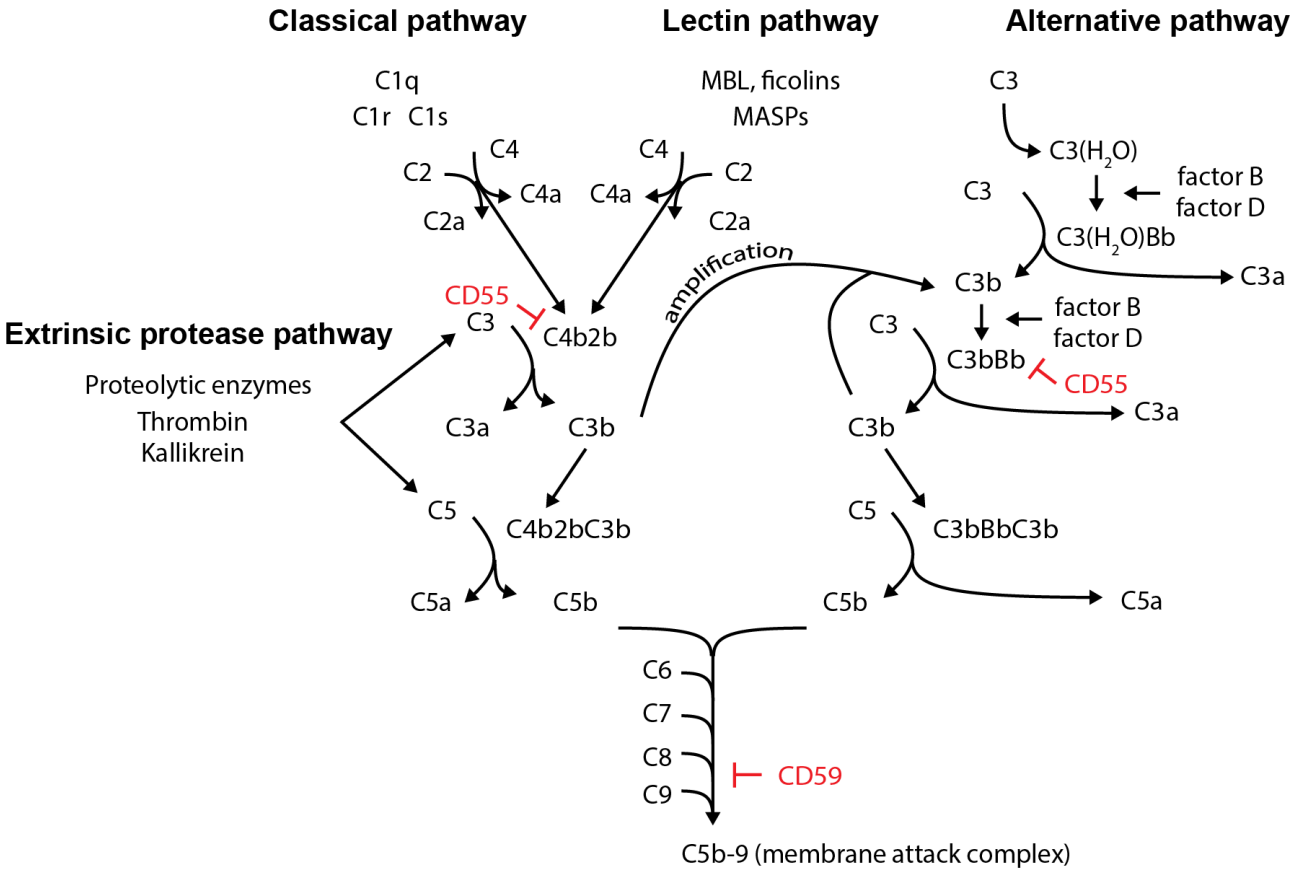
Classical pathway
Lectin pathway
Alternative pathway
C1q
C1r C1s
MBL, ficolins
MASPs
C3
C4
C2
C4a
C2a
C3(H2O)
factor B
factor D
C3(H2O)Bb
C3a
amplification
CD55
C3
C4b2b
C3b
Extrinsic protease pathway
Proteolytic enzymes
Thrombin
Kallikrein
C3bBb
C5
C4b2bC3b
C3bBbC3b
C5a
C5b
C6
C7
C8
C9
CD59
C5b-9 (membrane attack complex)

Figure 2. Activation pathways of complement.

The complement system can be activated through four pathways: the classical, lectin, alternative and extrinsic protease pathways. The classical pathway is triggered by the recognition of an antigen-antibody complex by C1q. C1q binds to a target surface, activating C1r and C1s that in turn cleaves C2 and C4 to form the C3 convertase C4b2b. The lectin pathway can be initiated by binding of mannose-binding lectins (MBLs) to certain carbohydrates expressed on the pathogen surface. This activates the MBL-associated serine protease (MASP) 2, cleaving C4 and C2 to form the C3 convertase of C4b2b. The alternative pathway is activated by spontaneous low level hydrolysis of C3 generating C3(H_2O) which forms a complex with factor B, allowing cleavage by factor D to generate the fluid-phase C3 convertase C3(H_2O)Bb. This fluid phase enzyme cleaves C3 and deposits C3b on surfaces. Factor B then binds to surface-bound C3b to form C3bB which can be cleaved by factor D to form the surface-bound C3 convertase C3bBb. Regardless of the pathway involved, activation of the complement cascade leads to the cleavage of C3 and C5, forming the anaphylatoxins C3a and C5a as well as the C5b fragment. C5b generation leads to the formation of the membrane attack complex (MAC), a lipophilic complex that forms pores in the pathogen membrane, leading to cell lysis. A fourth pathway where C3 and C5 can be directly cleaved by proteolytic enzymes such as thrombin and kallikrein has been detected and named the ‘extrinsic protease’ pathway.

CD55 and CD59 are the main complement regulatory proteins. CD55 promotes the degradation of C3- and C5-convertases, while CD59 inhibits C5b-9 (membrane attack complex) formation by interfering the binding of C9 units to C5b-8 complex.

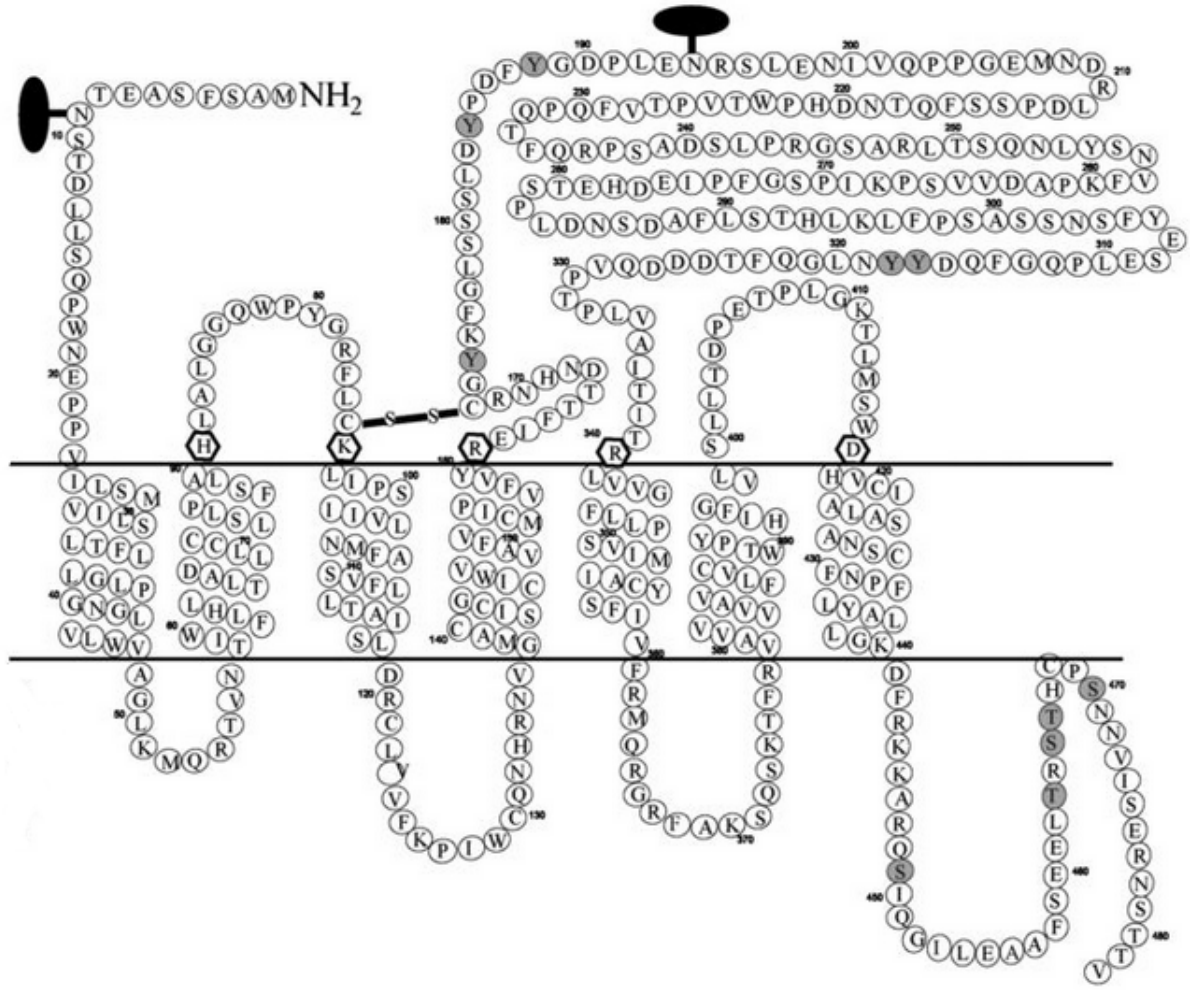

Figure 3. Structure of C3a receptor.

C3aR has a seven transmembrane helices structure. It contains a unique reduced N-terminal domain and a remarkable large second extracellular loop between the fourth and fifth transmembrane domains, which is required for C3a binding (Figure adapted from (Klos et al., 2013))

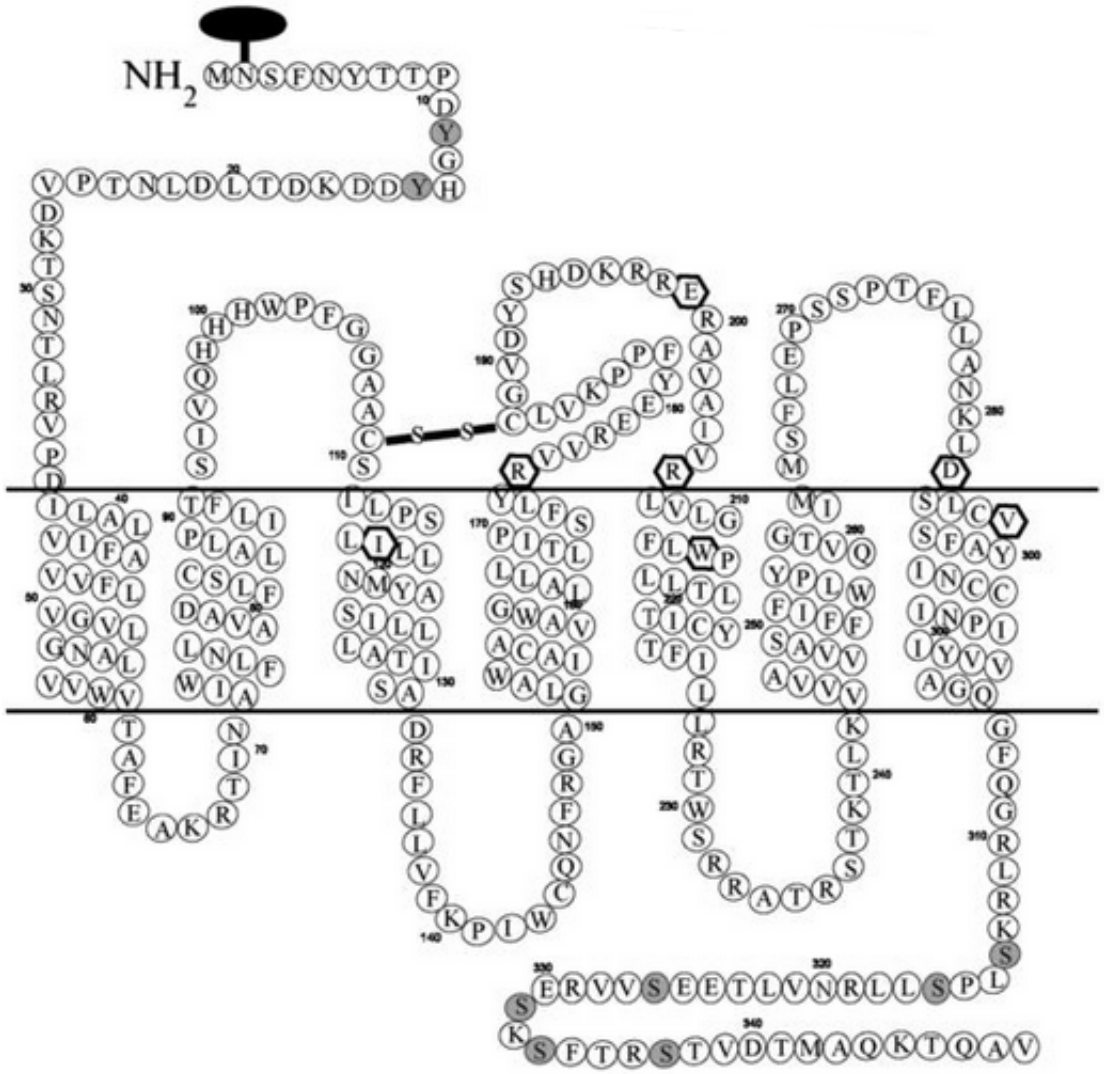

Figure 4. Structure of C5a receptor 1.

The seven transmembrane helices of C5aR1 contain different charged loop regions. The N-terminal extracellular domain of the receptor, and a secondary site, involving Glu199 and Arg206 of C5aR1, are involved in the interaction with C5a (Figure adapted from (Klos et al., 2013)).

Chapter 2

General Methodology

2.1 Mice

Transgenic hSOD1^{G93A} mice with high copy number (25 copies of hSOD1^{G93A} transgene) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were bred on C57BL/6J background to generate hSOD1^{G93A} mice and litter matched wild-type (WT) control mice. These hSOD1^{G93A} mice carry a high copy number of the mutated allele of the human (h) SOD1 gene where glycine at codon 93 is replaced by alanine. Homozygous C5aR1 deficient mice (C5aR1$^{-/-}$) were kindly provided by Dr Wetsel and described previously (Hollmann et al., 2008).

To generate hSOD1^{G93A} mice lacking C5aR1 (hSOD1^{G93A} × C5aR1$^{-/-}$) and C3aR (hSOD1^{G93A} × C3aR$^{-/-}$), transgenic heterozygous hSOD1^{G93A} males were first cross-bred with C5aR1$^{-/-}$ and C3aR$^{-/-}$ females to generate F1 progeny (hSOD1^{G93A} × C5aR1$^{+/-}$ and hSOD1^{G93A} × C3aR$^{+/-}$). hSOD1^{G93A} × C5aR1$^{+/-}$ and hSOD1^{G93A} × C3aR$^{+/-}$ males were cross bred with C5aR1$^{-/-}$ and C3aR$^{-/-}$ females to obtain F2 progeny (hSOD1^{G93A} × C5aR1$^{-/-}$ and hSOD1^{G93A} × C3aR$^{-/-}$).

The disease of amyotrophic lateral sclerosis (ALS) in hSOD1^{G93A} mice were categorised into four stages, pre-symptomatic (P30), onset (P70), mid-symptomatic (P130) and end stages (P175) according to the symptoms they display (Lee et al., 2013)(Table 1).

Female WT, hSOD1^{G93A}, WT × C5aR1$^{-/-}$, hSOD1^{G93A} × C5aR1$^{-/-}$, WT × C3aR$^{-/-}$ and hSOD1^{G93A} × C3aR$^{-/-}$ mice at onset, mid-symptomatic and end stages were employed in this study. All experimental procedures were approved by the University of Queensland Animal Ethics Committee (Permit Number 433-12), and complied ethical guidelines

regarding animal experimentation (Drummond, 2009). In addition, all procedures were conducted in accordance with the Queensland Government Animal Research Act 2001 and Protection Regulations (2002 and 2008), and conformed to the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition (National Health and Medical Research Council, 2013).

2.2 Immunohistochemistry

Transverse cryosections (10 µm) from the tibialis anterior (TA) and soleus (SOL) muscles of WT and mutant hSOD1^{G93A} mice were stained to localise the expression of C5aR1 and C3aR with specific cell-type markers for neuromuscular junctions (alpha-Bungaratoxin, 1:5000, Invitrogen/Life Technologies, Grand Island, NY, USA), Schwann cells (rabbit S100, 1:1000, DAKO, Kyoto, Japan), macrophages (rat CD11b, 1:250, Abcam, Cambridge, MA, USA), helper T cells (rat CD4, 1:250, Abcam, Cambridge, MA, USA; rabbit CD4, 1:50, Novus Biologicals, Littleton, CO, USA), cytotoxic T cells (rat CD8, 1:250, Abcam, Cambridge, MA, USA. Cryosections were blocked in phosphate-bufferd saline (PBS) containing 2% normal goat serum (Sigma, USA) or 3% donkey serum (Sigma, USA), and 0.2% Triton X-100 (Sigma, USA) at room temperature for 35 minutes and incubated with primary antibodies at 4 °C overnight. After incubation, the sections were washed in PBS and then incubated with an appropriate secondary antibody at room temperature for 2 hours: Alexa 555 goat anti-rat (1:1000, Invitrogen/Life Technologies, Carlsbad, CA, USA), Alexa 488 goat anti-rat (1:600, Invitrogen/Life Technologies, Carlsbad, CA, USA), Alexa 488 goat anti-rabbit (1:600, Invitrogen/Life Technologies, Mulgrave, VIC, Australia), Alexa 488 donkey anti-goat (1:600, Invitrogen/Life Technologies, Carlsbad, CA, USA) and Alexa 594 donkey anti-rat (1:1000, Invitrogen/Life Technologies, Carlsbad, CA, USA). All the primary and secondary antibodies were diluted in PBS with 2% bovine serum albumin and 0.2% Triton X-100. All sections were incubated with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen/Life Technologies) for 10 minutes at room temperature prior to mounted with Prolong Gold Anti-Fade medium (Invitrogen, Life Technologies). Fluorescent signals were observed using a Zeiss LSM Meta 510 upright confocal microscope with a Plan-Apochromat 63× oil objective (Carl Zeiss Inc., Oberkochen, Germany)

2.3 Quantification of peripheral immune cells

Serial sections of TA and SOL muscles were stained for macrophages (CD11b) and helper T cells (CD4) in WT, hSOD1^{G93A}, WT × C5aR1$^{-/-}$, hSOD1^{G93A} × C5aR1$^{-/-}$, WT×C3aR$^{-/-}$, and hSOD1^{G93A} × C3aR$^{-/-}$ mice (10 sections spaced 100 µm apart per animal, *n* = 3 mice/group). Five random regions (874 × 655 × 10 µm) from each section were selected without any knowledge of the presence of positive cells by viewing only in DAPI channel. Each selected region was imaged with standardised settings and then saved. The numbers of fluorescently labelled cells in each section were counted at 20× magnification and expressed as cells/mm^3.

