IDENTIFYING THE MOLECULAR BASIS FOR TREATMENT RESISTANCE IN A SUBSET OF MYASTHENIA GRAVIS PATIENTS OF AFRICAN ANCESTRY

Jennifer Mary Auret BSc (Hons)

ARTJEN003

Thesis presented for the Degree of

MASTERS (MEDICINE) in CELL BIOLOGY

In the department of Human Biology

UNIVERSITY OF CAPE TOWN

SOUTH AFRICA

July 2013

Supervisor: Assoc. Prof. Jeannine Heckmann

Co-supervisor: Assoc. Prof. Sharon Prince

Co-supervisor: Dr Amaal Abrahams

Declaration

I, Jennifer Mary Auret, hereby declare that the work on which this dissertation is based is my original work (apart from the normal guidance from my supervisor and except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any matter whatsoever.

SIGNATURE:

DATE:

This thesis is dedicated to the memory of Khula:

I was so lucky to have you in my life for those seven years. You were taken too soon, but your memory will forever live in my heart.

Acknowledgements

I would like to acknowledge and express my gratitude to the following people for their support during this thesis:

- My supervisor **Associate Professor Jeannine Heckmann** for her patience and dedication to this work. This thesis would not have been possible without the ideas she formulated, thanks must also be extended for her help with the writing of this thesis.

- My co-supervisor **Associate Professor Sharon Prince** for all her invaluable advice that extends to laboratory work as well as encouraging me to participate in local conferences.

- My co-supervisor **Dr Amaal Abrahams** who I am incredibly grateful for all her tremendous support she has given me throughout this degree. I must also thank her for helping me during the writing of this thesis.

- The **T-Box lab**, the entire group was incredibly supportive and gave wonderful technical advice in times of need.

- My dear friend in the laboratory, **Mubeen**: so many laughs, sushi-outings and happy memories were made.

- **Ms Ingrid Baumgarten** for her assistance with the EBV-transformation of the lymphoblasts.

- **My parents**, I am so thankful for your support. All I have achieved is due you; I love you both so much!

- **All my dearest friends** wow! You all are so special…Lolly, Emma, Jess, Kyla and Mieks thank you for all the good times!

- **Dirk**, you were there from the start of this degree, you listened to me, made me feel better on low days and always supported me. No words can express the extent of my gratitude I feel towards everything you have done for me.

- **NRF DAAD, UCT and the UCT Department of Neurology** for financial support.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

6-MP	6-mercaptopurine
%	percentage
℃	degrees Celsius
α	alpha
β	beta
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
ACh	acetylcholine
AChR	acetylcholine receptor
ADP	adenosine diphosphate
Amp	ampicillin
AMP	adenosine monophosphate
ARE	adenine-uracil rich elements
ATP	adenosine triphosphate
AZA	azathioprine
BCA	bicichoninic acid
bp	basepair
C	cytosine
C>G	c.-198C>G
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
Cl	chlorine
cm	centimetre
CR1	complement receptor 1
CRP	complement regulatory protein
CS	control sera
CsA	cyclosporine A
Ct	crossing point
Daf	mouse decay accelerating factor
DAF	human decay accelerating factor
DAPI	4', 6-Diamidino-2-Phenylindole
dH_2O	Distilled water
ddH_2O	Double distilled water
DEPC	diethyl pyrocarbonate
DLR	dual luciferase reporter
DMEM	Dulbecco's modified eagle's medium

DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
EAMG	experimental autoimmune myasthenia gravis
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	ethylenediaminetetra-acetic acid
EOM	extraocular muscle
FBS	fetal bovine serum
FCS	fetal calf serum
g	gram/gravity
G	guanine
gDAF	GPI-anchored DAF
GPI	glycosylphosphatidylinositol
GRs	glucococorticoid receptors
GREs	glucocorticoid response elements
GUS B	glucuronidase B
H_2O	water
Hrs	hours
hsp90	90 kDa heat shock protein
HuR	human antigen R
Inc	incorporated
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	Monopotassium phosphate
LB	Luria broth
LPS	lipopolysaccharide
M	molar
MAC	membrane-attack complex
MAPK	mitogen-activated protein kinase
MCP	membrane cofactor pathway
mg	milligram
MG	myasthenia gravis
MGS	treatment-naïve MG patient sera
$MgCl_2$	magnesium chloride
mins	minutes
MIRL	membrane inhibitor of cell lysis
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MTX	methotrexate
MTX_{Glu}	polyglutamated methotrexate

NaCl	sodium chloride
Na_2HPO_4	disodium phosphate
NFAT	nuclear factor of activated T-cells
ng	nanogram
nm	nanometre
NMJ	neuromuscular junction
OD	optical density
PBS	phosphate buffered solution
PCR	polymerase chain reaction
pmol	picamole
P/S	Penicillin-Streptomycin
qRT-PCR	quantitative real-time PCR
RIPA	radio-immunoprecipitation assay
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SA	South Africa
SCR	short concensus repeat
sDAF	soluble DAF
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
TBE	tris borate EDTA buffer
UK	United Kingdom
USA	United States of America
UTR	untranslated region
VEGF	vascular endothelial growth factor
WT	wild type
w/v	weight/volume

Abstract

Myasthenia gravis (MG) is an autoimmune disease in which pathogenic antibodies block, target or destroy the acetylcholine receptors of the muscle endplate resulting in failure of neuromuscular transmission and fatigable weakness. We have previously shown that a subpopulation of South African MG patients of African genetic ancestry develop a severe extraocular muscle (EOM) phenotype whilst receiving standard immunosuppressive drug therapies. This phenotype associates with a functional c.-198C>G SNP (C>G SNP) in the regulatory region of decay accelerating factor (DAF), a complement regulatory protein, which is essential for protection against complement-mediated damage. MG patients are treated with prednisone as the first-line immunosuppressant and frequently, an additional steroid-sparing agent, such as azathioprine, cyclosporin A or methotrexate. We hypothesised that MG patients with the C>G SNP when treated with immunosuppressant drugs, may have lower DAF protein levels contributing to increased susceptibility to autologous complement-mediated damage at their EOMs. This study tests this by comparing the effect of prednisone, azathioprine, cyclosporine and methotrexate individually and prednisone in combination with each of these steroid-sparing agents on wild-type and C>G DAF protein (western blotting) and mRNA (quantitative real time PCR) levels and promoter activity (luciferase reporter assays). These experiments were performed in EBV transformed lymphoblastoid cell lines from control individuals with wild-type DAF and MG patients carrying the C>G SNP. As a more representative model for this study the experiments were repeated in mouse skeletal muscle cells because there was no available EOM cell line. In support of the hypothesis of this study, prednisone, cyclosporin A and azathioprine individually was shown to repress C>G DAF protein levels while having either no effect on wild-type DAF or slightly activating it. Importantly, methotrexate was the only drug that was able to increase C>G DAF lymphoblast protein expression and therefore holds promise as a potentially effective treatment for MG patients with the C>G SNP. Moreover, using a calcein assay, clinically relevant doses of prednisone in combination with MG patient sera was shown to significantly increase the susceptibility of C>G DAF lymphoblasts to cell lysis (82% C>G DAF lymphoblasts vs. 9% wild-type DAF lymphoblasts). These results suggest that MG patient sera contain factor(s) that together with prednisone may also be responsible for the susceptibility of the EOMs in these patients to injury. The results show that the levels of DAF protein and mRNA did not always match which suggests that the drugs tested may regulate the DAF protein at a post-transcriptional level. In a mouse skeletal muscle model, Daf (equivalent to human wild-type DAF) protein expression was consistently repressed by prednisone treatment but activated by cyclosporin A, azathioprine and methotrexate. Furthermore, co-treatment of prednisone with either of the steroid-sparing drugs showed that azathioprine, and low doses of cyclosporin A and methotrexate

were able to overcome the repressive effect of prednisone-only treatment on Daf expression. These observations indicate that the regulation of Daf may be species and/or cell-type specific. Together the results from this thesis suggest that in EOMs, where DAF is already downregulated, compared to other muscles, our MG patients with the C>G SNP may have an increased susceptibility to complement-mediated damage when treated with prednisone, which further represses C>G DAF expression.