Given that the CD11b positive cells may also mark other peripheral immune cells, I used CD8 (cytotoxic T cell) and Ly6B (neutrophil) antibodies to distinguish CD11b positive macrophages. As minimal number of CD8$^+$ or Ly6B$^+$ cells were detected in either tibialis anterior or soleus muscles from hSOD1^{G93A}, hSOD1^{G93A} × C5aR1$^{-/-}$, hSOD1^{G93A} × C3aR$^{-/-}$ and wild-type mice (*data not shown*), the vast majority of CD11 positive cells shown in this study were macrophages (Christensen et al., 2001, Fink et al., 2014).

2.4 Real-time quantitative PCR

Total RNA was isolated from TA muscle of WT and hSOD1^{G93A} mice using Rneasy Lipid Tissue extraction kit (QIAGEN Inc., Alameda, CA, USA) according to the manufacturer's protocol. The total RNA was purified using Turbo DNAse treatment (Ambio, Life Techonologies) then converted to cDNA by means of a reverse transcription kit (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Target genes of interest were amplified using commercial TaqMan probes (Applied Biosystems, Life Technologies)(Table 2). 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} mice compared with expression in WT controls. (*n* = 5 mice/group)

2.5 Western blot analysis

Muscle homogenates from hSOD1^{G93A} and WT mice at different disease stages were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Pall, Ann Arbor, MI, USA). Membranes were blocked with 2.5% milk in Tris-buffered saline (TBS)-

Tween (TBST) solution (containing 1× TBS and 0.1% Tween 20) for one hour at room temperature and were subsequently incubated with anti-C5aR1 antibody overnight at 4 °C (1:2,500 dilution in 5% BSA-TBST; BMA Biomedical, Augst, Switzerland). Membranes were washed with TBST 3 × 10 minutes and then incubated with the goat anti-chicken horseradish peroxidase (HRP) (1:15,000 dilution in TBST, GE Healthcare, Pittsburgh, PA, USA) for one hour at room temperature. After a final wash with TBST for 6 × 5 minutes, signals were detected using the ECL system (GE Healthcare Biosciences, Pittsburgh, PA, USA). Blots were stripped and reprobed with anti-GAPDH (1:15,000; Millipore, Billerica, MA, USA) and then detected with sheep anti-mouse HRP (1:4,000; GE Healthcare) as loading control. Semi-quantitative densitometric analysis of these immunoreactive bands was carried out to determine differences in C5aR1 expression levels between $hSOD1^{G93A}$ and WT samples at different disease stages as described previously (Abramoff et al., 2004). (*n* = 4 mice/group)

2.6 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, $NaHCO_3$, 34 μM 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 (Livak and Schmittgen, 2001). Following the plate being blocked for 1 hour at room temperature with assay diluent (10% Fetal Calf Serum/PBS), C5a standard and TA muscle 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 TA muscle samples were adjusted to micrograms per protein and expressed as nanograms of C5a per microgram of protein. (*n* = 6 mice/group)

2.7 Statistical analysis

All measures were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). For the results from quantitative real time PCR, western blotting, ELISA, statistical differences between $hSOD1^{G93A}$ and WT mice were analysed using two-tailed *t*-

test at each stage of disease progression. The statistical differences between WT, hSOD1^{G93A}, WT × C5aR1$^{-/-}$, hSOD1^{G93A} × C5aR1$^{-/-}$, WT × C3aR$^{-/-}$ and hSOD1^{G93A} × C3aR$^{-/-}$mice for peripheral immune cell numbers were analysed using two-way analysis of variance (ANOVA). All data are presented as mean ± SEM and differences were considered significant when $P \leq 0.05$.

Table 1. Different stages defined in amyotrophic lateral sclerosis

Stage	Age	Phenotye
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 175 days postnatal	Full paralysis of lower limbs and loss of righting reflex

Table 2. Taqman probes used for quantitative PCR

Gene of interest	Catalogue number
C1qB	Mm01179619_m1
C3	Mm01232779_m1
C3aR	Mm01184110_m1
C4	Mm00437896_g1
C5	Mm00439275_m1
C5aR1	Mm00500292_s1
CD55	Mm00438377_m1
CD59a	Mm00483149_m1
C9	Mm00442739_m1
Factor B	Mm00433909_m1
Mannose binding lectin 1	Mm00495413_m1
Mannose binding lectin 2	Mm00487623_m1

Chapter 3

Expression of general complement components in the skeletal muscle of hSOD1^{G93A} mice

3.1 Introduction

The complement system comprises numerous plasma and membrane-bound proteins which serve to recognize and destruct invading pathogenic microorganisms while preserving normal cells (Walport, 2001). In mammals, the liver is the primary site of production of the most complement components. Over the last few decades, studies have shown various complement proteins synthesized by astrocytes, microglia, and neurons in the central nervous system (CNS) (Bonifati and Kishore, 2007, van Beek et al., 2003, Thomas et al., 2000). Under normal conditions, local synthesis of complement components in the CNS is relatively low. However, when stimulated with inflammatory cytokines, glial and neuronal cells in the CNS can assemble a wide variety of complement components (Levi-Strauss and Mallat, 1987, Thomas et al., 2000).

It has long been suggested that complement is involved in demyelination, neurodegenerative disorders and other CNS pathologies (Morgan and Gasque, 1996). In the last two decades, the complement system has been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS). Significant deposition of C3 was first found in the spinal cord and motor cortex of ALS patients (Donnenfeld et al., 1984). In addition, increased C3c in cerebrospinal fluid (CSF) and increased serum C4 has been detected (Apostolski et al., 1991, Kawamata et al., 1992). Moreover, up-regulation of C1q, C4d, C2, C3c, C3dg, and Factor H has been seen in various tissues in ALS patients (Tsuboi and Yamada, 1994, Trbojevic-Cepe et al., 1998, Grewal et al., 1999, Jiang et al., 2005,

Goldknopf et al., 2006). A recent study conducted by our lab has also shown elevated levels of C5a and C5b-9 in the plasma of ALS patients (Mantovani et al., 2014).

In addition to the findings of increased complement component in ALS patients, over-expression of complement factors has been shown in animal models of ALS. In the transgenic $SOD1^{G93A}$ mice, increased expression of C1q in ventral motor neurons from lumbar spinal cord was found using microarray analysis (Perrin et al., 2005). Another study demonstrated up-regulation of C1q in lumbar spinal cord motor neurons in two other mutant SOD animal models, $SOD1^{G37R}$ and $SOD1^{G85R}$ mice. Furthermore, expression of C1q protein was also found on motor neurons in these two SOD1 mutants (Lobsiger et al., 2007). Elevated C1q expression in SOD transgenic mice was further demonstrated by two other studies (Ferraiuolo et al., 2007, Fukada et al., 2007). Taken together, these results suggest that complement activation is involved in the disease progression of ALS mice models.

The role of complement activation in skeletal muscle during ALS pathogenesis has not been well studied. In $mSOD1^{G93A}$ mice lacking C4, decreased macrophage activation was observed. Since C4 is necessary for the activation of both the classical and lectin complement pathways, this result suggests that C4 deposition in skeletal muscle of ALS animal model may lead to the activation of downstream complement cascade and subsequent macrophage recruitment (Chiu et al., 2009). To further understand the role of complement plays in skeletal muscle, we quantified the expression levels of the major complement components in $hSOD1^{G93A}$ mice and compared to wild-type (WT) counterparts in this chapter.

3.2 Results

3.2.1 Up-regulation of major complement components in skeletal muscle of $hSOD1^{G93A}$ mice

Previous studies from our lab have shown up-regulation of major complement components in the lumbar spinal cord of $hSOD1^{G93A}$ mice, however it is unclear whether the complement system is activated in the skeletal muscle of $hSOD1^{G93A}$ mice during disease progression (Lee et al., 2013). To investigate this, the mRNA levels of the key initiating components (classical pathway - C1qB; lectin pathway – MBL-1/2; alternative pathway - factor B), a central protein for both classical and lectin pathways (C4), the central

component of all complement cascades (C3), and the major unit of the membrane attack complex (C9) were measured using quantitative real-time PCR in the tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice, respectively.

C1qB is the b chain of complement 1 subcomponent q (C1q). It reflects the expression level of C1q, the initiator of classical complement cascade. The results showed significant increase of C1qB transcripts by 2.1-fold and 6.5-fold at mid-symptomatic (P130) and end-stage (P175) of disease in hSOD1^{G93A} mice respectively when compared to wild-type mice (n = 5, *p < 0.05; Figure 5). Factor B, an initiator of the alternative complement activation pathway, also demonstrated increased transcript levels by 1.7-fold, 2.3-fold and 7.7-fold at onset, mid-symptomatic and end-stage disease in hSOD1^{G93A} mice when compared to age-matched wild-type mice (n = 5, *p < 0.05; Figure 6). C4 illustrated similar changes to that of C1qB and factor B, where its mRNA expression levels increased by 2.2-fold, 3.3-fold and 17.1-fold at onset, mid-symptomatic and end-stage disease in hSOD1^{G93A} mice (n = 5, *p < 0.05, **p < 0.01; Figure 7). Examination of mRNA levels on mannose binding lectin 1 and mannose binding lectin 2, initiators of the lectin pathway, were also performed. However, the results showed undetectable levels of MBL-1/2 mRNA in tibialis anterior muscle of either hSOD1^{G93A} or wild-type mice (*data not shown*).

In addition, mRNA expression levels of C3 were also examined. C3 plays a central role in the activation of all complement pathways. C3 displayed a marked increase in mRNA levels by 1.8-fold and 5.6-fold increase at mid-symptomatic (P130) and at end-stage (P175) of disease respectively (n = 5, *p < 0.05; Figure 8). In short, expression levels of initiator factor C1q, factor B, and C4, and central complement cascade component C3 were found significantly increased in hSOD1^{G93A} mice. These results suggest that activation of complement system in skeletal muscle of hSOD1^{G93A} mice may contribute to the disease progression of ALS.

3.2.2 Altered expression of complement regulators and receptors

Decay-accelerating factor ([DAF], CD55) is a glycosylphosphatidylinositol (GPI) linked membrane inhibitor of complement. It inhibits the activation of complement by interfering with the functional activity of C3 and C5 convertases in both the classical and alternative pathways. It is widely distributed on both vascular and non-vascular cell types (Lublin and Atkinson, 1989, Miwa and Song, 2001). Previous study from our laboratory has

demonstrated decreased expression of CD55 at both mRNA and protein levels in the spinal cord of hSOD1^{G93A} mice at end-stage disease (Lee et al., 2013). Here we examined the expression of CD55 in the tibialis anterior muscle of hSOD1^{G93A} mice and showed that its mRNA levels were significantly increased by 1.5-fold and 1.7-fold at onset and end-stage disease respectively when compared to age-matched wild-type mice (n = 5, *p < 0.05; Figure 9).

CD59a is the primary regulator of membrane attack complex assembly in mouse (Baalasubramanian et al., 2004). The mRNA levels of CD59a altered in a similar fashion to that of CD55; namely a 1.3-fold and a 2.4-fold increase of its mRNA expression were observed at onset and end-stage disease respectively in hSOD1^{G93A} mice when compared to wild-type controls (n = 5, *p < 0.05, **p < 0.01; Figure 10). Besides the regulator CD59a, the major component of membrane attack complex C9 was also measured. However, undetectable mRNA expression levels of C9 were found in tibialis anterior muscle of either hSOD1^{G93A} mice or wild-type counterparts (*data not shown*). Taken together, these results suggest that the homeostasis of the complement system was disrupted in the skeletal muscle of hSOD1^{G93A} mice.

3.3 Discussion

It has been well documented that activation of complement cascade contributes to the disease progression of ALS in both human patients and rodent models (Lee et al., 2012a). In the hSOD1^{G93A} transgenic mice, previous studies by our group have demonstrated that complement factors, including C1qB, factor B, C4 and C3, were strongly up-regulated in the central nervous system (Lee et al., 2013). However, little is known about the complement activation in the skeletal muscle of ALS mouse models. Since skeletal muscle is a direct target of SOD1 mutation, in this study, I sought to investigate the expression of the major complement factors and its regulator in the skeletal muscle of hSOD1^{G93A} mice (Dobrowolny et al., 2008).

The present study has for the first time demonstrated the up-regulation of mRNA expression in C1qB, factor B, C4 and C3, the initiators and central components of complement cascades, in the tibialis anterior muscle of hSOD1^{G93A} mice during disease progression. It has been identified that the mRNA expression levels of C1qB, factor B, C4 and C3 were up-regulated in the spinal cord of hSOD1^{G93A} mice throughout symptomatic

phases (Lee et al., 2013). Here I revealed that this up-regulation of complement factors is not restricted to the central nervous system, but also applies to skeletal muscle.

Skeletal muscle is an extremely complex and heterogeneous tissue composed of a spectrum of fibre types with different structure, molecular composition and functions. Recent evidence suggests that skeletal muscle is a primary target in hSOD1^{G93A} mice, and a retrograde and progressive sequential pattern of degeneration has been proposed where hSOD1^{G93A} mutation causes muscle atrophy, followed by fragmentation of their neuromuscular junction (NMJ), retrograde axonal degeneration and eventually motor neuron death (Dobrowolny et al., 2008, Dupuis and Loeffler, 2009, Zhou et al., 2010, Wong and Martin, 2010). In the present study, up-regulation of C1qB and C3 mRNA expressions was observed at mid-symptomatic (P130) age in tibialis anterior muscles. In addition, it has been previously shown that complement components C1q and C3b/iC3b are deposited at NMJ at pre-symptomatic (47 days), prior to the appearance of clinical symptoms, and remain detectable until mid-symptomatic (126 days) (Heurich et al., 2011). These findings implicate that activation of complement may contribute to the degenerations of distal axons in hSOD1^{G93A} mice during disease progression.

Besides C1qB and C3, factor B and C4 mRNAs transcripts were also significantly increased in the tibialis anterior muscles of hSOD1^{G93A} mice during disease progression, compared to wild-type controls. Factor B is a 93-kDa single peptide chain protein. It initiates the activation of alternative pathway by binding to the spontaneously hydrolysed C3. Upon activation, factor B is cleaved into the fragments Ba and Bb by factor D, generating C3 converatase C3bBb (Ricklin et al., 2010). C4 is a paralogous to C3 and C5, and shares up to 30% sequence identity with the two complement proteins. It is a central component in the classical and lectin pathways where its major fragment C4b plays an important role in mediating downstream complement activation (Mortensen et al., 2015). Significant up-regulations of both factor B and C4 mRNA levels were observed in tibialis anterior muscle of hSOD1^{G93A} mice, suggesting that all of the three main activation pathways within the complement cascade were activated in ALS disease progression in hSOD1^{G93A} mice. In combination with previous findings, where complement activation was demonstrated in the spinal cord of hSOD1^{G93A} mice, the results of the present study suggest that global complement pathway activation is a common feature of ALS pathology in hSOD1^{G93A} mice, and this activation is not restricted to CNS (Lee et al., 2013).

C1q acts as an opsonin in the immune system, marking apoptotic cells and debris for removal by phagocytosis. Recent evidence also suggests that C1q plays an active role in triggering developmental synapse elimination (Stevens et al., 2007, Chu et al., 2010, Stephan et al., 2012). In hSOD1^{G93A} mice, up-regulation of C1qB mRNA level has been shown in the spinal cord tissue while protein expression of C1 has been found on motor neurons and microglia. These findings suggest that C1q might assist in the removal of dying motor neurons through opsonisation in hSOD1^{G93A} mice (Lee et al., 2013). Combining the discovery of C1q deposition at the denervated NMJ from previous studies and the up-regulation of C1qB mRNA expression in tibialis anterior muscle shown by the present study, I postulate that C1q might drive the pruning of synapses at the denervated NMJ in hSOD1^{G93A} mice in a similar manner as in the developmental synapse elimination, leading to synapse degeneration.

C3 is the central compartment of the complement cascade where three primary activation pathways converged. It has been demonstrated that C3 deposits in spinal cord of transgenic SOD1^{G93A} murine models, and in spinal cord and motor cortex of ALS patients (Woodruff et al., 2008a, Lee et al., 2013, Donnenfeld et al., 1984). In the present study, significant increase in C3 mRNA levels were observed from mid-symptomatic (day 130) in the tibialis anterior muscle of hSOD1^{G93A} mice compared with wild-type mice. This may suggest that activation of complement pathways is a universal feature, not restricted to the central nervous system but also occurs in skeletal muscle, during the disease progression in the hSOD1^{G93A} mice. In addition to C3, deposition of C3b/iC3b, the active product of C3, has been found at the NMJ, promoting the opsonisation of those destructed NMJ by phagocytes (Heurich et al., 2011). Increased C3 mRNA expression in the skeletal muscle of hSOD1^{G93A} mice shown in this study supports this idea that complement facilitates the denervation of NMJ across ALS disease progression.

To investigate this up-regulation of complement in ALS affected muscles further, I examined the levels of complement regulators CD55 and CD59. CD55 inhibits the complement activation via suppressing the formation and promoting the catabolism of C3 and C5 convertases (Fujita et al., 1987). CD59 inhibits the formation of membrane attack complex by interfering the binding of C9 units to C5b-8 complex (Navenot et al., 1997).

Previous *in vitro* studies have shown that CD55 can protect neurons from degeneration

and apoptosis (Wang et al., 2010b). Decreased levels of CD55 mRNA and protein were observed in the spinal cord of hSOD1^{G93A} mice during later stages of disease (Heurich et al., 2011, Lee et al., 2013). By contrast, we showed a significant increase in mRNA level of CD55 in the tibialis anterior muscle of hSOD1^{G93A} mice at end-stage (day 175) compared with wild-type mice. Since CD55 inhibits the generation of C3 convertase, increases in CD55 mRNA expression level may lead to reduced production of active fragment C3b/iC3b, which in turn ameliorates denervation of NMJ in hSOD1^{G93A} mice. This may indicate a negative feedback mechanism to slow down the activation of complement. However, a significant increase of C3 mRNA level was shown in skeletal muscle of hSOD1^{G93A} mice at end-stage of disease as well. This finding gives rise to the question how is complement being activated in the absence of external pathogens in skeletal muscle?

It has been shown that mutant SOD1 toxicity can induce transcriptional up-regulation of C1q subunits (C1qA, C1qB and C1qC) in the motor neuron before the clinical symptoms, while restricted expression of mutant hSOD1^{G93A} on specific neurons is sufficient to induce motor neuron degeneration in mice (Lobsiger et al., 2007, Jaarsma et al., 2008, Wang et al., 2008). As a portion of mutant SOD1 may act from outside of the motor neurons (e.g. extracellular SOD), it is possible that the induced complement system recognizes the extracellular mutant SOD1 and marks the motor neuron for attack (Urushitani et al., 2006). These findings implicate mutant SOD1 toxicity in neurodegeneration via triggering activation of the complement system. Besides neuronal cells, mutant SOD1 is also expressed in skeletal muscle of hSOD1^{G93A} mice. Significant elevation of oxidation, accompanied by substantial compensatory antioxidant enzyme up-regulation has been observed in the skeletal muscle of hSOD1^{G93A} mice (Mahoney et al., 2006). Transgenic mice with selective-expression of *hSOD1^{G93A}* in skeletal muscle showed that accumulation of oxidative stress served as a signalling molecule to trigger muscle atrophy (Dobrowolny et al., 2008).

It has been well established that complement activation plays an important role in mediating tissue injury after oxidative stress. The lectin pathway mediates activation and deposition of complement after endothelial oxidative stress in the context of ischemia/reperfusion (I/R) injury (Collard et al., 2000). Complement activation and deposition also takes place following skeletal muscle I/R. The lectin and/or classical

pathway were implicated in the complement activation during skeletal muscle I/R (Weiser et al., 1996, Toomayan et al., 2003). Collectively, these data suggest that oxidative stress within skeletal muscle caused by mutant SOD1 toxicity might trigger the activation and deposition of complement within skeletal muscle of hSOD1^{G93A} mice.

In the previous study from our laboratory, CD59 displayed decreased mRNA expression level in the spinal cord of hSOD1^{G93A} mice at end-stage of disease (Lee et al., 2013). By contrast, the current study showed that CD59 mRNA expression was significantly increased in tibialis anterior muscle of end-stage hSOD1^{G93A} mice. This overexpression of CD59 observed in muscles of hSOD1^{G93A} mice could be beneficial as it may prevent muscle damage subsequent to deposition of MAC (Goncalves et al., 2002).

One limitation of my results is that I only examined mRNA transcript expressions but not protein expressions of the above complement factors. Given that our laboratory's previous published work has shown that the up-regulation of these complement factor transcripts does correlate with up-regulation of their proteins in the spinal cords of hSOD1^{G93A} mice, it does support my general conclusion for an up-regulation of these members of complement in skeletal muscle of hSOD1^{G93A} mice (Lee et al., 2013).

In summary, this research chapter has shown that complement is activated in the skeletal muscle of hSOD1^{G93A} mice during ALS disease progression, and the activation of complement may be as a consequence of increased oxidative stress within muscle, triggered by mutant SOD1 toxicity.

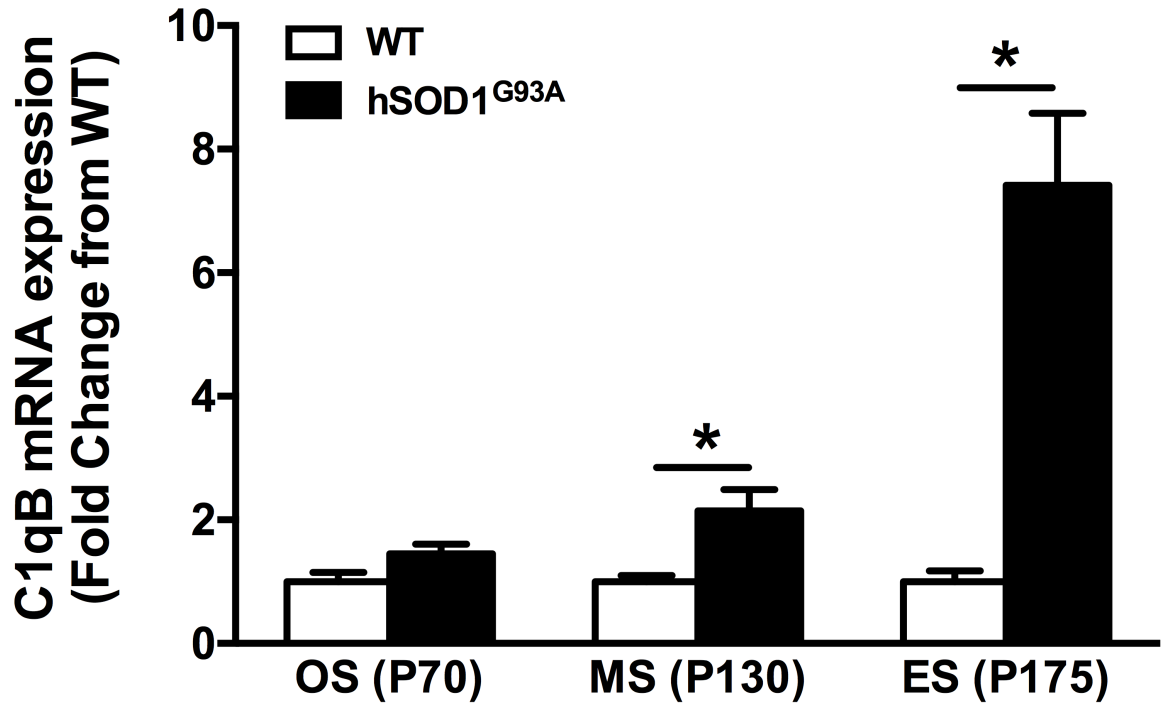

Figure 5. Expression of C1qB in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of C1qB in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR at three different ages. (n = 5; *p < 0.05, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

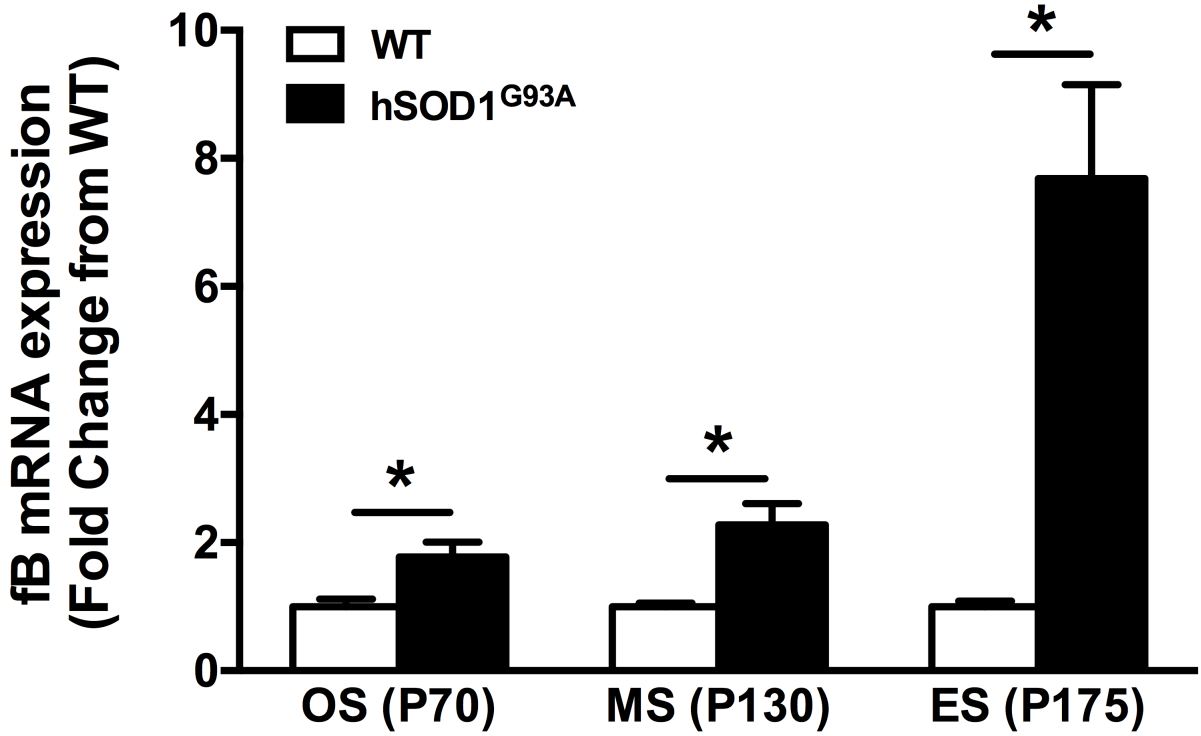

Figure 6. Expression of factor B in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of factor B in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR at three different ages. (n = 5; *p < 0.05, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

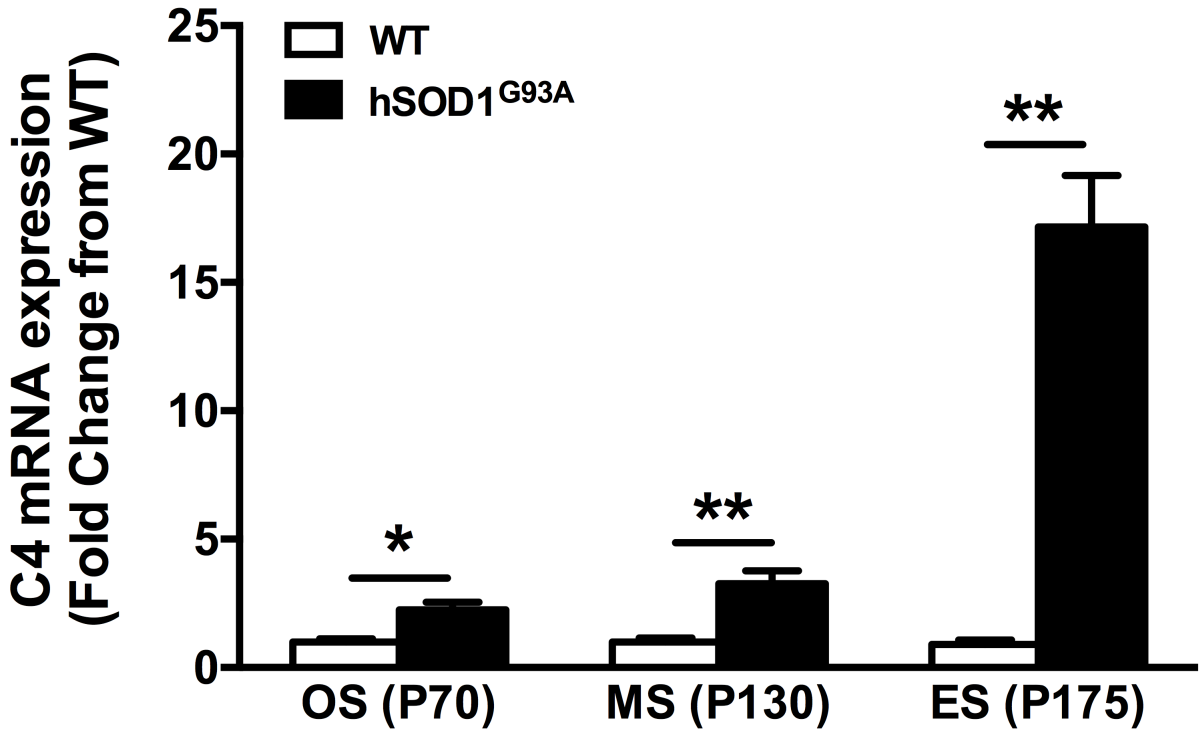

Figure 7. Expression of C4 in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of C4 in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR at three different ages. (n = 5; *p < 0.05, **p < 0.01, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

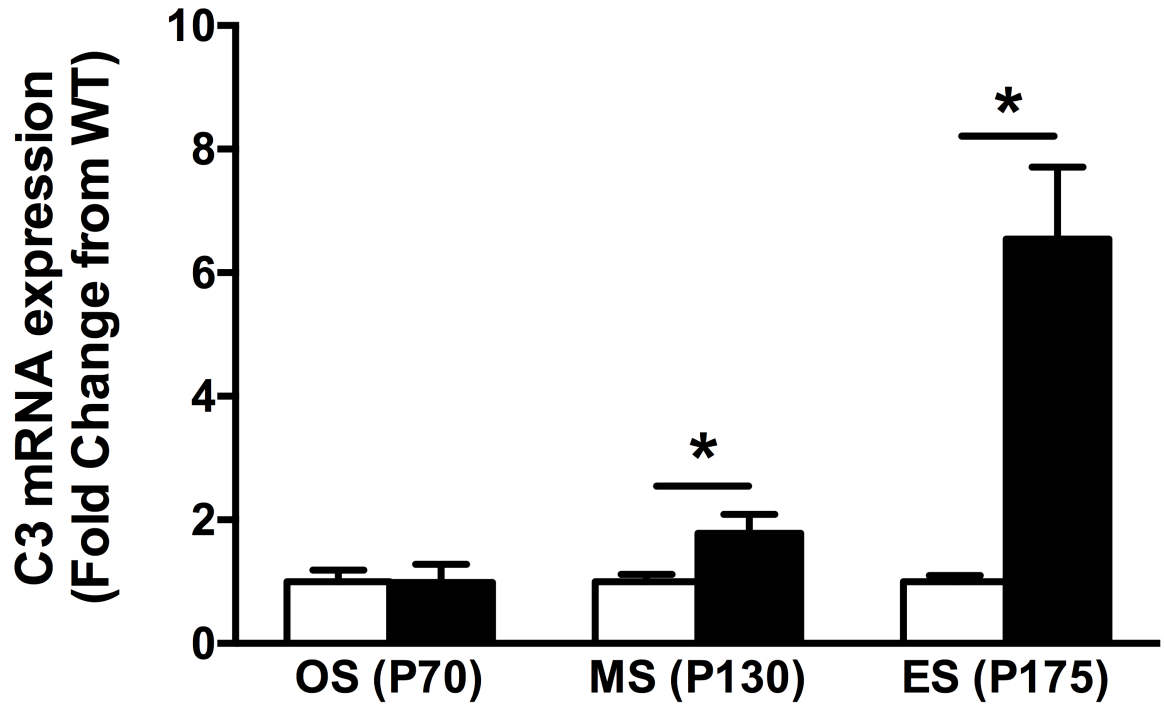

Figure 8. Expression of C3 in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of C3 in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR at three different ages. (n = 5; *p < 0.05, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

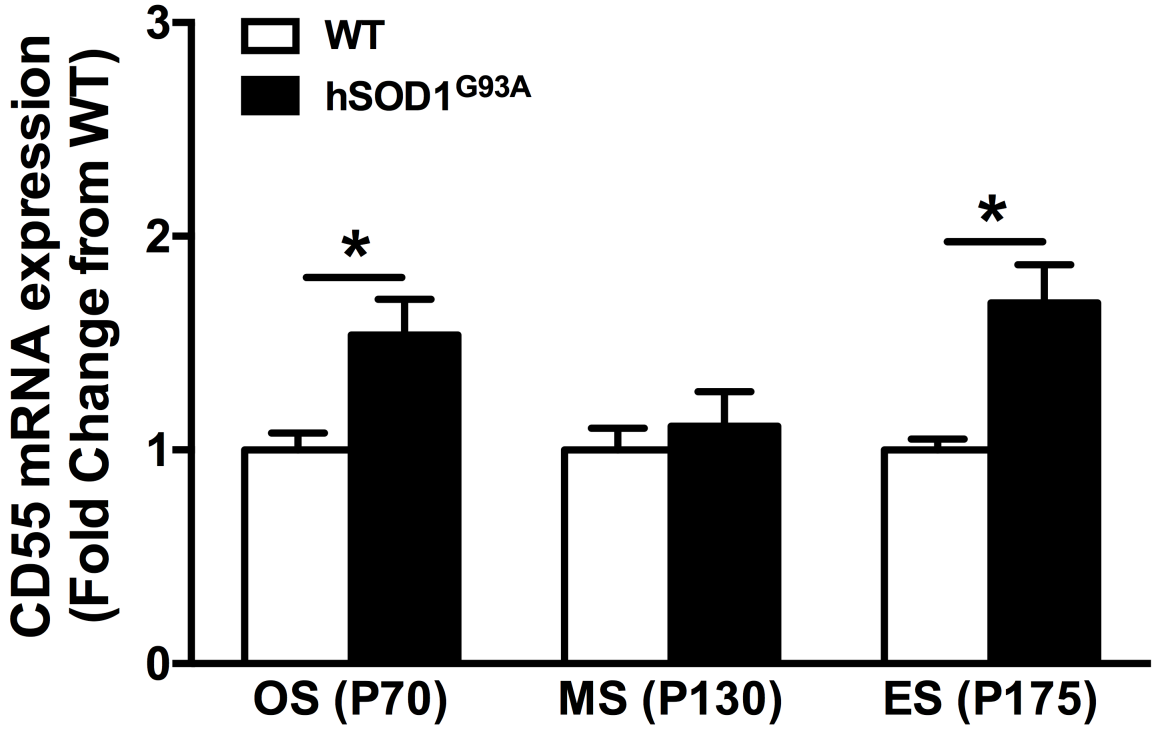

Figure 9. Expression of CD55 in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of CD55 in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR three different ages. (n = 5; *p < 0.05, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

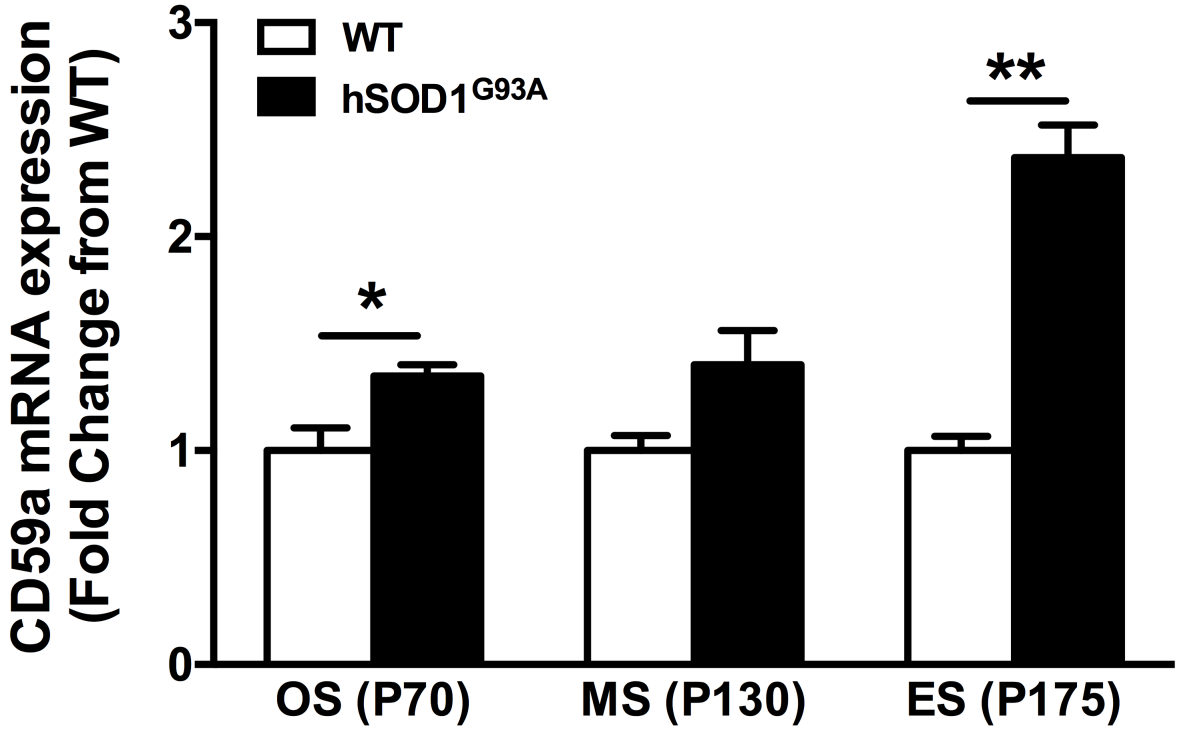

Figure 10. Expression of CD59a in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of CD59a in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR three different ages. (n = 5; *p < 0.05, **p < 0.01, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

Chapter 4

Role of C5a-C5aR1 signalling in regulating the infiltration of immune cells in hSOD1^{G93A} mice

4.1 Introduction

The C5 cleavage fragment C5a is the most potent complement peptide that recruits and activates immune cells, such as neutrophils, monocytes, eosinophils, and T lymphocytes. (Guo and Ward, 2005) Previous studies have demonstrated up-regulation of C5aR1, the major C5a receptor, in neurodegenerative states (Humayun et al., 2009, Woodruff et al., 2008a, Lee et al., 2013). Interestingly, our group has demonstrated the cellular localisation of C5aR1 to motor neurons and microglia in hSOD1^{G93A} mice (Lee et al., 2013). Moreover, our group has shown that hSOD1^{G93A} transgenic rats treated with PMX205, a selective C5aR1 antagonist, displayed a significant extension of survival and improved motor function compared to untreated animals (Woodruff et al., 2008a, Woodruff et al., 2014). Taken together, these findings suggest that complement system is *over-activated and the disease progression is associated with* C5a-C5aR1 signalling in these animal models of ALS.