CHAPTER 1: LITERATURE REVIEW

1.1 Myasthenia gravis

Myasthenia gravis (MG) is an autoimmune disorder in which auto-antibodies are directed towards the acetylcholine receptors located at the neuromuscular junction. As a result, neuromuscular transmission is impaired causing fluctuating muscle weakness that worsens after prolonged or continued use of muscle, but improves after periods of rest. Historically the term *myasthenia gravis* is of Greek origin with the word *myasthenia* derived from a combination of the nouns 'muscle' and 'weakness' and the addition of *gravis* that translates to severe (Keesey, 2002). It is believed that the first case of MG was documented in 1664 presenting in the American Indian chief Opechancanough; however, the first modern description of MG symptoms in patients would only be published more than two centuries later.

Today the global incidence rate of MG is estimated at 200-400 cases per million with the majority of patients initially presenting with fatigable weakness in their extraocular muscles (EOMs). Consequently, depending on the severity, patients' vision is often impaired as a result of eyelid drooping. The EOMs include a group of six small skeletal muscles which control movement of the eyeball in the socket as well as an additional muscle outside the socket, the levator palpebrae superioris, which is responsible for elevating the eyelid. In approximately 90% of MG patients the symptoms progress from the EOMs to additionally include the bulbar and limb muscles leading to what is known as generalised MG. However, if the symptoms remain confined to the EOMs for more than two years these individuals are classified as having ocular MG. For 15%-20% of MG patients a life-threatening complication known as "myasthenic crisis" occurs in which respiratory failure develops that requires hospitalization and mechanical ventilation.

Using serum-based radioimmunoassay analysis MG can be further grouped into either "seropositive" or "seronegative". Approximately 85%-90% of MG patients are seropositive as they contain antibodies in their serum that are directed towards the nicotinic acetylcholine receptor (AChR) of the neuromuscular endplate. Another cohort of MG patients contains autoantibodies that target the muscle-specific kinases although this type is very uncommon. The remaining 10% are

classified as seronegative, as with standard assays no detectable anti-AChR antibodies are in their serum. In the Cape region of South Africa the incidence of seropositive MG was found to be similar to that of other developing countries although the remaining areas in South Africa showed a lower apparent incidence. The latter is likely the result of under recognition and diagnosis of MG where access to health care may be suboptimal (Bateman et al., 2005).

1.2 The neuromuscular junction and MG (see Figure 1.1)

The neuromuscular junction is the area of contact between terminal nerve endings and muscle fibres and is also known as the muscle endplate. In seropositive MG, the pathogenic antibodies are targeted to the alpha subunit of AChR which is located in the plasma membrane of the muscle fibre of the neuromuscular junction. The axons of the nerve endings contain synaptic vesicles that are filled with the neurotransmitter acetylcholine which is released when the action potential arrives at the axonal terminal. The acetylcholine then diffuses across the synapse and binds to the AChRs located on the crests of the postsynaptic folds of the muscle endplate. Upon binding, Ca^{2+} channels are opened, resulting in depolarization along the muscle fibre causing the muscle to contract (Wray and Porter, 1993). Previous studies have shown that the neuromuscular junctions of different skeletal muscle types show anatomical and physiological variation, some of which may influence their susceptibility to MG. In particular, there is extensive evidence that the unique properties of the EOMs render them susceptible to involvement in certain disorders including MG (Soltys et al., 2008)

1.3 MG and extraocular muscle susceptibility

The EOMs are a complex specialised group of muscles required for rapid, accurate movement of the orbits for both voluntary and reflex responses. Their high specialization contributes to distinctive properties including fast contractile rates, high resistance to fatigue and ability to maintain contractions (Soltys et al., 2008). As a result of this uniqueness they differ from skeletal muscles both anatomically and physiologically. The EOMs are comprised both of singly innervated fibres, like those present in skeletal muscle, and in addition multiple innervated fibres that are known for their high oxidative activity (Wasicky et al., 2000). A further unique property of EOMs is the co-expression of both fetal and adult forms of the AChR subunits in their neuromuscular junctions (Kaminski et al., 1996). Furthermore, the neuromuscular junctions have been shown to

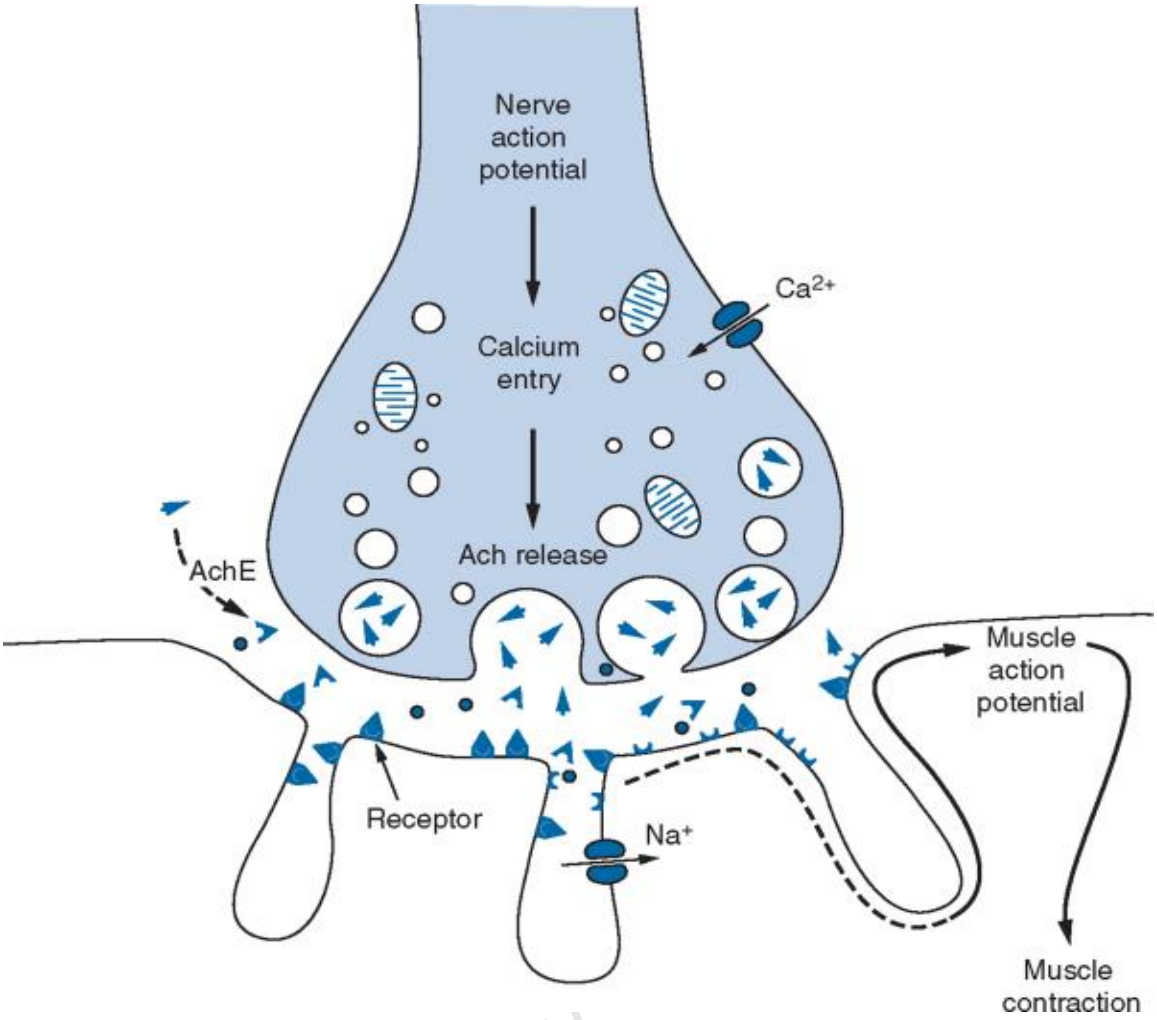


Figure 1.1: Structure of the neuromuscular junction. Vesicles at the terminal end of the axon releases acetylcholine (Ach) which diffuses across the synaptic cleft and attaches to the acetylcholine receptors (AChRs) on the postsynaptic membrane leading to muscle contraction (adapted from http://www.collegepaeds.ac.za).