Since complement has been initiated in the skeletal muscle of ALS murine animal model (Chapter 3), and that C5a-C5aR1 signalling is activated in CNS, it is reasonable to hypothesize that the C5a-C5aR1 signalling may also be enhanced in the skeletal muscle of hSOD1^{G93A} mice. C5a has been recognized as a potent chemoattractant for immune cells, elevated expression of its major receptor C5aR1 might also lead to increased

infiltration of immune cells in to skeletal muscle, and this immune cell invasion would differ in accordance to the level of muscle damage and muscle type (fast- or slow-twitch muscle) in hSOD1^{G93A} mice, during ALS progression. To investigate and test these ideas, in this chapter, the expression levels of C5 and its major receptor C5aR1 in the skeletal muscle of hSOD1^{G93A} mice were examined. To further elucidate the function of C5a-C5aR1 signalling in the skeletal muscle during disease progression of ALS, the extent of immune cell infiltration, namely CD11b^{+} macrophages and CD4^{+} helper T cells, was determined and compared in respect to the type of muscle in muscle.

4.2 Results

4.2.1 Up-regulation of C5a ligand and its receptor C5aR1 in the skeletal muscle of hSOD1^{G93A} mice

The downstream complement factor C5a, the ligand for C5aR1, can be rapidly generated in response to insult or injury in the CNS (Woodruff et al., 2011). In order to determine whether downstream complement factor C5a is also activated in skeletal muscle of ALS affected animal model in response to muscle destruction, protein expression levels of C5a were examined the in the tibialis anterior muscle of hSOD1^{G93A} and wild-type mice using ELISA. The results showed marked increases in C5a at onset (P70), mid-symptomatic (P130) and end-stage (P175) of disease by 1.8-fold, 1.5-fold and 1.7-fold when compared with wild-type mice respectively (n = 6, *p<0.05 and ***p<0.001; Figure 11). Previous studies have shown increases in C5aR1 expression in the CNS of multiple rodent models of ALS (Woodruff et al., 2011, Lee et al., 2013, Humayun et al., 2009). Given C5a expression has increased in the tibialis anterior muscle during ALS disease progression in hSOD1^{G93A} mice, I next examined the expression levels of its signalling receptor C5aR1 during these disease stages in the skeletal muscle of hSOD1^{G93A} and compared to age-matched wild-type controls.

The mRNA and protein expression levels for C5aR1 in the tibialis anterior muscle of wild-type and hSOD1^{G93A} mice were examined using quantitative real-time PCR and western blot, respectively. C5aR1 mRNA expression was significantly increased by 1.9-fold at mid-symptomatic and by 4.6-fold at end-stage of disease respectively when compared to wild-type mice (n = 5; *p < 0.05, **p < 0.01; Figure 12). This change in mRNA expression was confirmed at protein level using western blot analysis, where a 45kDA C5aR1 immuno-reactive band was observed in hSOD1^{G93A} and wild-type mice at end-stage of disease

(Figure 13A). Semi-quantitative analyses of these bands relative to GAPDH loading control showed increased C5aR1 protein levels in the tibialis anterior muscle of hSOD1^{G93A} mice by 8.2-fold at end-stage of disease when compared to WT mice (n = 6, ** p < 0.01; Figure 13B).

Altogether, the results above support my hypothesis that downstream factors of complement cascade were activated in skeletal muscle of ALS animal model during disease progression. More importantly, C5a-C5aR1 signalling appeared to be activated in ALS-affected skeletal muscle, implicating an active role of C5a-C5aR1 signalling mediating ALS disease progression in hSOD1^{G93A} mice.

4.2.2 Cellular localisation of C5aR1

I next investigated the cellular localisation of C5aR1 to see where C5a-C5aR1 signalling might exert its functions in skeletal muscle tissue during ALS disease progression. To achieve this, I performed immunohistochemistry for C5aR1 on tibialis anterior muscle from hSOD1^{G93A} and wild-type mice. Transverse sections of tibialis anterior muscles from end-stage animals were immuno-stained for C5aR1, along with specific cellular markers to identify neuromuscular junction (α-Bungarotoxin), Schwann cells (anti-S100), macrophages (anti-CD11b) and helper T cells (anti-CD4).

I first examined the co-localisation of C5aR1 on neuromuscular junction (NMJ), the site of communication between motor nerve axons and muscle fibres, which undergoes destruction during ALS disease progression, and Schwann cell, the neuroglia at NMJ that guides synaptic homeostasis and repair. I demonstrated that in wild-type and hSOD1^{G93A} mice, C5aR1 was not present on either neuromuscular junction (yellow arrows in Figure 14A) or Schwann cells (yellow arrows in Figure 14B), suggesting that increased expression of C5a and its signalling receptor C5aR1 may be in response to gross changes within the muscle (e.g. increased oxidative stress levels within muscle fibres) during disease progression, rather than a focal response of NMJ damage.

Following this demonstration, I then examined whether peripheral immune cells, possibly migrating into tibialis anterior muscle via the chemoattraction function of C5a, were responsible for the increased expression of C5aR1 within this tissue. Two major populations of immune cells in skeletal muscle, macrophages and T lymphocytes, were

selected for investigation of C5aR1 localisation. I showed that C5aR1 was expressed predominantly on CD11b positive macrophages in both the wild-type and hSOD1^{G93A} mice (yellow arrows in Figure 15A) and some on CD4 positive helper T cells (yellow arrows in Figure 15B). Overall, the data suggest that C5a is not directly involved in the degeneration of NMJ, but acting as a strong chemoattractant by recruiting immune cells into skeletal muscle of hSOD1^{G93A} mice, that are presumably displaying increased levels of damage due to the toxic effects of mutant SOD1 within muscle fibres.

4.2.3 hSOD1^{G93A} mice lacking C5aR1 have a reduced number of macrophages and helper T cells when compared to hSOD1^{G93A} mice during disease progression of ALS

Several studies have shown that the infiltration of macrophages and T cells in spinal cord of ALS patients may contribute to ALS pathology (Troost et al., 1989, Graves et al., 2004). Given that I showed a marked C5aR1 accumulation in infiltrating macrophages and helper T cells, attracted by C5a via its signalling receptor C5aR1, in tibialis anterior muscles of hSOD1^{G93A} mice. I investigated whether absence of C5aR1 in hSOD1^{G93A} mice would have any effect on the infiltration of peripheral macrophages and helper T cells in the tibialis anterior muscle. Transverse sections of tibialis anterior muscle from wild-type, hSOD1^{G93A}, and hSOD1^{G93A} x C5aR1$^{-/-}$ mice at onset (P70), mid-symptomatic (P130) and end-stage (P175) of disease progression were stained for markers of macrophages (anti-CD11b) and helper T cells (anti-CD4), and were quantified.

The number of macrophages in tibialis anterior muscle of hSOD1^{G93A} mice was significantly increased at onset, mid-symptomatic and end-stage of disease when compared to wild-type mice (n = 3, *p < 0.05 and **p < 0.01; Figure 16A). By contrast the number of macrophages in tibialis anterior muscle of hSOD1^{G93A} mice lacking C5aR1 was significantly reduced at mid-symptomatic and end-stage of disease when compared to hSOD1^{G93A} mice (n = 3, **p < 0.01 and *** p < 0.001; Figure 16A). Similar to macrophages, I showed that the number of helper T cells was significantly increased in tibialis anterior muscle of hSOD1^{G93A} mice at all stages of disease when compared to age-matched wild-type mice (n = 3, **p < 0.01 and ***p < 0.001; Figure 17A). Interestingly only at mid-symptomatic ALS disease stage there was a further increased in the number of helper T cells. This increase did not occur in the hSOD1^{G93A} mice lacking C5aR1, suggesting that the spike in helper T cell numbers at this stage is influenced by the

presence of C5aR1 (n = 3, $^{**}p < 0.01$; Figure 17A). Taken together, these data confirmed my speculation that C5a-C5aR1 signalling mediates the infiltration of pre-immune cells, macrophages and T helper cells, into ALS affected skeletal muscle of hSOD1^{G93A} mice, and these infiltrations were largely attenuated in hSOD1^{G93A} mice lacking C5aR1.

It has been demonstrated that fast-twitch muscle fibres are preferentially affected in ALS (Frey et al., 2000, Atkin et al., 2005, Pun et al., 2006). As massive invasions of immune cells were shown in tibialis anterior muscles that are vulnerable in ALS, this raises the question of the extent of immune cells invasions into muscles that are less vulnerable in ALS, like slow-twitch soleus muscles. To address this, I next examined the number of macrophages and T helper cells in the soleus muscles from wild-type, hSOD1^{G93A}, and hSOD1^{G93A} x C5aR1$^{-/-}$ mice at onset (P70), mid-symptomatic (P130) and end-stage (P175) of ALS disease progression. The number of macrophages in soleus muscles of hSOD1^{G93A} mice significantly increased at mid-symptomatic and end-stage of disease when compared to WT mice (n = 3, $^{***}p < 0.001$; Figure 16B). By contrast there was a significant decrease in the number of macrophages in the soleus muscles of hSOD1^{G93A} mice lacking C5aR1 (n = 3, $^{***}p < 0.001$; Figure 16B). Interestingly, the number of macrophages in soleus muscles of hSOD1^{G93A} mice was much lower than the number of macrophages in tibialis anterior muscles of hSOD1^{G93A} mice, which supports other studies that showed fast-twitch muscles are preferentially affected in hSOD1^{G93A} mice when compared to slow-twitch muscles (Figure 16) (Frey et al., 2000, Atkin et al., 2005). This idea was also supported by the quantification of T helper cells, where no significant differences of T helper cell numbers were observed in soleus muscles across wild-type, hSOD1^{G93A}, and hSOD1^{G93A} x C5aR1$^{-/-}$ mice during ALS disease progression, but the numbers of T helper cells in tibialis anterior muscles of hSOD1^{G93A} muscles were markedly higher than their counterparts in soleus muscles (Figure 17). To summarise, these results demonstrated that immune cells invade skeletal muscle of hSOD1^{G93A} mice in a muscle type-dependent manner, where fast-twitch muscles were more vulnerable to immune cell infiltration, compared to the slow-twitch soleus muscle.

4.3 Discussion

Among the complement factors, C5a is one of the most potent complement peptide with a range of functions (Guo and Ward, 2005). C5a exerts its effects through its two receptors, C5aR1 and C5aR2 (Sarma and Ward, 2012). Previous studies have demonstrated up-

regulation of C5aR1 within the CNS of $SOD1^{G93A}$ rodents, suggesting that C5a-C5aR1 signalling plays a role in the pathology of ALS (Woodruff et al., 2008a, Lee et al., 2013). In the present study, the expression of C5aR1 in $hSOD1^{G93A}$ TA muscle was elevated at both mRNA and protein levels. In addition to C5aR1, its ligand C5a also showed increased protein expression. These results demonstrated that downstream complement effectors, in particular C5a and its signalling receptor C5aR1, may be actively involved in the disease progression of ALS through both CNS and skeletal muscle. As C5a is a potent chemotactic agent regulating migration of immune cells, I examined the cellular localisation of C5aR1, the receptor which C5a exert its functions through, and quantified the number of immune cells, in particular $CD11b^{+}$ macrophages and $CD4^{+}$ helper T cells, infiltrated into skeletal muscle of $hSOD1^{G93A}$ mice (Guo and Ward, 2005).

The neuromuscular junction (NMJ) is the synapse where the axon terminal of a motor neuron communicates with the motor endplate. It has been demonstrated that endplates are denervated much earlier than the axons and the cell body loss during the pathogenesis of ALS in $hSOD1^{G93A}$ mice, and this degeneration process may be driven by the initiation of complement system in the skeletal muscle (Fischer et al., 2004, Heurich et al., 2011). Here I found no deposition of C5aR1 at neuromuscular junction in tibialis anterior muscles of either wild-type or $hSOD1^{G93A}$ mice. The results suggest that C5a-C5aR1 signalling may not be directly involved in the destruction of NMJ, but rather a response to muscle damage caused by accumulated oxidative stress in the muscle, triggered by the expression of mutant SOD1 within these muscle fibres.

It has long been known that perisynaptic Schwann cells regulate morphological stability, integrity and repair of the NMJ. So far, the involvement of Schwann cells in ALS pathology is still unclear. In spite of that Schwann cells are incapable of triggering disease independently, they may modulate ALS pathogenesis and progression like other glial cells, including astrocytes, microglia and oligodendrocytes (Clement et al., 2003, Boillee et al., 2006, Yamanaka et al., 2008, Wang et al., 2010a, Kang et al., 2013). This was addressed by previous study showing that knock-down of G37R expression in Schwann cells of $SOD1^{G37R}$ mice shortened the late phase of disease and survival, suggesting that the neuroprotective effect of G37R in Schwann cells was greater than its toxicity (Lobsiger et al., 2009). Another study showed that in contrast to microglia and astrocytes, accumulation of G93A within Schwann cells is not pathological to spinal cord motor neurons or

deleterious to disease course in transgenic hSOD1^{G93A} mice (Turner et al., 2010). Furthermore, knockdown of G85R in Schwann cells of SOD1^{G85R} mice showed delayed onset and extended survival, suggesting that G85R expression in Schwann cells is neurotoxic (Wang et al., 2012). In a more recent study, impairments in Schwann cells functions were observed in SOD1^{G37R} mice. As the alternations in the synaptic transmission decoding ability of Schwann cells are detrimental to NMJ repair, it was suggested that Schwan cells could play an important role in NMJ maintenance and progression of ALS disease (Arbour et al., 2015). In the present study, I showed no co-localisation of C5aR1 to Schwann cells in TA muscles of hSOD1^{G93A} mice, suggesting that C5a-C5aR1 signalling is unlikely to contribute to denervation of NMJ through altering the properties of perisynaptic Schwan cells.

In ALS patients, substantial numbers of infiltrating macrophages and T cells are found in the spinal cord (Troost et al., 1989, Kassmann et al., 2007). Infiltration of T cells into spinal cord has also been observed in hSOD1^{G93A} transgenic mouse model of ALS disease (Chiu et al., 2008, Beers et al., 2008). Since C5a is a potent chemoattractant for immune cells, and its receptor C5aR1 was not present on either NMJ or Schwann cells, it seems reasonable to postulate that C5a signals through C5aR1 expressed on the pre-immune cells, which infiltrated into skeletal muscles. Hereby I examined C5aR1 expression on immune cells. The results showed C5aR1 was co-localised to macrophages and T helper cells, indicating that immune cells are the likely sources of C5aR1 generation in skeletal muscle during disease progression.

It has been well defined that microglia and astrocytes are activated in the CNS in mouse model of ALS, while T cells infiltrate the spinal cord (Hall et al., 1998, Chiu et al., 2008, Beers et al., 2008). However, the role of the innate immunity in the skeletal muscle and its target tissues have not been well characterised. A recent study has shown that activated macrophages accumulate along the length of degenerating motor nerve fibres in ventral roots, sciatic nerves and muscles in mutant SOD1^{G93A} and mutant SOD1^{G37R} mice muscle (Chiu et al., 2009). In this study, I found a striking infiltration of CD11b positive macrophage cells in tibialis anterior muscles, a fast-twitch muscle that is vulnerable to denervation, of hSOD1^{G93A} mice from disease onset when compared with wild-type mice. Surprisingly, this infiltration was largely attenuated in hSOD1^{G93A} mice lacking C5aR1.

Numerous studies have shown that macrophages are essential for skeletal muscle regeneration (Summan et al., 2006, Arnold et al., 2007, Bryer et al., 2008). After acute injury, M1 or proinflammatory macrophages, arise from exposure to inflammatory stimuli such as T helper (Th) 1 cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α, infiltrate to the site of damage in association with recruited monocytes, and participate in the phagocytic removal of cellular debris (Mantovani et al., 2004). Macrophages also exhibit a spectrum of non-inflammatory phenotypes, also referred as M2 macrophages. The population of M2 macrophages is more complex than that of M1 macrophages. M2a or 'alternatively activated' macrophage, results from stimulation of Th2 cytokines interleukin (IL)-4 and IL3, are associated with tissue repair, wound healing and fibrosis (Bhattacharjee et al., 2013). Meanwhile, anti-inflammatory M2c macrophages induced by IL-10, release anti-inflammatory cytokines to deactivate the M1 phenotype and promote the proliferation of non-myeloid cells (Sica and Mantovani, 2012). Consequently, invading macrophages transit from a proinflammatory, which is usually found in the early stages after muscle injury, to an anti-inflammatory type (Arnold et al., 2007, Bryer et al., 2008).

In the present study, I showed increasing trends in the number of infiltrating macrophages into tibialis anterior muscles of hSOD1^{G93A} mice from disease onset (day 70) when compared to wild-type controls. Given that muscle denervation occurs at early disease stage in hSOD1^{G93A} mice, it is plausible to postulate that invading macrophages from disease onset in tibialis anterior muscles is in response to muscle damage and subsequent regeneration (Fischer et al., 2004). To understand which phenotypes of macrophages contribute to muscle repair in ALS hSOD1^{G93A} mouse model, further study is needed to clarify the composition of macrophage populations during disease progression. Interestingly, the accumulation of macrophages was not observed in tibialis anterior muscles of hSOD1^{G93A} mice lacking C5aR1, suggesting that C5a-C5aR signalling is required to recruit macrophages into damaged muscles of hSOD1^{G93A} mice during ALS progression. This is supported by other studies that show C5a is a strong chemoattractant for immune cell invasion including macrophages into peripheral tissues (Guo and Ward, 2005).

Several studies have addressed the infiltration of T cells in the CNS from both ALS patients and transgenic mouse models (Holmøy, 2008, Chiu et al., 2008, Beers et al., 2008). The present study showed a substantial invasion of CD4 positive helper T cells into

tibialis anterior muscle of hSOD1^{G93A} mice across the three disease stages where a reduction of helper T cell numbers was observed in hSOD1^{G93A} × C5aR1$^{-/-}$ mice. Previous studies have indicated a neuroprotective role of T cells or CD4^{+} T cells in hSOD1^{G93A} transgenic mice as depleting functional T cells or CD4^{+} T cells led to the acceleration of disease progression (Chiu et al., 2008, Beers et al., 2008). Knocking out CD4^{+} T cells decreased microglial reactivity suggesting a direct interaction between CD4^{+} T cells and glial activation (Beers et al., 2008). Another study showed that motor neuron degradation was accelerated in mutant SOD1 mice lacking functional CD4^{+} T cells, suggesting the importance of CD4^{+} T cells for neuroprotective effects in ALS (Holmøy, 2008). In this study, we showed an elevated and diminished number of CD4^{+} helper T cells in tibialis anterior muscle of hSOD1^{G93A} and hSOD1^{G93A} × C5aR1$^{-/-}$ mice, respectively. Recent studies showed that T cells also participate in the skeletal muscle regeneration. Improved muscle regeneration has been shown in the absence of T cells in the animal model of dysferlinopathy (Farini et al., 2012). As hSOD1^{G93A} mice with fully deleted C5aR1 demonstrated a significant extension in survival, it is possible that diminished T cells infiltration facilitates muscle regeneration in hSOD1^{G93A} × C5aR1$^{-/-}$ mice, which in turn ameliorate muscle denervation (Woodruff et al., 2014). However, the exact role of T cells in ALS pathology awaits further investigation.

C5a is a strong chemoattractant and is involved in the recruitment of inflammatory cells such as T cell, eosinophils, neutrophils, monocytes and macrophages (Guo and Ward, 2005, Don et al., 2007). Since a massive influx of macrophages and helper T cells, accompanied by increased expression of C5a and its receptor C5aR1, has been quantified in hSOD1^{G93A} tibialis anterior muscles, suggests that C5a-C5aR1 signalling mediates the recruitment of these peripheral immune cells during the ALS disease progression. This is confirmed by the observation of reduced numbers of infiltrating macrophages and helper T cells in hSOD1^{G93A} mice lacking C5aR1.

In addition to tibialis anterior muscles, the numbers of infiltrating macrophages and helper T cells were also quantified in soleus muscle, a slow-twitch muscle that is less vulnerable to ALS pathogenesis. A similar infiltration profile of macrophage in soleus muscles was discovered where significantly increased quantities of macrophages were observed in hSOD1^{G93A} mice when compared with wild-type mice, and this influx of macrophages was diminished in hSOD1^{G93A} mice lacking C5aR1. Meanwhile, minimal infiltrations of helper T

cells were detected in soleus muscle of wild-type, hSOD1^{G93A} and hSOD1^{G93A} × C5aR1$^{-/-}$ mice. Together, these results suggest that infiltrations of pre-immune cells occur in a muscle type-dependent manner as fewer macrophages and helper T cells were observed in the soleus muscles, a muscle less affected in ALS.

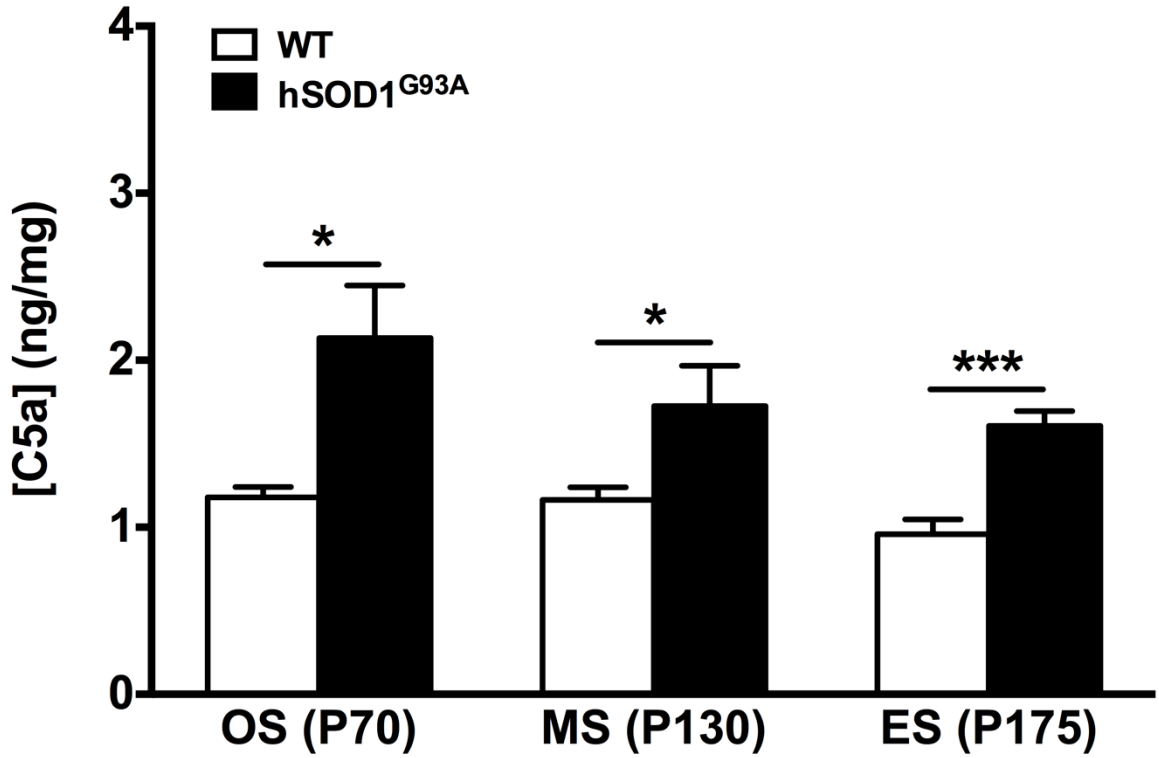

Figure 11. Expression of C5a in hSOD1^{G93A} and wild-type mice during disease progression.

Protein expression of C5a in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR at three different ages. (n = 6; *p < 0.05, ***p < 0.001; Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

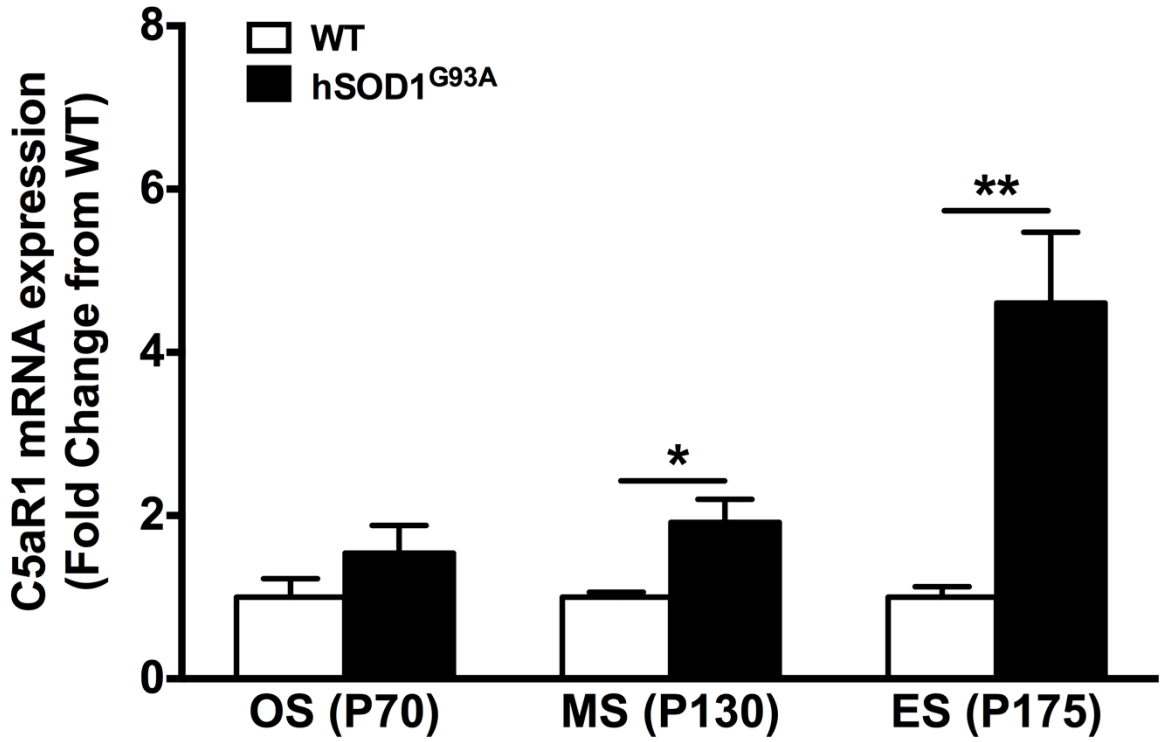

Figure 12. Expression of C5aR1 in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of C5aR1 in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR at three different ages. (n = 5; *p < 0.05, **p < 0.01, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

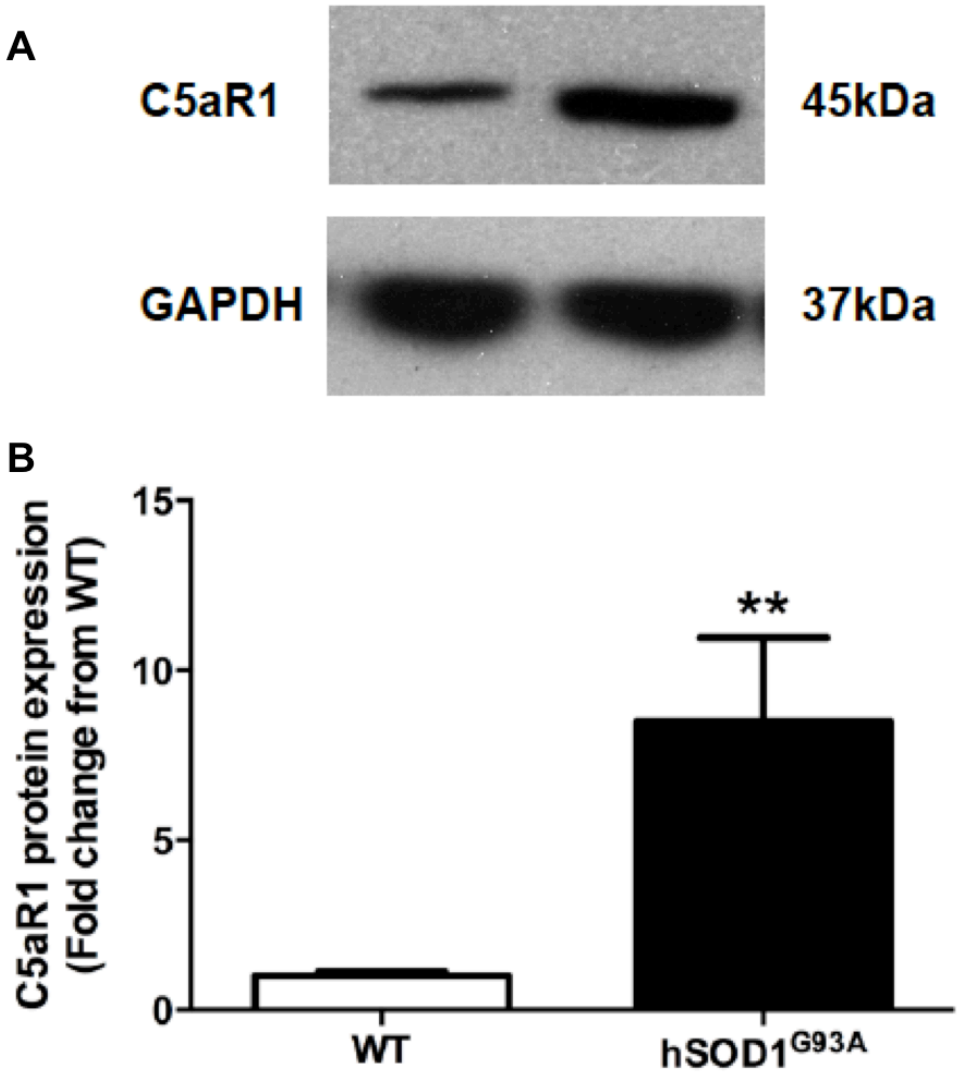

Figure 13. Protein expression of C5aR1 in hSOD1^{G93A} and wild-type mice at end-stage.

Protein expression of C5aR1 in tibialis anterior (TA) muscle of end-stage wild-type (WT) and hSOD1^{G93A} mice was detected by western blot (A) and the expression levels were semi-quantified (B). (n = 4; $^{**}p < 0.005$, Student t test). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels were the same across genotype indicating equal loading of protein. Bars represent the mean ± SEM.

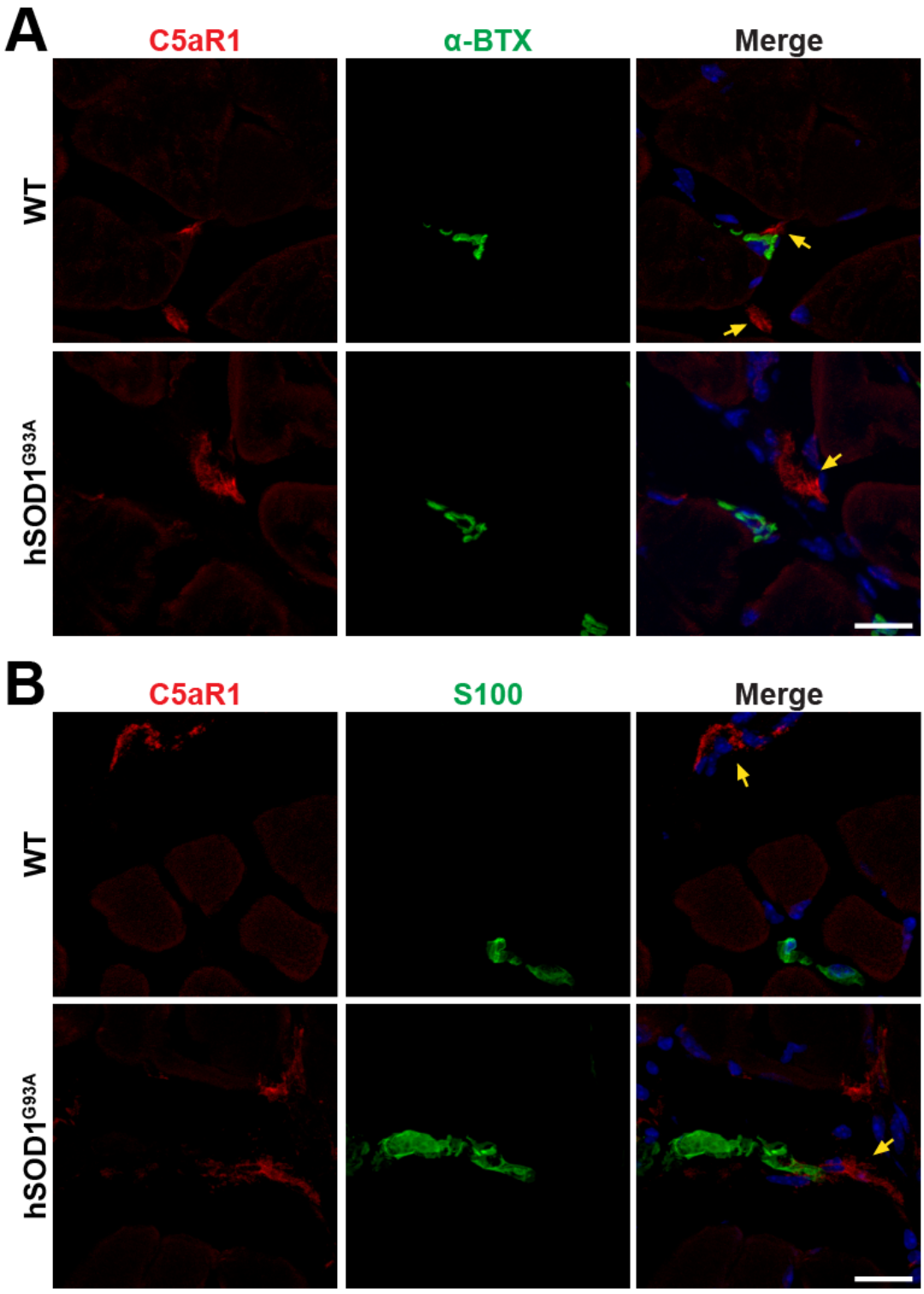

Figure 14. C5aR1 is not localised at neuromuscular junction or Schwann cells in hSOD1^{G93A} mice.

C5aR1 is not expressed on either neuromuscular junction or Schwann cells. Immunostaining of C5aR1 (red, yellow arrows) with α-Bungarotoxin (green, **A**) for motor endplate and S100 (green, **B**) for Schwann cells in TA muscle of WT and hSOD1^{G93A} mice at end stage. Nuclei were visualized with DAPI (blue). Scale bars, 20 μm.