have less subjunctional folds compared to skeletal muscle (Spencer and Porter, 1988). The anatomical and physiological differences identified between EOMs and skeletal muscles have further been extended to a transcriptional level where differences in gene and protein expression patterns have been demonstrated at both synaptic and extrasynaptic regions (Soltys et al., 2008). However, it still remains to be elucidated why EOMs are more susceptible to certain diseases but not others. For example, they are spared in Duchenne's muscular dystrophy and motor neuron disease yet are prominently involved in Graves' disease and MG. In addition to altered muscle pathway proteins, genes involved in the immune response were also shown to have varying degrees of expression when comparing EOMs and skeletal muscle (Porter et al., 2001). Most notably, Kaminski et al (2004) showed that the complement regulator protein, decay accelerating factor (DAF) is expressed at a low level in the EOMs which could be contributing to their susceptibility to complement-mediated injury in MG.

1.4 The role of the complement pathway in MG

The complement pathway is a critical component of the innate immune system. It is a cascade of biochemical reactions composed of over 30 different serum and membrane proteins, which act synergistically to clear pathogens from the body and help immune cells to fight cancers and infections. It does this through a number of processes including cell lysis of pathogens, enhancement of phagocytosis and the attraction of inflammatory cells. Complement can be activated by three major pathways namely the alternative, lectin and classical pathways (see **Figure 1.2**). The alternative pathway is spontaneously activated on cell membranes, in plasma and other body fluids. Antibody binding to the target antigen results in the activation of the classical pathway whereas the lectin pathway is triggered when mannose-binding lectin binds to the surface of target microorganisms. Upon activation, all three pathways ultimately result in a complement cascade involving the cleavage of C3 and C5 into fragments mediated by the proteolytic enzymes known as the C3/C5 convertases, followed by a series of activation steps and the formation of the membrane-attack complex (MAC), a multimeric protein complex that produces cell lysis.

The importance of complement in MG disease pathogenesis has long been documented and extensive data suggests the classical pathway is responsible for AChR loss at the neuromuscular junctions in experimental autoimmune MG (EAMG) and MG (Kusner et al., 2008). An early study by Nastuk et al (1959), identified that serum from MG patients had less complement factors

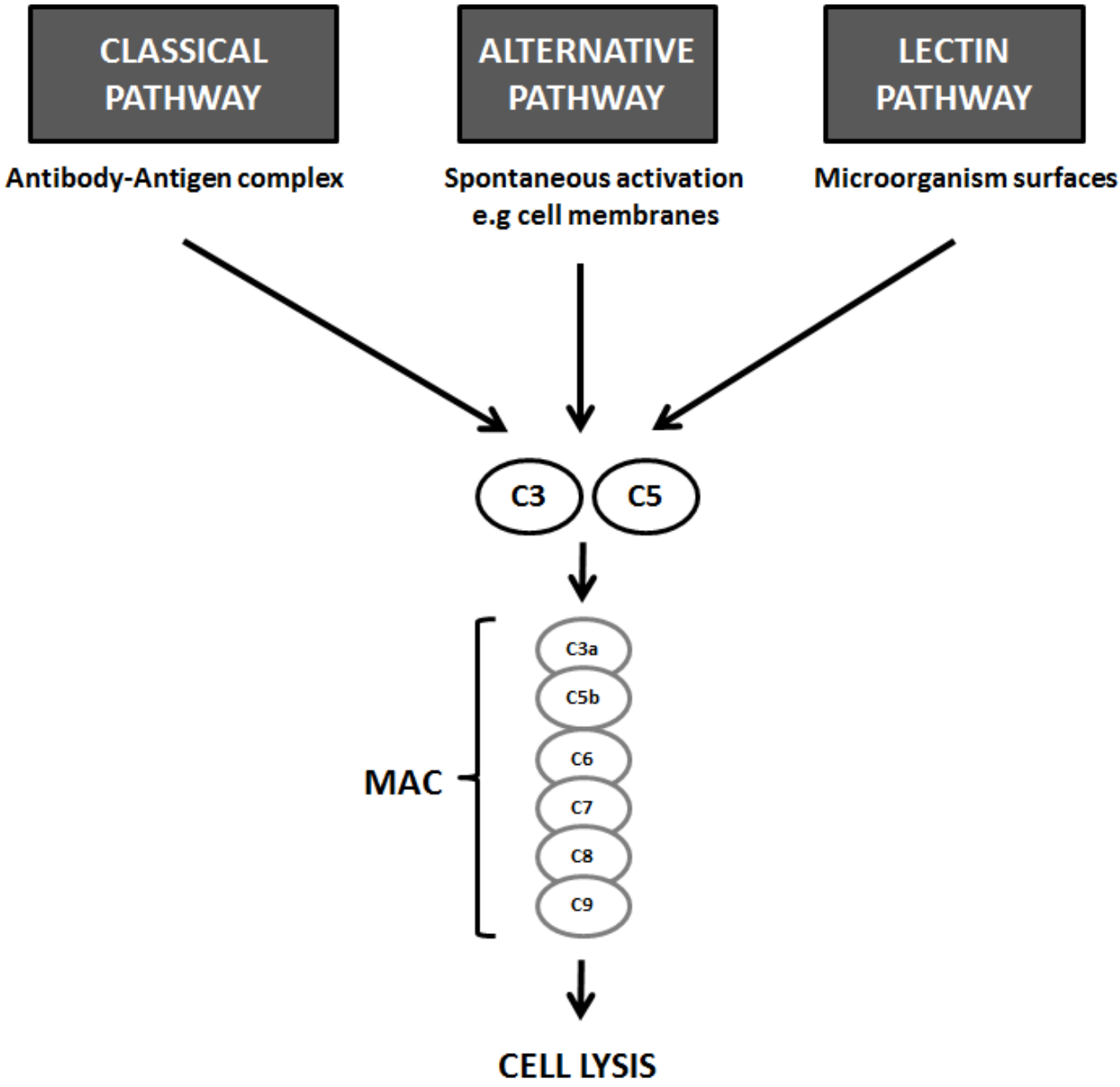


Figure 1.2: The alternative, classical and lectin pathways are involved in the complement system. For MG, the classical pathway is most relevant because of its activation by the antibodies that attack the acetylcholine receptors. All three pathways converge on the C3 and C5 leading to their cleavage which results in the activation of the membrane-attack complex (MAC) causing cell lysis and membrane destruction.

than serum isolated from control individuals. Follow on studies reported that complement-fixing globulins from myasthenic sera bound to muscle sections (McFarlin et al., 1966) which were later shown to bind specifically to the neuromuscular junctions (Engel et al., 1977). The complement activation fragments of C3, C9 and the MAC have also been found at the degenerating postsynaptic neuromuscular junctions in animal models of EAMG and MG patients (Nakano and Engel, 1993; Sahashi et al., 1980). Furthermore, the C3 component was shown to be localised at the motor end-plate in animals with EAMG (Sahashi et al. 1980). The assembly of the MAC was shown to cause damage at the postsynaptic surface of MG muscle contributing to failure of neuromuscular transmission (Soltys et al. 2009). Studies using EAMG models in mice showed those deficient in C4 or C5 had less severe MG (Christadoss, 1988; Tüzün et al., 2003) while genetically modified mice not expressing cell-surface complement regulator proteins develop severe weakness with EAMG (Lin et al., 2002). Based on these and other reports it is now recognized that the consequences of complement-mediated damage at the neuromuscular junction as a result of autoantibody activation is the main effector mechanism in the pathogenesis of MG.

1.5 Decay accelerating factor

It is critical for the human body to tightly regulate the complement system in order to avoid activated autologous complement that would otherwise result in damage to self-tissues. For this reason a group of proteins known as complement regulatory proteins are ubiquitously expressed on the surface of most cell types. There are a number of complement regulatory proteins that have been identified in humans including: decay accelerating factor (DAF, or CD55), membrane cofactor protein (MCP, or CD46), complement receptor 1 (CR1, or CD35) and membrane inhibitor of cell lysis (MIRL, or CD59). Specifically, MCP serves to inactivate complement components C3b and C4b (Riley-Vargas et al., 2004), CR1 mediates the transport of immune complexes to phagocytes (Zorzetto et al., 2003) and CD59 acts as an inhibitor of the structuration of the complement membrane attack complex (Meri et al., 1990). Structurally, all the complement regulatory proteins contain variable numbers of complement control protein domains comprising approximately 70 amino acid repeating motifs, also known as short consensus repeat domains.

Hoffman was the first to describe DAF in human erythrocytes in 1969, and DAF was later isolated and characterised in 1982 by Nicholson-Weller et al. In humans there are two major variants of DAF derived from alternative splicing of the *DAF* gene: the first is anchored to the plasma membrane by

a glycosylphosphatidylinosital anchor (gDAF) (**Figure 1.3**) and the second is a soluble form (sDAF) (Medof et al., 1987). The gDAF variant is ubiquitously expressed in different cell types whereas the sDAF isoform is expressed at lower levels in body fluids such as tears, saliva, plasma and urine (Medof et al., 1987). DAF functions to regulate complement by inhibiting the assembly of and accelerating the degradation of pre-existing C3 and C5 convertases (Lublin and Atkinson, 1989; Medof et al., 1984). This prevents autologous complement activation and formation of the MAC, thereby protecting cells from complement-mediated injury.

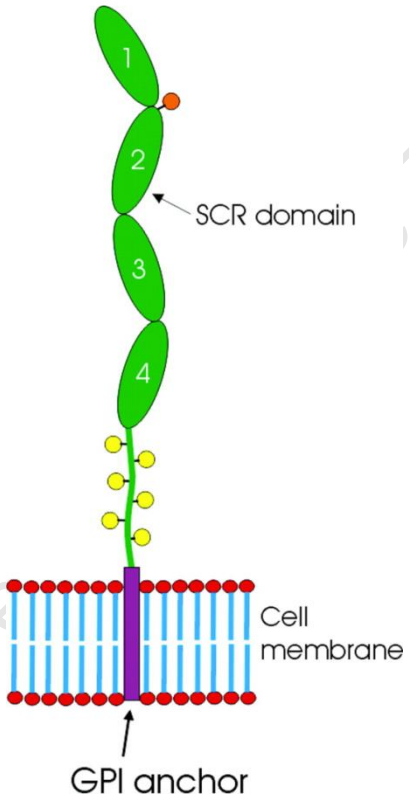


Figure 1.3: Structure of the human gDAF isoform. gDAF is attached to the cell membrane via a glycosylphosphatidylinositol (GPI)-anchor (adapted from He et al., 2002). SCR= short consensus repeat.

Expression of DAF is known to be influenced by both tissue (Lin et al., 2001) and cell type (Thomas and Lublin, 1993). Previous studies have reported that DAF is primarily regulated at a transcriptional level (Thomas and Lublin, 1993) although there is evidence that tissue-specific factors and inflammation can influence DAF mRNA stability (Andoh et al., 2001). Moreover, DAFs expression can be regulated by cytokines such as IL-1, IL-6, TNF-alpha and IFN-gamma (Spiller et al., 2000; Ahmad et al., 2003), prostaglandins (Holla et al., 2005), bacterial lipopolysaccharide (LPS)(Cauvi et al. 2006), and tissue-specific factors (Andoh et al., 2001). For example, endothelial murine Daf (equivalent to the human gDAF isoform) protein and mRNA levels were increased four-fold above basal levels after 24 to 48 hours stimulation with TNF-α. This Daf upregulation was modulated by, amongst others, nuclear factor-KB (NFκB) dependent pathways (Ahmad et al., 2003).

Apart from its role as a complement regulatory protein, DAF has other functions including protecting against natural killer-mediated lysis (Finberg et al., 1992), acting as a receptor for microorganisms and certain viruses (Pham et al., 1995), serving as a ligand for intracellular adhesion by primarily interacting with CD97 leukocytes (Hamann et al., 1996) as well as a negative modulator of T-cell immunity (Liu et al., 2005).

1.6 Problem identification

Previously, the Groote Schuur MG clinic in Cape Town reported variations in clinical outcomes of MG amongst the different ethnic groups in South Africa (Heckmann et al., 2007). It has been observed that a subpopulation of MG patients who are African or of recent African genetic descent develop a severe extraocular muscle (EOM) complication (**Figure 1.4**) characterised by ophthalmoplegia (paralysis of all the extraocular muscles) and ptosis (eyelid drooping) which does not respond to immunosuppressive drug treatment (Heckmann et al., 2010; Heckmann et al., 2012). Previous data from a study in our laboratory showed that a c.-198C>G single nucleotide polymorphism (SNP) located in the regulatory region of DAF is associated with the development of this treatment-resistant EOM phenotype (Heckmann et al., 2010). In this study the c.-198C>G SNP was identified in eight out of 136 MG patients and six out of 167 controls. All of the c.-198C>G SNPs were detected in individuals of African ancestry (Black and mixed ancestry) and was absent in both the White MG patients and controls. Overall, the c.-198C>G SNP was found to be significantly more associated with the African ancestry MG patients having the severe EOM phenotype (p=0.009). The MG subjects belonging to the Black subpopulation were more likely to develop paresis of at least

one EOM if they have the c.-198C>G promoter SNP. The Black MG patients with the c.-198C>G SNP also had increased likelihood of developing pareses of at least 5 EOMs ($p < 0.01$). Similarly, in patients of mixed ancestry (M/A) the SNP was significantly associated with the development of one or more EOM dysfunction ($p=0.022$) but not with the developing five or more EOM. However, when the findings for the patients of African genetic-ancestry (Black and M/A) were combined, the likelihood of developing EOM with at least five or more dysfunctions was significantly increased in the patients with the SNP ($p=0.009$). None of the White MG subjects had the c.-198C>G. The same study also established that the c.-198C>G SNP inhibits DAF upregulation in response to immune stimuli such as LPS. Using a bioinformatics approach this SNP was shown to result in the potential gain of an NFkB binding site and a loss of a Sp1 binding site (Heckmann et al., 2010). These binding sites may therefore be involved in mediating the LPS-induced influence on DAF expression.