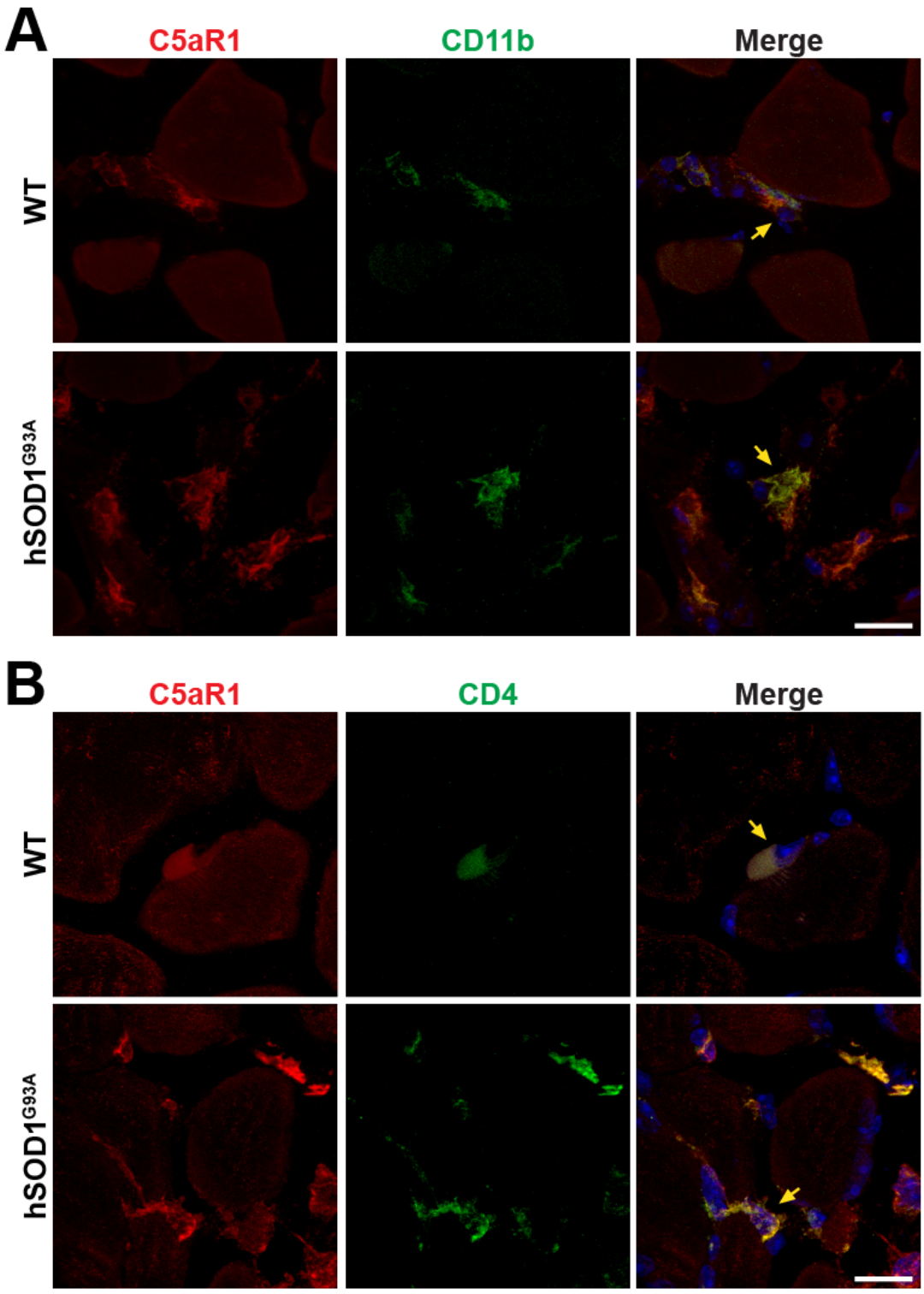

Figure 15. C5aR1 is localised to macrophages and helper T cells in hSOD1^{G93A} mice. Cellular localization of C5aR1 on macrophages and helper T cells. Immunostaining of C5aR1 (red) with CD11b (green, **A**) for macrophages and CD4 (green, **B**) for helper T cells in tibialis anterior (TA) muscle of WT and hSOD1^{G93A} mice at end stage. C5aR1 (yellow arrows) is co-localised with CD11b-positive macrophages and CD4-positive helper T cells. Nuclei were visulised with DAPI (blue). Scale bars, 20 µm.

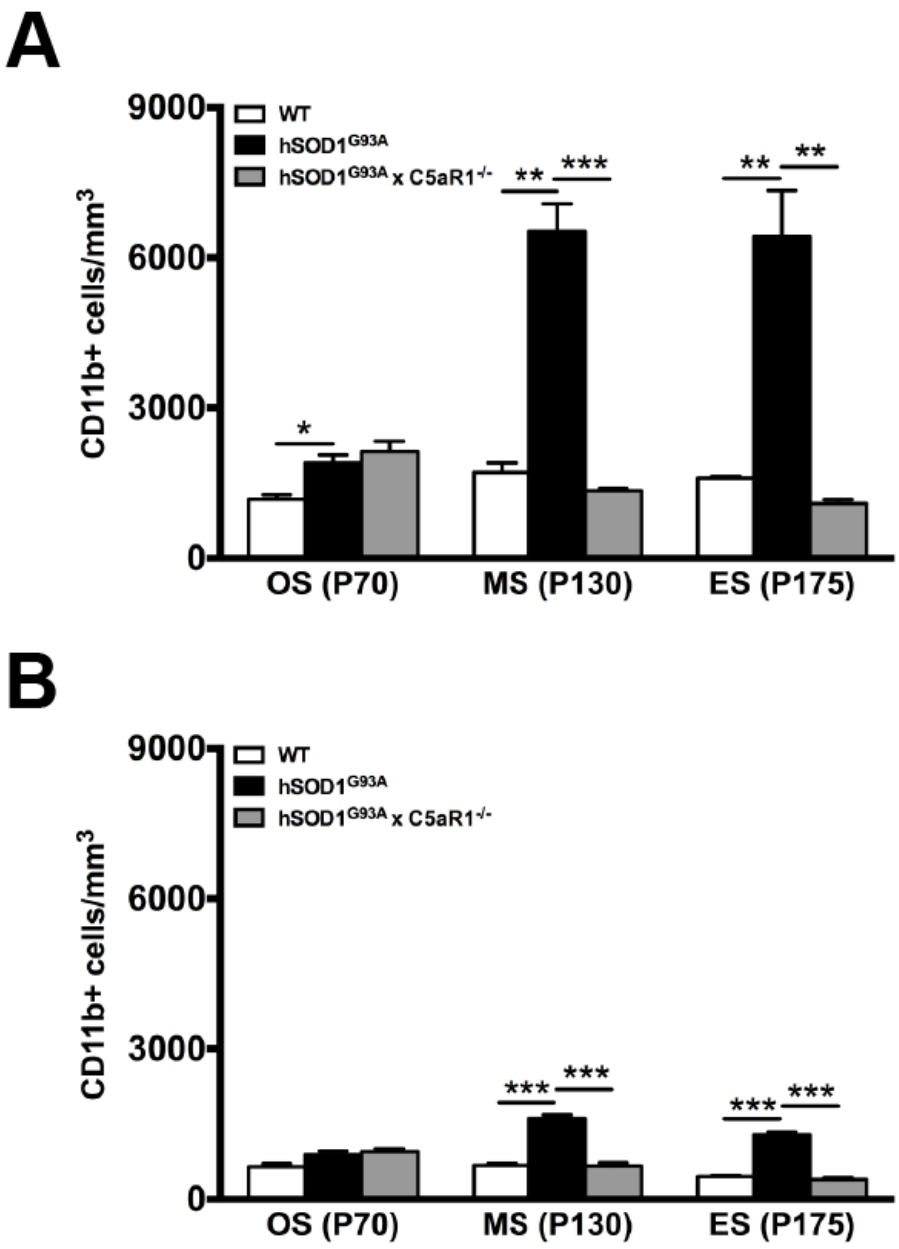

Figure 16. Quantification of macrophages in skeletal muscle of WT, hSOD1^{G93A} and hSOD1^{G93A} × C5aR1$^{-/-}$ mice.

Infiltration of macrophages into tibialis anterior or soleus muscle groups at various stages. Cryosections of tibialis anterior (**A**) and soleus (**B**) muscle from WT, hSOD1^{G93A} and hSOD1^{G93A}×C5aR1$^{-/-}$ mice were stained for CD11b and quantified as described in Materials and Methods. More CD11b^{+} macrophages were present in MS and ES hSOD1^{G93A} mice than WT mice, while less CD11b^{+} macrophages were found in MS and ES hSOD1^{G93A}×C5aR1$^{-/-}$ mice compared with hSOD1^{G93A} mice (n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, two-way ANOVA). Bars represent the mean ± SEM. WT, wild-type; OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

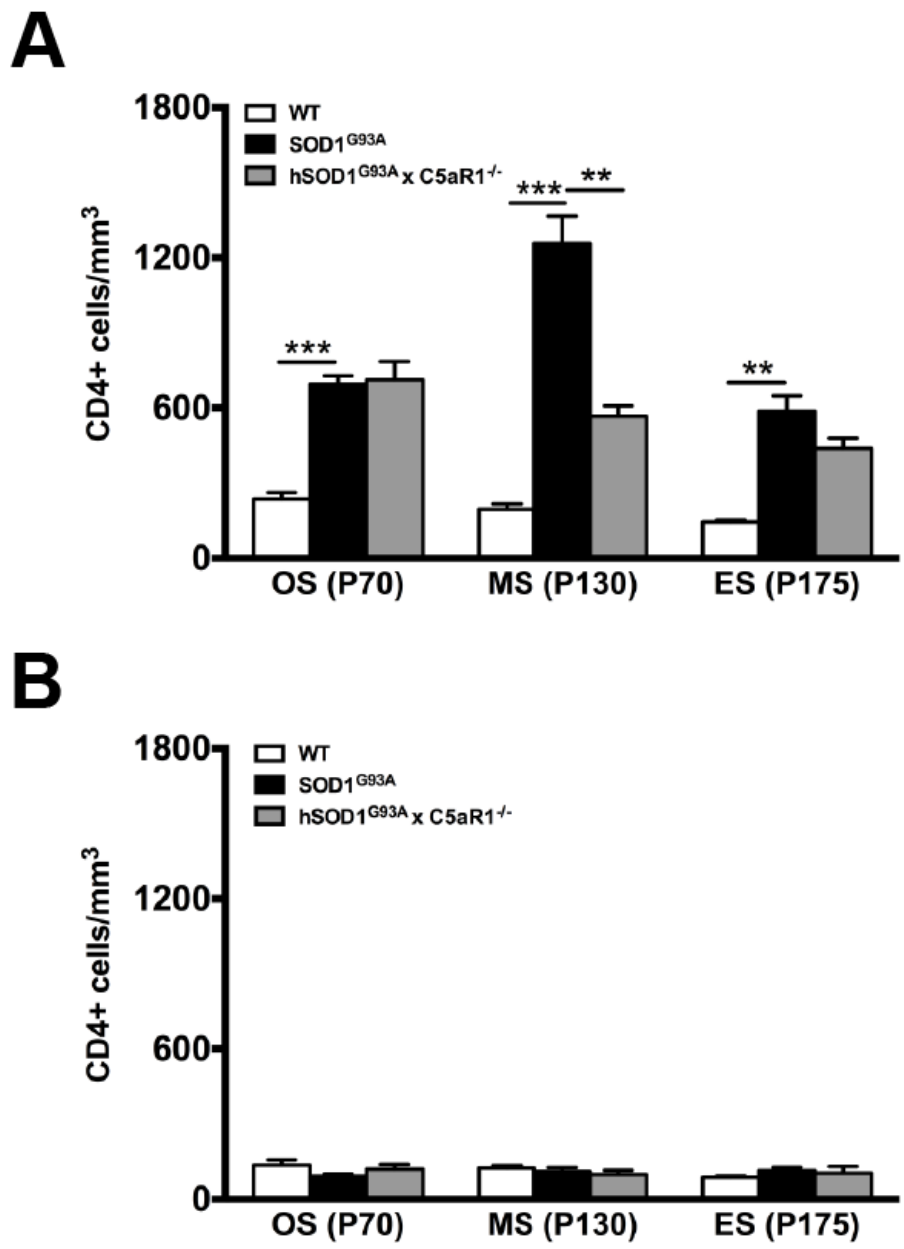

Figure 17. Quantification of helper T cells in skeletal muscle of WT, hSOD1^{G93A} and hSOD1^{G93A} × C5aR1$^{-/-}$ mice.

Infiltration of helper T cells into tibialis anterior or soleus muscle groups at various stages. Cryosections of tibialis anterior (**A**) and soleus (**B**) muscle from WT, hSOD1^{G93A} and hSOD1^{G93A}×C5aR1$^{-/-}$ mice were stained for CD4 and quantified as described in Materials and Methods. More CD4^{+} helper T cells were present in hSOD1^{G93A} mice than WT mice, while less CD4^{+} helper T cells were found in MS hSOD1^{G93A}×C5aR1$^{-/-}$ mice compared with hSOD1^{G93A} mice in TA muscle (A). There are no significant changes of CD4^{+} helper T cells numbers in SOL muscle (B). (n = 3; $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA). Bars represent the mean ± SEM. WT, wild-type; OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

Chapter 5

Role of C3aR signalling in regulating the infiltration of immune cells in hSOD1^{G93A} mice

5.1 Introduction

Complement protein C3 is the central component of the complement system (Figure 2). Cleavage of the C3 by the convertase C4b2b of the lectin and classical pathways, and the convertase C3bBb of the alternative pathway, results in the generation of the C3a and C3b fragments (Lambris, 1988). C3a is a versatile peptide with diverse functions in immune response. It mediates both proinflammatory and anti-inflammatory activities upon binding to its receptor C3aR (Wetsel et al., 2000).

C3aR is a G-protein-coupled receptor. It is widely expressed on myeloid and lymphoid cells, including mast cells, eosinophils, neutrophils, monocytes/macrophages, microglia, dendritic cells, T cells and B cells (Klos et al., 2009). It is also found expressed on several types of neuronal cells (Davoust et al., 1999). The biological functions of C3aR mediated signalling system, such as exocytotic release of granula from basophils or eosinophils, and the up-regulation and release of the monocyte chemoattractant protein-1 (MCP-1, also: chemokine CCR ligand 2, CCL2), are tightly controlled (Bischoff et al., 1990, Takafuji et al., 1994, Ahamed et al., 2001).

In inflamed human CNS, C3aR has been found on both astrocytes and microglia in the area of pathology and infiltrating macrophages in multiple sclerosis and meningitis (Gasque et al., 1998). Deposition of C3/C3b has been shown in spinal cord and skeletal muscle of hSOD1^{G93A} transgenic murine ALS models (Woodruff et al., 2008a, Heurich et

al., 2011, Lee et al., 2013). Together these observations suggest an involvement of C3 in CNS inflammation.

Given that C3 participates in neuroinflammatory diseases and accumulates in the skeletal muscle of hSOD1^{G93A} transgenic mice, raises the idea that C3 plays an active role in the pathogenesis of ALS in the skeletal muscle of hSOD1^{G93A} transgenic mice. Support for this idea comes from studies that show C3 fragment C3a is a chemotactic factor for immune cells, hence it is possible that C3a exert its function through signalling its receptor C3aR on circulating immune cells, promotes their infiltration into damaged muscle such as seen in ALS (Ricklin and Lambris, 2013). To address these questions, I assessed the expression of C3aR, and quantified the number of infiltrating immune cells in the skeletal muscle of hSOD1^{G93A} mice with comparison to WT and hSOD1^{G93A} mice lacking C3aR.

5.2 Results

5.2.1 Expression of C3aR

C3aR, the receptor of C3, is expressed by hippocampal, cortical and motor neurons in the normal CNS and plays roles in regulating neurogenesis (Davoust et al., 1999, Rahpeymai et al., 2006, Ducruet et al., 2012). However, the roles of C3aR in neurodegenerative diseases are ill defined. Therefore, I first examined the expression of C3aR in the skeletal muscle of hSOD1^{G93A} mice. The mRNA levels of C3aR in the tibialis anterior muscle of wild-type and hSOD1^{G93A} mice were determined by qPCR. The results showed that the mRNA level of C3aR significantly increased by 1.6-fold, 2.6-fold and 7.6-fold at onset (P70), mid-symptomatic (P130) and end-stage (P175) disease in tibialis anterior muscle of hSOD1^{G93A} mice when compared with wild-type mice (n = 5; *p < 0.05; Figure 18), suggesting an activation of C3a-C3aR signalling within muscles, during the ALS disease progression in the hSOD1^{G93A} ALS model.

5.2.2 Cellular localisation of C3aR

Since elevation of C3aR mRNA expression was observed, I next examined cellular localisation of C3aR within skeletal muscle tissues of hSOD1^{G93A} and wild-type mice using immunohistochemistry. Transverse sections of tibialis anterior muscles at end-stage were stained for C3aR1 along with specific cellular markers to identify neuromuscular junction (α-Bungarotoxin), Schwann cells (anti-S100), macrophages (anti-CD11b) and helper T cells (anti-CD4).

My results showed that C3aR was not expressed at neuromuscular junction or surrounding Schwann cells (Figure 19). In order to determine whether C3a-C3aR signalling contributes to the recruitment of infiltrating immune cells during skeletal muscle regeneration, I examined the expression of C3aR on invading macrophages and helper T cells. I found predominate expression of C3aR on CD11b positive macrophages in tibialis anterior muscles from hSOD1^{G93A} mice, compared to that of aged-matched wild-type controls (Figure 20). Together, these data suggest that C3a-C3aR signalling is not involved in the destruction of NMJ, the focal site of muscle damage, but rather for macrophage migration by chemotaxis into muscles from hSOD1^{G93A} mice during disease progression, presumably trigged by muscle responding to the toxic effects of mutant SOD1 expressed within their cytoplasm.

5.2.3 hSOD1^{G93A} mice lacking C3aR have reduced number of macrophages and helper T cells when compared to hSOD1^{G93A} mice during disease progression of ALS

In the previous chapter, I showed significant invasion of macrophages and helper T cells induced by C5a-C5aR1 signalling in tibialis anterior muscles of hSOD1^{G93A} during disease progression (Figures 16 and 17). Since C3a also strongly promotes chemotaxis of immune cells, I investigated whether C3a-C3aR1 signalling modulates the infiltration of macrophages and helper T cells in the tibialis anterior muscles of hSOD1^{G93A} mice. Transverse tibialis anterior muscle sections from wild-type, hSOD1^{G93A}, and hSOD1^{G93A} lacking C3aR (hSOD1^{G93A} x C3aR$^{-/-}$) mice at onset (P70), mid-symptomatic (P130) and end-stage (P175) of ALS disease progression were stained for markers of macrophages (anti-CD11b) and helper T cells (anti-CD4). The presences of such stained cells within tibialis anterior muscles from these mice and were quantified.

I observed significant invasion of macrophages and helper T cells in tibialis anterior muscle of hSOD1^{G93A} mice (Figures 21A and 22A). By contrast, I noted significant reductions in the number of macrophages shown in tibialis anterior muscles of hSOD1^{G93A} mice lacking C3aR at mid-symptomatic (P130) and end-stage (P175) of disease when compared to hSOD1^{G93A} mice (n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$; Figure 21A). Interestingly, hSOD1^{G93A} mice lacking C3aR showed an initial increased number of macrophages at disease onset (P70) when compared to hSOD1^{G93A} mice (n = 3, p = 0.24; Figure 21A). As for helper T cells, notable decreases were observed in tibialis anterior muscles of

hSOD1^{G93A} x C3aR$^{-/-}$ mice at mid-symptomatic and end-stage when compared to hSOD1^{G93A} mice (n = 3, **p < 0.01; Figure 22A). In short, the data supports my hypothesis that C3 is involved in the pathogenesis of ALS by modulating the infiltrating immune cell in skeletal muscle through C3a-C3aR signalling.

In Chapter 4, I showed that immune cells migrated into skeletal muscle of hSOD1^{G93A} mice in a muscle type-dependent manner, namely there were significantly less macrophages and helper T cells invasion into soleus muscles – a slow-twitch muscle, compared to tibialis anterior muscles, which has fast-twitch properties (Figures 16 and 17). Here, I compared the numbers of macrophages and helper T cells in the soleus muscles of wild-type, hSOD1^{G93A}, and hSOD1^{G93A} x C3aR$^{-/-}$ mice at onset (P70), mid-symptomatic (P130) and end-stage (P175) of disease progression. In the soleus muscles from hSOD1^{G93A} mice lacking C3aR, a similar trend of changes in the numbers of macrophage were observed as in the tibialis anterior muscles, where fewer macrophages were found as disease progresses (n = 3, **p < 0.01, ***p < 0.001; Figure 21B). The extent of macrophage invasion in soleus muscles is far less severe than in tibialis anterior muscles in hSOD1^{G93A} mice lacking C3aR (Figure 21). As for helper T cells, I found no obvious changes in helper T cell numbers in soleus muscles from wild-type, hSOD1^{G93A}, and hSOD1^{G93A} x C3aR$^{-/-}$ mice (Figure 22B). Similarly, the overall numbers of helper T cells in soleus muscles were far fewer than its counterparts in tibialis anterior muscles in hSOD1^{G93A} x C3aR$^{-/-}$ mice. These results suggest that immune cell infiltrations mediated by C3a-C3aR signalling also follows the muscle type-dependent pattern as shown in hSOD1^{G93A} x C5aR1$^{-/-}$ mice, where fewer infiltrating immune cells were found in soleus muscles that appeared to be more resistant to degenerative effects.

5.3 Discussion

C3a is an anaphylactic peptide formed by the cleavage of complement factor C3. It can induce proinflammatory and anti-inflammatory biological actions through binding to its G-coupled receptor C3aR. Previous studies suggest that C3a might play multiple roles in promoting neuronal development, regeneration and repair (Yanamadala and Friedlander, 2010). It has been reported that blocking C3aR has beneficial therapeutic effects in CNS lupus by reducing neuronal apoptosis and gliosis in brains of MRL/*lpr* mouse of lupus model (Jacob et al., 2010). However, the role of C3a-C3aR signalling in ALS remains unclear.

In the present study, I showed increased mRNA expression of C3aR in tibialis anterior muscles of hSOD1^{G93A} mice during disease progression. In addition, immunolocalisation of C3aR was found on macrophages, but not at the neuromuscular junction, or on Schwann cells and helper T cells. Quantifications of CD11b positive macrophages and CD4 positive helper T cells demonstrated that C3a-C3aR signalling participates in the recruitment of those immune cells into damaged muscle tissue, where significant drops in the number of macrophages and helper T cells were seen in tibialis anterior muscles of hSOD1^{G93A} lacking C3aR. In brief, these findings indicate that C3a-C3aR contributes to the pathogenesis of ALS in hSOD1^{G93A} transgenic mice by regulating the migration of immune cells in skeletal muscle.

C3 is the central component of complement cascades. In the skeletal muscle, deposition of C3 and its cleaved fragment C3b/iC3b at the NMJ has been shown in both patients and animal models of myasthenia gravis (MG), the most common autoimmune disorder of neuromuscular transmission (Soltys and Wu, 2012). Deposition of C3b/iC3b at denervated NMJ has also been observed in hSOD1^{G93A} transgenic mice, implicating the classical complement pathway in degeneration of distal axons (Heurich et al., 2011). As increased mRNA of C3 in skeletal muscle during ALS disease progression of hSOD1^{G93A} transgenic mice was observed in Chapter 3, it is necessary to identify whether C3 fragment C3a will mediate inflammatory response at NMJ, causing further tissue damage. The finding that C3aR was not detected on either NMJ or perisynaptic Schwann cells, the glial component of the NMJ, in tibialis anterior muscles of hSOD1^{G93A} mice in this study, suggests that C3a-C3aR signalling is unlikely to be involved in the destruction of NMJ (Auld and Robitaille, 2003).

Like complement peptide C5a, C3a attracts immune cells to sites of activation via binding to its receptor C3aR. It is assumed that C3a-C3aR signalling is responsible for recruiting immune cells into muscle injury sites during ALS disease progression. To verify this assumption, cellular localisation of C3aR on macrophages and helper T cells was examined, as well as the number of immune cells in skeletal muscle of hSOD1^{G93A} transgenic mice. Previous studies have demonstrated expression of C3aR on infiltrating macrophages in multiple sclerosis, and C3a is a chemotaxin for mouse macrophages (Gasque et al., 1998, Zwirner et al., 1998b). In another study, high C3aR expression has been found on infiltrating macrophages in the adipose tissue of mice, and depletion of

C3aR showed a string decrease in macrophage infiltration (Mamane et al., 2009). These findings indicate that C3a-C3aR may contribute to the accumulation of macrophages at the site of inflammation. In the present study, I found expression of C3aR on macrophages in tibialis anterior muscles of hSOD1^{G93A} mice. In addition, significant reduction in macrophage influx has also been observed in tibialis anterior muscles of hSOD1^{G93A} mice lacking C3aR. Overall, my results are consistent with findings of previous studies showing that C3a-C3aR signalling regulates the invasion of macrophages (Mamane et al., 2009). Moreover, C3a-C3aR signalling may play a beneficial role in ALS pathogenesis of hSOD1^{G93A} mice as these infiltrating macrophages are essential for muscle regeneration after injury (Summan et al., 2006, Arnold et al., 2007, Bryer et al., 2008). In Chapter 4, I showed that immune cells infiltrate skeletal muscle of hSOD1^{G93A} x C5aR1$^{-/-}$ mice in a muscle type-dependent manner, where fewer macrophages and helper T cells were presented in soleus muscles compared to tibialis anterior muscles. These differences in the numbers of immune cells between tibialis anterior and soleus muscles were also seen in hSOD1^{G93A} x C3aR$^{-/-}$ mice, suggesting that the extent of immune cell invasion in hSOD1^{G93A} is regulated by complement signalling and determined by the twitch-type specificity of skeletal muscle.

It has been shown that functional C3aR is expressed on the CD4^{+} and CD8^{+} blood- or skin-derived T cell cones from patients with atopic dermatitis (Werfel et al., 2000). Expression of C3aR on T cells also provides co-stimulatory signals that enhance effector T cells (Teff) and limit natural regulatory T cells (nTreg) function (Strainic et al., 2008, Kwan et al., 2013). Moreover, genetic deficiency or pharmacological blockade of C3aR signalling augments murine and human induced regulatory T cells (iTreg) stability (van der Touw et al., 2013). Double immunostaining showed that C3aR expression was absent from CD4^{+} T cells in tibialis anterior muscles of wild-type and hSOD1^{G93A} mice, suggesting the absence of signalling into CD4^{+} cells via C3aR. A recent study discovered that the absence of C3aR signalling in CD4^{+} cells diverts naïve T cells into Foxp3^{+} iTreg cells, indicating that complement has a crucial role in modulating the induction and function of Treg cells. In the same study, striking up-regulation of C5aR2 expression on iTreg cells was observed in dendritic cells lacking C3a and C5a, whereas less C5aR2 was found on iTreg cells treated with exogenous transforming growth factor (TGF)-β1 (Strainic et al., 2013). TGF-β1 is up-regulated in muscle of muscular dystrophies, such as Duchene muscle dystrophy (DMD), congenital muscular dystrophy, and inflammatory myositis, and mutant SOD1 induces

marked up-regulation of TGF-β1 mRNA expression in muscles of hSOD1^{G93A} mice (Bernasconi et al., 1999, Ishitobi et al., 2000, Galbiati et al., 2012). Combining these findings, it is possible that C5aR2 expression decreases in tibialis anterior muscles from hSOD1^{G93A} x C3aR$^{-/-}$ mice, resulting from the synergistic effect of C3aR signalling and cytokine TGF-β1 where the absence of C3aR signalling in CD4^{+} T cells induces of iTreg cells and TGF-β1 down-regulates C5aR2 expression on those iTreg cells. Since C5aR2 is the alternative receptor of C5a, C5a-C5aR2 signalling could regulate the infiltration of helper T cells in skeletal muscle in a similar manner as C5a-C5aR1. Given that the absence of C5a-C5aR1 signalling attenuates CD4^{+} T cells influx in tibialis anterior muscles of hSOD1^{G93A} mice, the reduced CD4^{+} cells migrations observed in tibialis anterior muscles of hSOD1^{G93A} x C3aR$^{-/-}$ mice in this study might be due to the impaired C5a-C5aR2 signalling. To verify these speculations, further studies would be required to examine the cytokine expression and C5aR2 signalling in skeletal muscle of transgenic hSOD1^{G93A} mice.

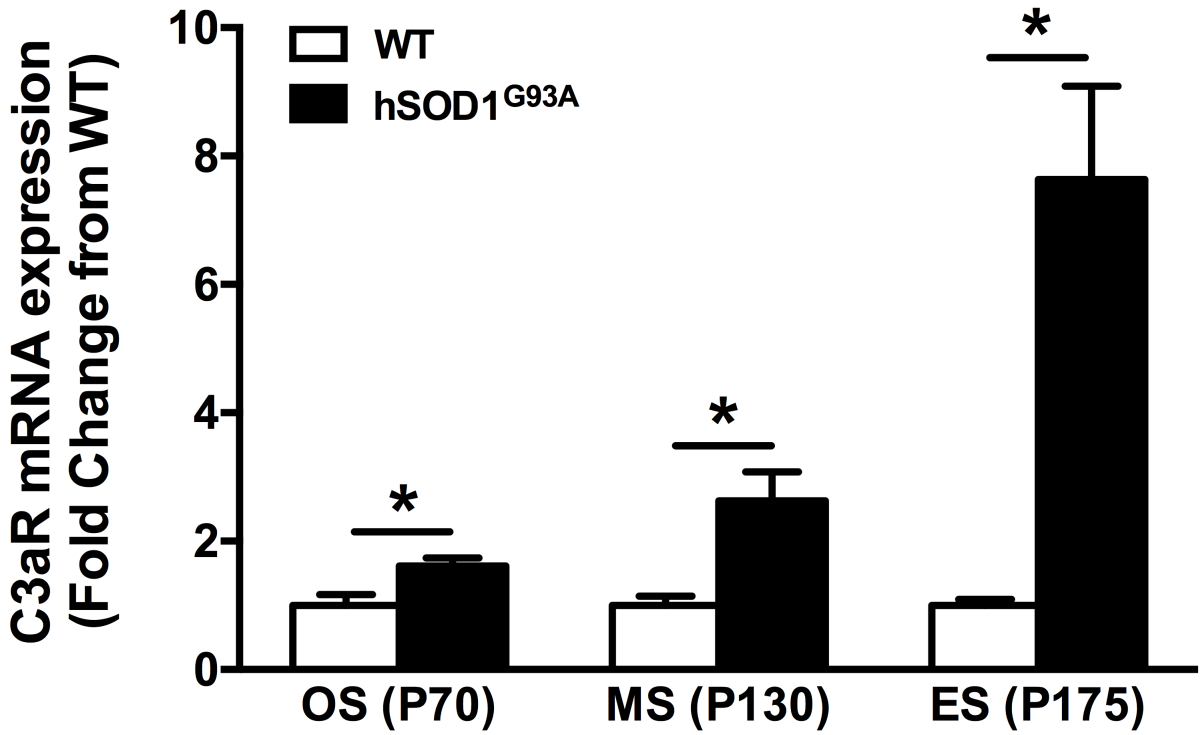

Figure 18. Expression of C3aR in hSOD1^{G93A} and wild-type mice during disease progression.

mRNA expression of C5aR1 in tibialis anterior (TA) muscle of wild-type (WT) and hSOD1^{G93A} mice were quantified by quantitative real-time PCR at three different ages. (n = 5; *p < 0.05, **p < 0.01, Student t test). Bars represent the mean ± SEM. OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

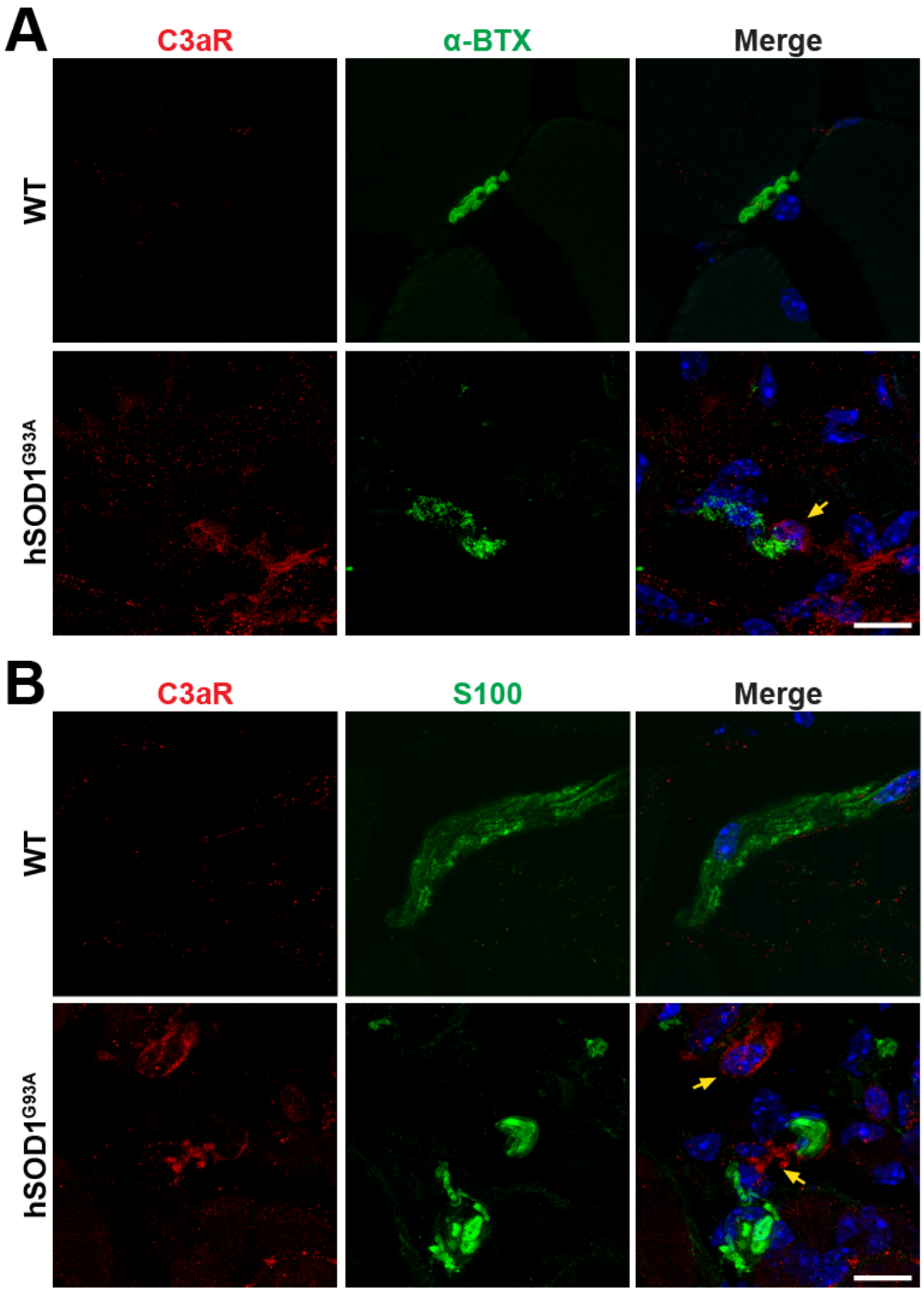

Figure 19. C3aR is not localised at neuromuscular junction or Schwann cells in hSOD1^{G93A} mice.

C3aR is not expressed on either neuromuscular junction or Schwann cells. Immunostaining of C3aR (red, yellow arrows) with α-Bungarotoxin (green, **A**) for motor endplate and S100 (green, **B**) for Schwann cells in TA muscle of WT and hSOD1^{G93A} mice at end stage of disease. Nuclei were visualized with DAPI (blue). Scale bars, 20 μm.

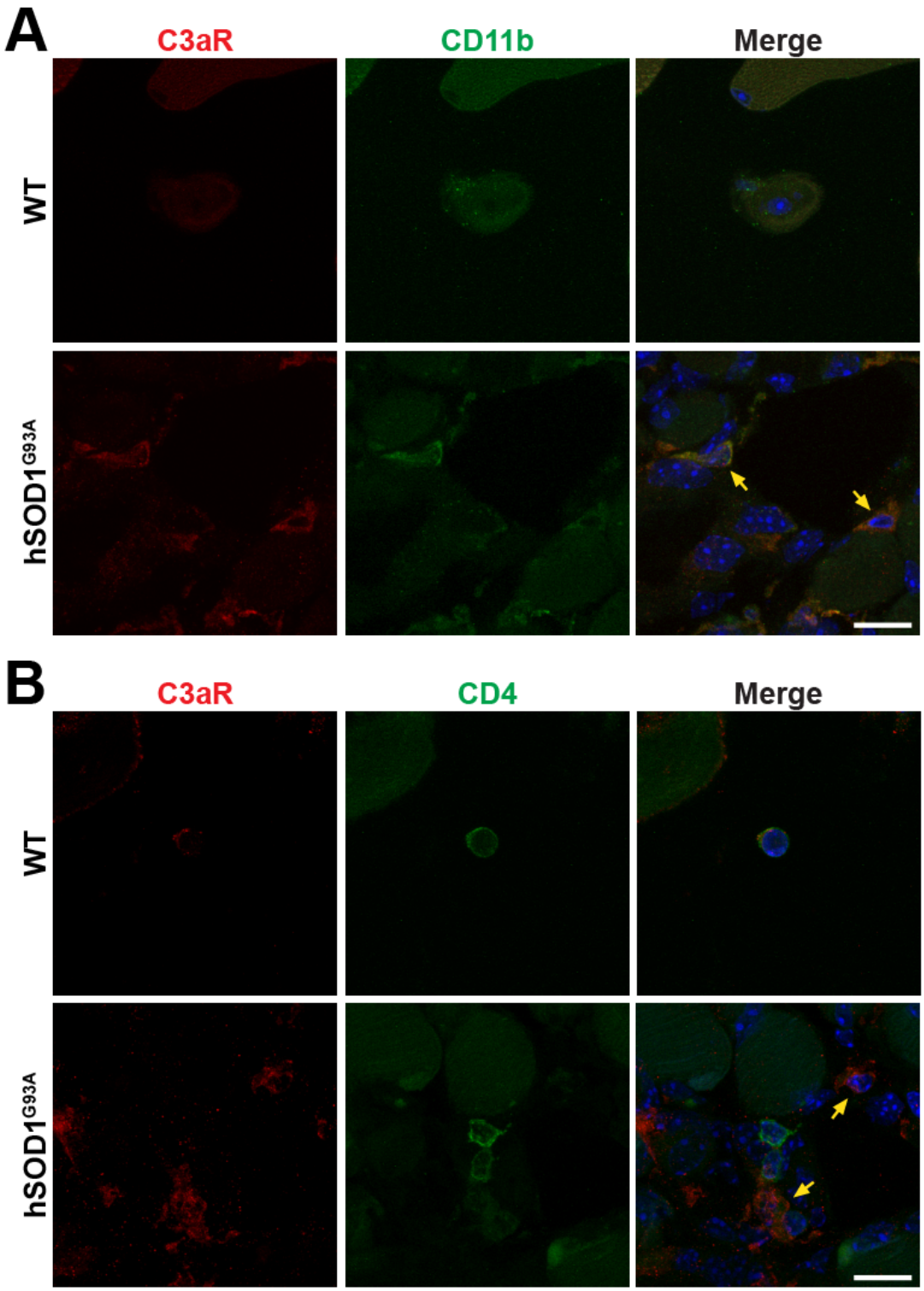

Figure 20. C3aR is localised to macrophages, but not helper T cells in hSOD1^{G93A} mice.