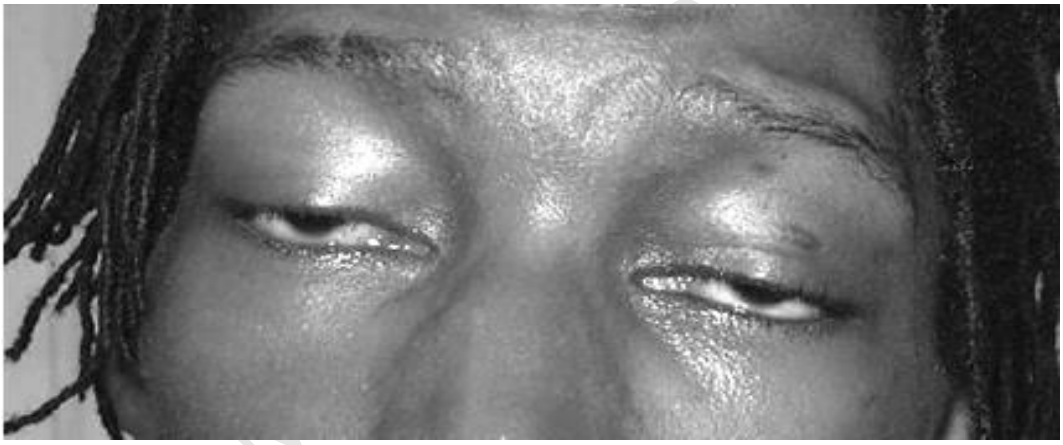


Figure 1.4: Photograph of MG patient displaying the treatment-resistant extraocular muscle phentotype (with permission from the patient) (Heckmann et al., 2007).

1.7 Sp1 and NFκB influence on DAF expression

The transcription factor Sp1 is ubiquitously expressed in the nuclei of all cells and is regarded as a house-keeping transcription factor that regulates target gene transcription by binding to Sp1-binding sites (Brivanlou and Darnell, 2002). In a murine model Daf was reported to have several Sp1 transcription binding sites in its regulatory region that regulate both its constitutive expression as well as LPS-induced expression (Cauvi et al., 2006). Interestingly, in the same study the Sp1 site lost

in the human c.-198C>G SNP was shown to not influence constitutive expression of either Daf or LPS-induced Daf upregulation. Sp1 binding sites have been found to be recognised by transcription factors other than Sp1 and indeed, Sp1 binding sites are often found in the regulatory region of NFκB-regulated genes (Hirano et al., 1998). Interestingly, it has previously been reported that functional coupling exists between Sp1 and NFkB, as Sp1 was shown to bind with high affinity to a subset of NFkB binding sites (Hirano et al., 1998).

The NFkB/Rel family of transcription factors are highly conserved and play critical roles in a wide range of physiological and pathological processes. In particular, NFkB serves an important role in the immune system by regulating both innate and immune responses (Hayden et al., 2006). The subunits of NFkB/Rel family of transcription factors, including p65, p50, p52, RelB and c-Rel, form various homo- and heterodimers that translocate to the nucleus, bind DNA thereby regulating target gene transcription. Even small differences in NFkB binding sites have been reported to favour certain dimer combinations which further can induce differences in gene regulation (Hayden and Ghosh, 2004). While there is limited information showing the effect of NFkB on DAF expression, NFkB has been shown to indirectly increase murine Daf expression via TNF-α in vascular endothelial cells (Ahmad et al., 2003).

1.8 MG drug treatments

To date there is no cure for MG and treatment approaches include symptomatic therapy and often, in addition, the administration of immunosuppressive agents. In the 1930's the first rational treatment of MG began when Mary Walker noticed that MG symptoms were similar to that of curare poisoning (Walker, 1935). She administered the physostigmine drug, an anticholinesterase, and patients showed a rapid improvement in response to treatment. Several decades later the finding that MG had an autoimmune etiology (Nastuk et al., 1959; Simpson, 1960) prompted the use of immunosuppressive drugs. Today, corticosteroids and other immunosuppressive drugs constitute standard treatment for MG patients not responding to symptomatic treatment.

Presently, symptomatic treatment using acetylcholinesterase inhibitors include neostigmine or pyridostigmine. These drugs target the hydrolytic enzyme acetylcholinesterase that usually regulates levels of acetylcholine by rapidly degrading it. Often MG symptoms respond to these anticholinesterases which block the active site of acetylcholinesterase so the enzyme can no longer break down the acetylcholine molecules before they reach the postsynaptic membrane receptors.

In MG there are too few functional acetylcholine receptors so with the acetylcholinesterase blocked, the efficiency of acetylcholine binding to these receptors is improved. If MG is severe, in that there are too few AChRs to "translate" the signal at the endplate, then the focus of the treatment shifts to immunosuppressive management of this antibody-mediated disease. As MG is a disease that may fluctuate in severity, the ultimate goal of MG therapy is to reverse the symptoms of the disease, achieve remission and maintain this state. The immunosuppressive drugs that are used to reverse MG symptoms include prednisone, cyclosporin A, azathioprine and methotrexate.

1.8.1 Prednisone

Prednisone (or prednisolone) is a corticosteroid (or glucocorticoid) drug that is the first-line immunosuppressive therapy in MG (Gilbert et al. 2007). The drug's potent anti-inflammatory, immunosuppressive and anti-allergic actions have led to its extensive use as a treatment in a variety of diseases for over 60 years. Treatment strategies using corticosteroids usually begin with administering a high dose of the drug; however for MG patients, they are initially prescribed low doses in the range of 10-20 mg prednisone per day. Generally, if patients are experiencing bulbar or respiratory symptoms they are first pre-treated with plasma exchange therapy which decreases the risk of increased weakness that is often experienced with corticosteroid treatment.

Extensive research has given an insight into the complexities of the mechanism of action of corticosteroids. Broadly, their anti-inflammatory properties are mediated by the repressive effect the drug has on multiple inflammatory genes encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors and proteins. Specifically, corticosteroids are known to suppress inflammatory cytokines such as IL-1b, IL-2, IL-3, IL-6, IL-11 and TNF-α and chemokines such as IL-8, MCP-1, MCP-3 and MCP-4 which are required to attract inflammatory cells to sites of inflammation. They are also known to play a role in increasing expression of anti-inflammatory proteins. However, the pathways targeted by the drug that result in these beneficial effects have yet to be fully elucidated.

The beneficial effects of corticosteroid drugs, such as prednisone, were initially thought to be mediated by its genomic effects such as receptor-mediated regulation of gene expression. Herein, prednisone rapidly diffuses across the cell membrane and then binds to glucocorticoid receptors (GRs) located in the cell cytoplasm, thereby activating them. Two versions of GRs are known to exist and are a result of alternative splicing. The GRα form binds the corticosteroids, whilst the less

expressed GRβ form is not activated by corticosteroids but is able to bind to DNA (Lu and Cidlowski, 2004). Inactive GRs are bound to various inhibitory proteins and in addition, 90 kDa heat shock protein (hsp90) that acts as a chaperone thereby preventing nuclear translocation (Barnes, 1998). Upon activation by prednisone, hsp90 dissociates allowing the GR-corticosteroid complex to translocate into the nucleus. Homodimers consisting of two GR-corticosteroid complexes form in the nucleus which act as transcription factors by binding to glucocorticoid response elements (GREs) in the 5' regulatory region of target genes that are responsive to corticosteroid treatment (Barnes, 1998) (**Figure 1.5**). This binding usually results in activation of the gene (*trans*-activation); however, negative GRE binding sites are also known to exist which repress the associated genes (*cis*-represion) (Dostert and Heinzel, 2004).