Cellular localization of C3aR on macrophages, but not helper T cells. Immunostaining of C3aR (red) with CD11b (green, **A**) for macrophages and CD4 (green, **B**) for helper T cells in tibialis anterior (TA) muscle of WT and hSOD1^{G93A} mice at end stage. C33aR (yellow arrows) is co-localised to CD11b-positive macrophages, but not to CD4-positive helper T cells. Nuclei were visulised with DAPI (blue). Scale bars, 20 µm.

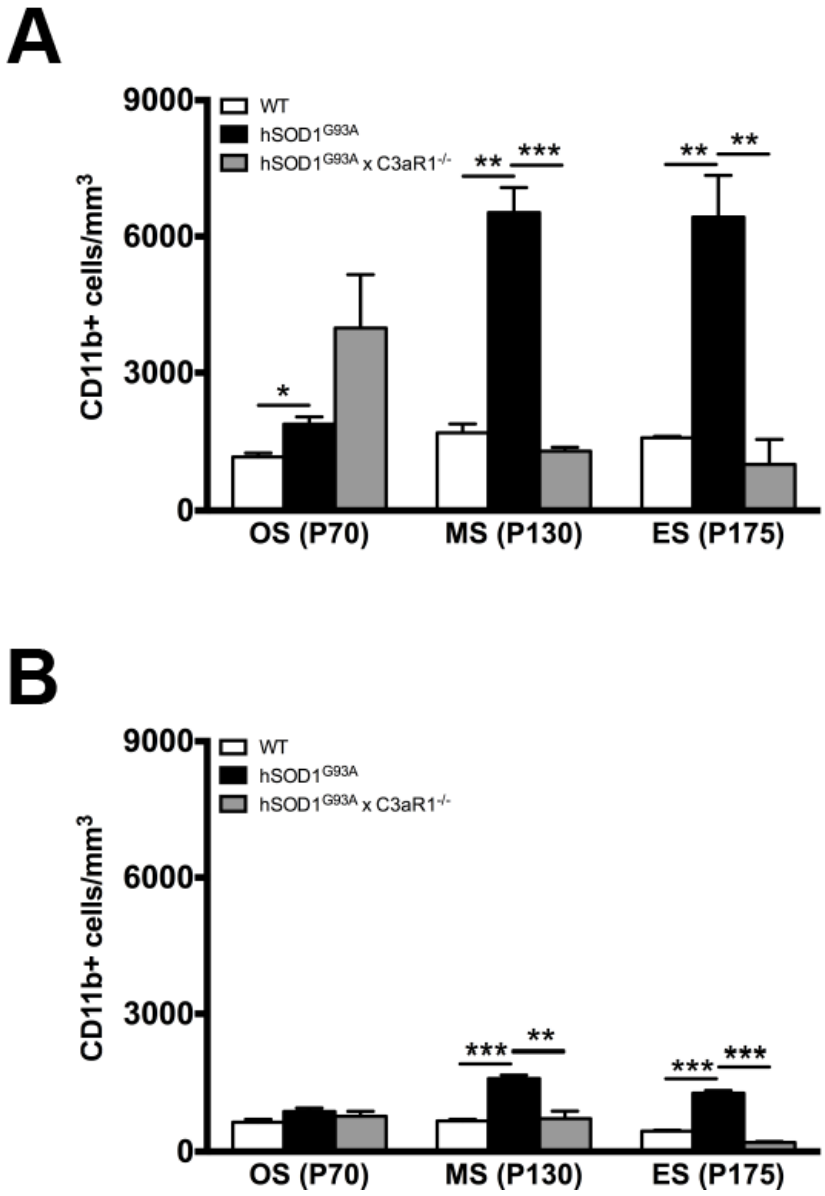

Figure 21. Quantification of macrophages in skeletal muscle of WT, hSOD1^{G93A} and hSOD1^{G93A} × C3aR$^{-/-}$ mice.

Infiltration of macrophages into tibialis anterior or soleus muscle groups at various stages. Cryosections of tibialis anterior (**A**) and soleus (**B**) muscle from WT, hSOD1^{G93A} and hSOD1^{G93A}×C3aR$^{-/-}$ mice were stained for CD11b and quantified as described in Materials and Methods. Fewer CD11b^{+} macrophages were found in MS and ES hSOD1^{G93A}×C3aR$^{-/-}$ mice compared with hSOD1^{G93A} mice (n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, two-way ANOVA). Bars represent the mean ± SEM. WT, wild-type; OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

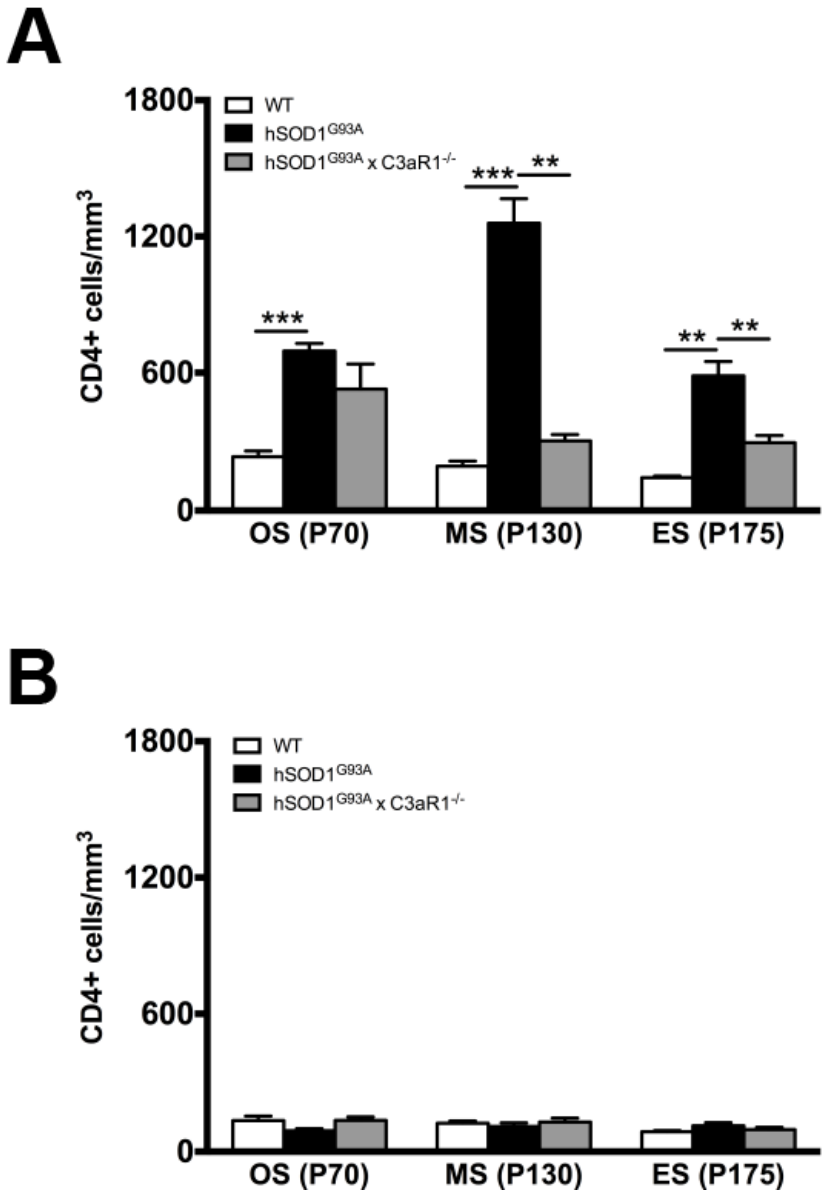

Figure 22. Quantification of helper T cells in skeletal muscle of WT, hSOD1^{G93A} and hSOD1^{G93A} × C3aR$^{-/-}$ mice.

Infiltration of helper T cells into tibialis anterior or soleus muscle groups at various stages. Cryosections of tibialis anterior (**A**) and soleus (**B**) muscle from WT, hSOD1^{G93A} and hSOD1^{G93A}×C3aR$^{-/-}$ mice were stained for CD4 and quantified as described in Materials and Methods. Fewer CD4^{+} helper T cells were found in MS and ES hSOD1^{G93A}×C3aR$^{-/-}$ mice compared with hSOD1^{G93A} mice in TA muscle (**A**). There are no significant changes of CD4^{+} helper T cells numbers in SOL muscle (**B**). (n = 3; $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA). Bars represent the mean ± SEM. WT, wild-type; OS, onset = postnatal day 70 (P70); MS, mid-symptomatic = postnatal day 130 (P130); ES, end stage = postnatal day 175 (P175).

Chapter 6

General discussion

Amyotrophic lateral sclerosis is a late onset fatal neurodegenerative disease. It is well characterised by the progressive loss of upper and lower motor neurons in the central nervous system accompanied by muscle weakness and atrophy (Wijesekera and Leigh, 2009). Over the years, many theories of ALS pathogenesis have been proposed, including oxidative stress, excitotoxicity, mitochondrial dysfunction, axonal transport defects and abnormal protein aggregation (Parakh et al., 2013). In addition to these mechanisms, evidence from several studies have suggested involvement of complement system in human ALS pathology and animal models of ALS (Lee et al., 2012a). A previous study from our lab has demonstrated local activation of complement in the central nervous system of hSOD1^{G93A} mice, a well-defined transgenic mouse model of ALS that carries mutant human *SOD1* gene, during the disease progression (Lee et al., 2013). However, the roles of complement in the skeletal muscle in ALS pathology are poorly understood. The aim of this study was to determine the expression and biological function of complement factors in the skeletal muscle of hSOD1^{G93A} mice. My working hypothesis was that complement is activated in the skeletal muscle of hSOD1^{G93A} mice, and it contributes to ALS disease progression through modulating immune cell migrations via complement receptors C3aR and C5aR1 signalling.

The first major finding from the present study is that complement activation in the skeletal muscle of hSOD1^{G93A} mice. Using quantitative PCR, I detected up-regulation in mRNA levels of C1qB, factor B and C3, the initiators of classical and alternative pathway and central compartment of complement cascade, showing that complement has been activated. These findings are consistent with previous studies that have demonstrated in spinal cord and neuromuscular junction of hSOD1^{G93A} mice, suggesting that complement

activation is not restricted to CNS, but also displayed in skeletal muscle of ALS disease model (Heurich et al., 2011, Lee et al., 2013). To date, the initiating site of neurodegeneration is still under debate. The “dying-back” theory has been suggested where pathological changes in skeletal muscles, motor axons and motor nerve terminals appear to precede motor neuron degeneration and clinical symptoms (Dadon-Nachum et al., 2011, Moloney et al., 2014). This hypothesis is supported by the finding of C1q and C3b/iC3b deposition at the neuromuscular junction at in hSOD1^{G93A} mice of pre- (day 47) and mid-symptomatic (day 126) disease stage (Heurich et al., 2011). As significant increases of C1qB and C3 were observed in tibialis anterior muscles of hSOD1^{G93A} mice at disease onset (day 70), my findings may favour the “dying-back” pattern of ALS pathology in hSOD1^{G93A} mice where activation of complement in skeletal muscle may contribute to the degenerations of the NMJ and its motor axons.

The second main finding of this study is that complement receptor C5aR1 is responsible for the recruitment of immune cells in hSOD1^{G93A} mice. Complement peptide C5a is the active fragment cleaved from C5. It possesses a wide spectrum of biological functions, including induced chemotaxis of immune cells like neutrophils, monocytes, macrophages, and T lymphocytes. C5a exert is functions via binding to its signalling receptor C5aR1 or alternative receptor C5aR2 (Guo and Ward, 2005). Studies from our lab have demonstrated that up-regulation of C5aR1 within the CNS of SOD1^{G93A} rodents, and selectively blocking C5aR1 activity with specific antagonist or depleting *C5aR1* in transgenic SOD animals ameliorates disease symptoms and extends life span. These data suggest that C5a-C5aR1 signalling plays a detrimental role in the pathology of ALS (Woodruff et al., 2008a, Lee et al., 2013, Woodruff et al., 2014). Herein I showed elevated expressions of C5aR1 and its ligand C5a in tibialis anterior muscles of hSOD1^{G93A} mice, and immunohistochemistry results demonstrated that C5aR1 is localised to CD11b positive macrophages and CD4 positive helper T cells, but not at NMJ or on Schwann cells.

NMJ is the synapse that connects axon terminals to motor endplates. It undergoes destruction much earlier than the axons and cell body loss in hSOD1^{G93A} mice during disease progression (Fischer et al., 2004, Heurich et al., 2011). Schwann cells, the principle glia of the PNS, regulates the morphological stability, integrity and repair of NMJ (Darabid et al., 2014). Multiple studies showed either neuroprotective or neurotoxic effect

of Schwann cells by manipulating the expression of mutant *SOD1* gene within Schwann cells (Lobsiger et al., 2009, Turner et al., 2010, Wang et al., 2012, Arbour et al., 2015). The absence of C5aR1 on NMJ and Schwann cells indicates that C5a-C5aR1 signalling is not involved in the process of triggering phagocytosis of Schwann cells, or inducing perisynaptic Schwann cell-guided NMJ reinnervation during ALS disease progression as seen in nerve injury (Reichert et al., 1994, Kang et al., 2014). As other types of phagocytes, like macrophages, can be attracted to remove nerve debris during axonal degeneration, the recruitments of other immune cells by C5a-C5aR signalling have also been examined. Cellular localisation of C5aR1 to infiltrating macrophages and helper T cells was demonstrated, and mass influx of macrophage and helper T cells were also observed in tibialis anterior muscles of hSOD1^{G93A} mice.

Macrophages have long been implicated in muscle regeneration after injury (Summan et al., 2006, Arnold et al., 2007, Bryer et al., 2008). Depending on their phenotypes, M1 or M2, macrophages can either participate in the removal of cellular debris or be actively involved in the tissue repair (Summan et al., 2006). Interestingly, the infiltration of macrophages is significantly attenuated in tibialis anterior muscles of hSOD1^{G93A} mice lacking C5aR1. A similar trend was also observed in soleus muscles of hSOD1^{G93A} mice lacking C5aR1, but to a lesser extent. These results suggest that C5a-C5aR1 signalling plays a pivotal role in recruiting macrophages into skeletal muscles of hSOD1^{G93A} mice during disease progression. Similarly, significant reductions in infiltrating helper T cells numbers were also seen in tibialis anterior muscles of hSOD1^{G93A} mice lacking C5aR1. Several studies have illustrated infiltration of T cells in the CNS of both ALS patients and transgenic mouse models, and suggested a neuroprotective property of functional CD4^{+} T cell in transgenic SOD1 mice (Beers et al., 2008, Chiu et al., 2008, Holmøy, 2008). The roles of T cells present in skeletal muscle of ALS animal models including hSOD1^{G93A} mice remains unclear. By contrast, studies in other muscle diseases such as MD show that depleting T and B lymphocytes resulted in an improvement of muscle regeneration (Farini et al., 2012). Given that hSOD1^{G93A} mice lacking C5aR1 display a significant extension in survival (Woodruff et al., 2014) and muscle performance in grip-strength (*unpublished observations*), I postulate that diminished T cell infiltration in tibialis anterior muscles of hSOD1^{G93A} mice lacking C5aR1 may contributes to muscle regeneration. In brief, my results suggest that C5a-C5aR signalling is actively involved in the pathogenesis of ALS in skeletal muscle, facilitating muscle regeneration by mediating immune cell infiltrations into

skeletal muscle.

Depending on the composition of muscle fibre types, skeletal muscle can be basically categorised into fast-twitch and slow-twitch subtypes. By contrast to fast-twitch tibialis anterior muscles, slow-twitch muscle like soleus muscles is more resistant to denervation. Minimal invasions of macrophages and helper T cells were found in soleus muscles of hSOD1^{G93A} when compared to their counterparts in tibialis anterior muscles, indicating that the infiltration of immune cells in skeletal muscle occurs in a muscle type-dependent manner. Denervation in ALS occurs in a muscle fibre type-specific pattern, where fast-twitch type II fibres undergo destruction first, followed by slow-twitch type I fibres (Frey et al., 2000, Hegedus et al., 2007, Pun et al., 2006). Skeletal muscle is the major site of dietary glucose disposal. Defects in skeletal muscle glucose uptake are associated with insulin resistance. Altered muscle metabolism in hSOD1^{G93A} mice has also been proved to be fibre type related, where decreased insulin-stimulated glucose uptake occurred in fast-twitch muscle first at middle stage of disease (Smittkamp et al., 2014). Recent reports show that macrophage numbers within muscle are elevated during obesity, which associated with insulin resistance, and that muscle cells *in vitro* can mount autonomous inflammatory responses under metabolic challenge (Pillon et al., 2013). This cross-talk between skeletal muscle and immune cells implicates that the differences in the extent of immune cell invasion between tibialis anterior muscles and soleus muscle might be related to the altered muscle metabolism in hSOD1^{G93A} mice.

In addition to C5a-C5aR1 signalling, the present study also investigated C3a-C3aR signalling in ALS pathology. C3a is an active fragment of complement factor C3. It can initiate both pro- and anti-inflammatory responses via binding to its receptor C3aR (Yanamadala and Friedlander, 2010). However, the role of C3a-C3aR1 signalling in ALS has not been well elucidated. Increased mRNA level of C3aR was seen in tibialis anterior muscles of hSOD1^{G93A}, suggesting the existence of C3aR signalling in skeletal muscle. Similar to C5aR1, there was no co-localization of C3aR to NMJ or on perisynaptic Schwann cells, suggesting that C3a-C3aR signalling does not participate in the denervation of NMJ. Interestingly, similar trends of immune cells migration was discovered, whereas hSOD1^{G93A} mice lacking C3aR showed a striking reduction in macrophage and helper T cell numbers in tibialis anterior muscles when compared to hSOD1^{G93A} mice. However, expression of C3aR was found absent on $CD4^+$ T cells in

tibialis anterior muscles of hSOD1^{G93A} mice. Previous studies showed that the absence of C3aR signalling in CD4^{+} T cells induces transition of naïve T cells to iTreg cells, and iTreg cells expressing less C5aR2 under exposure to excessive TGF- β1, a cytokine whose mRNA level is largely increased in muscle of hSOD1^{G93A} mice due to mutant SOD1 toxicity (Strainic et al., 2013, Galbiati et al., 2012). Since C5aR2 is the alternative receptor for C5a, it is possible that C5a-C5aR2 signalling is reduced as a consequence of absence of C3aR on CD4^{+} T, which in turn recruits fewer T cells into tibialis anterior muscles as seen in hSOD1^{G93A} mice lacking C5aR1. This finding requires further study to investigate the expression of cytokines that are related to T cell chemotaxis and subpopulation of T cells in skeletal muscle of hSOD1^{G93A} mice.

In conclusion, the current study demonstrates activation of complement in skeletal muscle of transgenic hSOD1^{G93A} mice, and signalling of complement receptor C5aR1 and C3aR are involved in the pathogenesis of ALS in skeletal muscle. These findings suggest that complement is actively involved in the disease progression of ALS, and its impact is not limited to CNS, but also applies to skeletal muscle, which may yield new mechanistic insights into the ALS pathology.

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