Past research has highlighted that many of the genes repressed or activated by glucocorticoid drugs, such as prednisone, do not contain GREs in their regulatory regions. This led to the discovery that glucocorticoids can regulate inflammatory gene expression by various indirect mechanisms. For example, in the case of the potent pro-inflammatory transcription factor, NFκB, glucocorticoids have been shown to upregulate the expression of its inhibitor, IκBα, thereby preventing its translocation to the nucleus (Scheinman et al., 1995). Furthermore, it was suggested by Mukaida et al (1994) that prednisone inhibits NFkB-driven gene activation by binding and sequestering it and thus preventing NFkB binding to DNA. More broadly, prednisone is known to have a complex effect on multiple signal transduction pathways including an inhibitory effect on c-Jun N-terminal kinases (JNK) which are necessary for the activation of NFkB (Caelles et al., 1997). Lastly, corticosteroids are thought to inhibit NFkB by increasing the levels of glucocorticoid-induced leucine zipper protein, a known inhibitor of NFkB (Barnes, 1998). All these mechanisms prevent the increase in inflammatory gene expression by NFkB, and are a result of the glucocorticoids acting upstream of NFkB binding. However, there are reports that glucocorticoids influence chromatin remodelling mechanisms

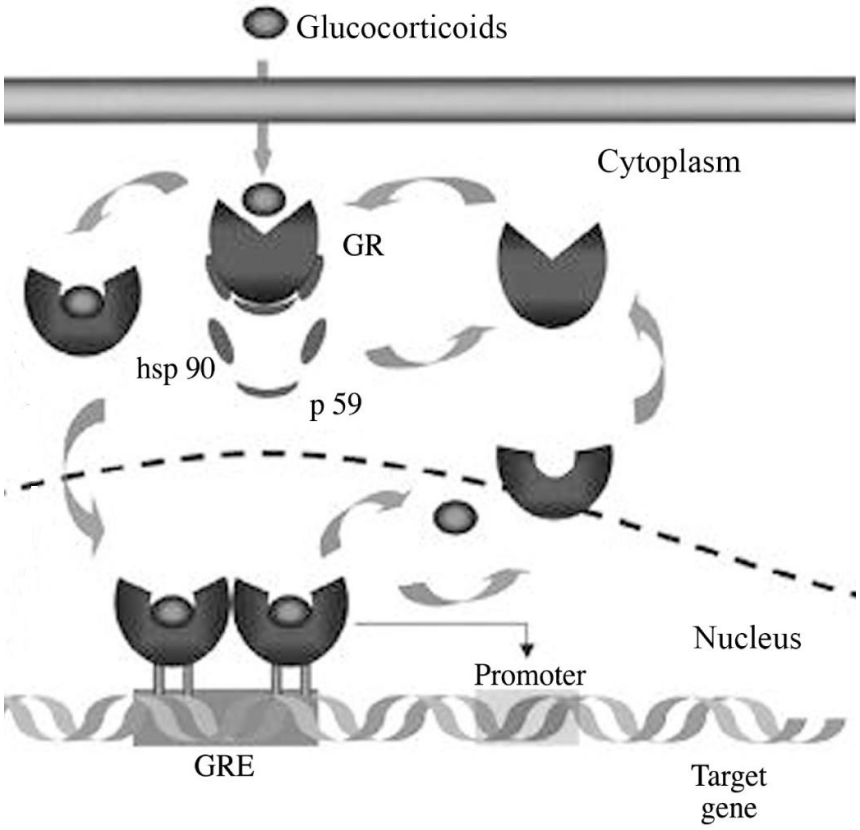


Figure 1.5: Corticosteroid mechanism of action. Corticosteroids such as prednisone rapidly diffuse across the cell membrane and bind to glucocorticoid receptors (GR) which are complexed with the 90 kDa heat shock protein (hsp90). Upon ligand binding, GR dissociates from hsp90 and translocates to the nucleus, where it binds as a dimer to a glucocorticoid response element (GRE) located within the promoter of target genes (adapted from Lee, 2007).

downstream of NFkB-DNA binding (Hart et al., 2000). Specifically, activated glucocorticoids have been shown to stop expression of inflammatory genes by reversing the unwinding of DNA (Ito et al., 2000). These diverse mechanisms further highlight the broad regulatory effects that corticosteroids have on gene expression.

Research conducted over the last two decades, has also revealed the post-transcriptional actions of glucocorticoids. For example glucocorticoids are known to have a regulatory effect on mature cytoplasmic mRNA stability thereby affecting protein synthesis. It is known that the expression levels of certain inflammatory proteins are regulated at an mRNA level (Anderson et al., 2004). This is mediated by adenine-uracil rich elements (ARE) in the 3'-untranslated regions (3' UTR) of the genes where proteins, such as human antigen R (HuR), bind to and are thought to stabilize the mRNA transcripts (Dean et al., 2004). It is suggested that glucocorticoids may inhibit the proteins that stabilise mRNA thereby increasing the rate of mRNA breakdown and reducing protein levels (Smoak and Cidlowski, 2006). Interestingly, the 3' UTR of DAF is known to harbour an ARE and thereby potentially affecting mRNA stability (Gray et al., 2010).

Overall, much remains to be understood regarding the precise molecular mechanisms by which prednisone mediates its potent anti-inflammatory and immunosuppressive effects. Clinically, adverse side-effects associated with prednisone such as adrenal suppression and hyperglycaemia necessitates the use of low doses or additional steroid-sparing immunosuppressant's. In MG treatment regimens, the steroid-sparing agents which are commonly used include azathioprine, cyclosporine A, methotrexate (all used in this thesis) and mycophenolate mofetil (Heckmann et al., 2007; Heckmann et al., 2011).

1.8.2 Cyclosporin A

Cyclosporin A (CsA), isolated from the fungus *Hypocladium inflatum gams,* was found by Borel et al (1976) to have potent immunosuppressive properties. Since its discovery, CsA has been used extensively for the prevention of organ transplant rejection. The molecular mechanisms of its immunosuppressive actions have mostly been investigated in T-cells in which it was initially shown to inhibit T-cell activation and also prevent certain B-cell responses (Klaus and Hawrylowicz, 1984).

T-cell activation requires both a signal from the T-cell receptor and a co-stimulatory signal involving CD28 and CD80 on T-cells. The mechanism by which CsA inhibits T-cell activation by these signals

involves the inhibition of cytoplasmic protein calcineurin, a serine-threonine phosphatase which is activated by calcium (Dolmetsch et al., 1997). Once in the cell, CsA binds to ubiquitously expressed cyclophilins in the cell cytoplasm; thereafter, this complex binds to and inhibits calcineurin which is known to be involved in T-cell signalling pathways necessary for gene expression and biological responses to external stimuli such as foreign antigens (Stankunas et al., 1999). In the absence of CsA, calcineurin is known to dephosphorylate nuclear factor of activated T-cells (NFAT) family members allowing their translocation into the nucleus and therefore their regulation of gene expression (Ho et al., 1996). The CsA-cyclophilin complex inhibits calcineurin by suppressing its phosphatase activity and thus preventing its activation of NFAT and consequently activation of NFAT regulated genes which include the cytokines IL-2 and IL-4. This inhibition of the calcineurin/NFAT pathway is one of the mechanisms by which CsA is thought to mediate its immunosuppressive actions as IL-4 is required for B-cell help while IL-2 is necessary for T-cell proliferation (Rao et al., 1997).

Apart from the inhibition of the calcineurin/NFAT pathway by CsA, studies have indicated that CsA influences the mitogen-activated protein kinase (MAPK) signalling pathway by specifically blocking JNK and p38. When T-cells are activated by ligand binding of both T-cell and CD28 costimulatory receptor, CsA was shown to prevent activation of JNK and p38 signalling pathways (Su et al., 1994; Matsuda et al., 1998). Importantly, activated JNK and p38 together with ERK signalling pathways are known to activate transcription factors such as AP-1 and NFκB. Therefore, the downstream inhibition of AP-1 and NFκB activation contributes to CsA's anti-inflammatory properties. In addition, there is evidence suggesting CsA may also have inhibitory effects upstream of the JNK and p38 signalling pathways (Matsuda et al., 1998).

There is a paucity of information on the effect of CsA treatment on DAF expression. A study using human endothelial cells, found that initially DAF was upregulated by vascular endothelial growth factor (VEGF) thereby protecting against complement-mediated injury. However, this VEGF-induced activation of DAF was suppressed after CsA treatment (Mason et al., 2004). Importantly, CsA treatment alone had no effect on basal expression of DAF in these cells. In an additional study using a kidney endothelial cell model, CsA was shown to directly increase both DAF and CD49 expression (Kim et al., 2007). The effects of CsA treatment on DAF expression therefore suggest potential cell-type specific effects. Interestingly, CsA has been shown to increase the synthesis of TGF-β *in vitro* and *in vivo* (Li et al., 1991; Khanna et al., 1994). Moreover TGF- β is known to upregulate DAF on

primary cultures of orbital fibroblasts (Cocuzzi et al., 2001). However, no studies have as yet investigated the effect of CsA on DAF expression in a myofibroblast lineage.

Despite CsA's popularity as an immunosuppressive drug to prevent organ transplant rejection, its extreme potency leads to severe side effects such as nephrotoxicity, neurotoxicity and hepatotoxicity (Kahan, 1989; Hojo et al., 1999). However, the beneficial effects of CsA include its relatively short onset of action of between one and three months compared with the more delayed effects of the other steroid-sparing agents discussed below.

1.8.3 Azathioprine

Azathioprine (AZA) is one of the oldest immunosuppressive drugs which has been used for almost 60 years primarily to prevent organ transplant rejection. Other diseases which commonly use AZA as a treatment include rheumatoid arthritis, hematologic malignancies and inflammatory bowel disease.

Azathioprine is a synthetic purine and falls under the thiopurine drug classification. Once ingested, AZA is readily absorbed by the gut and converted to its active metabolite form 6-mercaptopurine (6-MP). Moreover 6-MP is further metabolized by three competing pathways, of which only one pathway produces an active compound known as 6-thioguanine (Belgi and Friedmann, 2002). The drug's mechanism of action, although not yet well defined at a molecular level, was initially attributed to its inhibitory effect on purine nucleotide biosynthesis (Lennard, 1992). Initial reports suggested the active compound 6-thioguanine is randomly incorporated into DNA which thus effects cells with high proliferation rates, such as T-cells, thereby suppressing them (Lennard, 1992). However, the doses of AZA used in these early studies were far higher than the dosage given to inflammatory bowel disease patients (Cuffari et al., 1996). Later an *in vitro* study showed clinically relevant doses of AZA were able to mediate significant apoptosis of T lymphocytes (Tiede et al., 2003). Furthermore, a new mechanism was described in which AZA targets the small GTPase, Rac1 resulting in apoptosis seen in T lymphocytes. Overall, small GTPases have roles in a range of cellular processes such as cell growth and differentiation (Atreya and Neurath, 2009). The AZA metabolite, 6-thioguanine was shown to bind to Rac1 (Tiede et al., 2003) preventing interaction with its guanosine exchange factor, VAV, which ultimately reduced Rac1 activation. This prevented Rac1 to interact with its target genes including, MEK, NFκB and Bcl-x_L which led to a mitochondrial pathway of apoptosis (Tiede et al., 2003).

Specifically in the case of MG patients, the beneficial effects of AZA also include the ability to reduce anti-AChR antibody levels (Mantegazza et al., 1988). Although AZA has an apoptotic effect on T-cells, the drug appears not to influence the release of cytokines from dividing or non-dividing T-cells which have not yet undergone T-cell mediated apoptosis (Ben-Horin et al., 2009). Some have suggested this may be the reason why AZA has a delayed clinical response which may take up to 15 months before steroid-sparing effects are seen.

1.8.4 Methotrexate

Methotrexate (MTX) is a potent anti-inflammatory and immunosuppressive agent which is regarded as the gold standard for rheumatoid arthritis treatment. The drug is a folic acid antagonist that is known to inhibit both purine and pyrimidine synthesis. These effects are mediated by MTX inhibiting enzymes such as dihydrofolate reductase, thymidylate synthase and 5-aminoimidazole-4-carbox-amide ribonuleotide (AICAR) transformylase which all ultimately lead to inhibition of purine and pyrimidine biosynthesis. This therefore greatly inhibits cells with high proliferation rates such as T-cells. Other molecular mechanisms that contribute to the drugs potent anti-inflammatory effects include promotion of adenosine release that results in adenosine-mediated suppression of inflammation (Cronstein, 2005).

MTX is usually administered to patients either orally or intramuscularly, and is readily absorbed by cells after which it rapidly undergoes polyglutamation (MTX_{Glu}). The MTX_{Glu} then inhibits AICAR transformylase, resulting in the accumulation of AICAR transformylase that inhibits AMP deaminase. This ultimately leads to the release of intracellular adenine nucleotides and adenosine into the extracellular matrix (Tian and Cronstein, 2007). The extracellular AMP, ADP and ATP are then dephosphorylated to adenosine (Tian and Cronstein, 2007) which is able to bind to specific adenosine receptors expressed on the cell membrane. The mechanisms by which adenosine mediates anti-inflammatory responses is still largely unknown. However, in a mouse model of rheumatoid arthritis, the anti-inflammatory effects of MTX were completely reversed when the mice were treated with the adenosine receptor antagonists, theophylline and caffeine (Montesinos et al., 2007). An additional mechanism by which MTX mediates its anti-inflammatory effects is thought to be through inhibition of NFκB. A study by Majumdar and Aggarwal (2001) in Jurkat T-cells and fibroblasts, suggested that MTX inhibits IκBα phosphorylation and degradation, which in turn, prevents NFκB activation.

The effects of MTX on purine and pyrimidine biosynthesis contribute to its potential toxicities which may include bone marrow suppression, liver toxicity and stomatitis. However, patients are generally administered folic acid that has been shown to help prevent MTX-associated toxicities. The low cost of the drug and its anti-inflammatory effects has led to its use in MG treatment in South Africa. Preliminary data suggests the drug is an effective steroid-sparing agent in MG (Heckmann et al., 2011).

1.9 Objectives and aims

The broad objective of this study was to explore the molecular mechanism(s) that govern extraocular muscle treatment-resistance in a subset of myasthenia gravis patients of African origin. This was achieved by the following specific **aims**:

1. To determine the effect(s) of prednisone, the first line immunosuppressant therapy for MG treatment, on wild-type versus C>G DAF expression.

2. To investigate the effect(s) of the steroid-sparing drugs cyclosporin A, azathioprine and methotrexate alone or in combination with prednisone on wild-type versus C>G DAF expression.

CHAPTER 2: Materials and Methods

2.1 Generation of Epstein-Barr Virus transformed B lymphocytes

Lymphoblastoid cell lines were derived from healthy non-MG patient control individuals (n=3 and expressing WT DAF) or MG patients (n=6 and expressing C>G DAF) attending the University of Cape Town MG clinic. Consent for the experimental use of the lymphocytes was obtained from the control individuals or MG patients. Lymphoblastoid cell lines were established by transforming lymphocytes from peripheral blood using Epstein-Barr virus (EBV) described below. Briefly, isolated peripheral blood was spun at 3000 x rpm using a bench top centrifuge (Centrifuge 5810R, Eppendorf, Germany) for 5 mins at room temperature (RT). The buffy coat, containing leucocytes and platelets, and serum layers was gently removed from the centrifuged blood and added to a layer of Histopaque (Sigma, USA) which enables separation of cells based on a density gradient. The mixture was spun at 3500 x rpm for 20 mins to collect the red blood cells at the bottom of the tube leaving a serum layer at the top containing the lymphocytes and platelets. The serum was carefully removed and added to a fresh tube and washed twice with culture media, containing Dulbecco's Modified Eagle's Medium (DMEM) (Appendix A) + 10% heat-inactivated foetal calf serum (FCS) (Gibco, UK) and 1% Penicillin-Streptomycin (P/S) (Appendix A), and then incubated in 5 mls of EBV containing culture media.

The EBV was obtained by culturing B95.8 cells, a marmoset blood leukocyte line that releases high titres of transforming EBV, under acidic conditions. Media was collected and transferred to a 50 ml conical tube, then spun down at 400 x g for 10 mins. The supernatant was removed and filtered through a 0.22 µm filter. The filtrate containing the EBV particles without any donor cells was mixed with an equal amount of fresh culture medium, stored at 4°C for a maximum of 6 months and was used to transform blood lymphocytes.

To obtain only B-lymphocyte cell lines, lymphocytes obtained as described above were cultured for 3 days at 37°C in EBV media containing phytohaemagglutinin (Welcome Reagents Ltd, UK), which kills contaminating T lymphocytes. Transformed B lymphoblasts were monitored twice a week and the media changed depending on the cell growth.

2.2 Genotype sequencing of lymphoblasts

The genomic DNA of healthy control or MG patients was screened to ensure lymphoblast cell lines with the correct genotype, WT decay accelerating factor (DAF) or c.-198C>G SNP (C>G SNP) DAF, was used for the duration of this study.

2.2.1 Isolation of genomic DNA from lymphoblasts

Genomic DNA was extracted from lymphoblastoid cells generated from healthy control individuals or MG patients, using the QIAamp DNA Blood Midi Kit (Qiagen, USA) as per manufacturers' instructions. Briefly, lymphoblasts were pelleted using a benchtop centrifuge (Centrifuge 5810R, Eppendorf, Germany) at 1000 x rpm for 5 mins at RT. The culture media-infused supernatant was removed and the pellet washed twice in sterile 1 x phosphate buffered saline (PBS) (Appendix A) by centrifugation at 300 x g for 5 mins. Twenty microliters of Proteinase K and 200 µl AL Buffer was added to the cells, pulse vortexed for 15 seconds, then left to incubate at 56°C for 30 mins in a heating block. Following incubation, 200 µl ethanol was added to each tube and mixed by pulse-vortexing for 15 seconds followed by brief centrifugation. The mixture was then applied to a spin column and centrifuged at 13 000 x rpm for 1 min at RT. The column was placed into a clean collection tube and 500 µl AW 1 Buffer was added to the column and centrifuged for 1 min at 13 000 x rpm at RT. Again the column was inserted into a clean collection tube, 500 µl AW 2 Buffer was added and the column centrifuged for 3 mins at 12 000 x rpm at RT. The flow through was then discarded and the column centrifuged for 1 min at 12 000 x rpm. Finally the column was inserted into a fresh 1.5ml microcentrifuge tube, 100 µl AE buffer added to the top of the column, and left to incubate at RT for 10 mins. Thereafter, the DNA was collected in the microcentrifuge tube by centrifugation at 12 000 x rpm for 1 min and stored at -20°C.

2.2.2 Measuring DNA concentration

The concentration of extracted DNA was measured using a NanoDrop ND-1000 UV-visible spectrophotometer (NanoDrop Technologies, USA). The NanoDrop ND-1000 3.3 software (Coleman Technologies Inc., USA) displays the concentration in ng/µl and an estimation of the purity of DNA is assessed by 260/280 ratio. A ratio of 1.8-2.0 is accepted as pure for DNA.

2.2.3 Conventional polymerase chain reaction (PCR)

To screen for the C>G SNP in the DAF gene, PCR was performed under the following conditions: initial denaturation at 94°C (5 mins) followed by 25 amplification cycles comprising denaturation at 94°C (30 seconds), annealing at 60°C (30 seconds), extension of 72°C (30 seconds), followed by a final extension at 72°C (7 mins) in a 2720 Thermal Cycler (Applied Biosystems, USA). The forward and reverse primers were designed by Dr Robea Ballo and were specifically designed to amplify the region of DNA in the DAF gene where the c.-198C>G SNP is situated. **Table 2.1** shows the optimised PCR reaction mixture.

Table 2.1: PCR reaction mixture for DAF gene amplification.

Reagent	Amount
Template DNA	100 ng/µl
Forward Primer (20 µM)	1 µl
Reverse Primer (20 µM)	1 µl
2 x Failsafe Buffer (Epicentre Biotechnologies, USA)	7.5 µl
GoTaq DNA polymerase (Promega, USA)	0.2 µl
Nuclease free H_2O	4.3 µl

2.2.4 Agarose gel electrophoresis

The size of the amplified PCR product was checked by electrophoresis on a 2% agarose gel (Appendix A). Once the 2% gel solution was solidified, the gel comb was removed from the casting platform and placed in the electrophoresis tank (OMEG Scientific, SA) filled with 1 x TBE buffer (Appendix A). A total volume of 8 µl of sample was loaded per well containing 5 µl of PCR product mixed with 3 µl of agarose loading dye. In this study, the loading dye was spiked with the fluorescent stain, SYBR gold (Invitrogen, USA) in the ratio 1:2000 to allow for visualisation on a UV transilluminator (Spectroline Model TR-312A Transilluminator, USA). A 100 bp molecular weight marker (Appendix C) was also loaded onto the gel.

2.2.5 Sequencing

Sequencing of PCR products was performed by Inqaba biotech (SA). The sequences obtained were confirmed manually and analysed for any changes by aligning them with the known reference sequence using the Clustal Alignment facility in BioEdit version 7.0.0 (data not shown).

2.3 Amplification of plasmid constructs

The DAF promoter region (-724 to +80) cloned as a Bgl II and Hind III fragment in the pGL3 firefly luciferase reporter vector was kindly donated by Ray DuBois (Holla et al., 2005). Using the WT DAF reporter construct as a template, site directed mutagenesis was performed to mutate the C at position -198 to G (performed by Dr Huajiang Teng, UCT).

2.3.1 Making competent cells

DH5α E.coli cells were inoculated into 5 ml of Luria Broth (LB) (Appendix A) and incubated overnight at 37°C with shaking. One millilitre of this culture was then used to inoculate 100 ml of LB and cells were grown for 2 to 3 hrs until cell growth reached the log phase (OD600nm=0.5-0.8). Cells were centrifuged at 3000 x g for 10 mins at RT (Centrifuge 5810R, Eppendorf, Germany); the bacterial pellet resuspended in 100 mM ice-cold calcium chloride ($CaCl_2$) solution and incubated on ice for 1 hr. Following centrifugation at 3000 x rpm for 1 min, the supernatant was discarded and 1 ml of ice-cold $CaCl_2$ was used to resuspend the pellet. $CaCl_2$ permeabilises the cell wall of the bacteria to allow entry of foreign DNA but this causes the cells to be very fragile and care was taken in successive processes so as not to damage the cells. Finally 100 µl of competent cells were either used immediately for DNA transformation or they were frozen in autoclaved glycerol at -80°C for long term storage.

2.3.2 Transformation

To 100 µl of competent cells, 1 µl of DNA plasmid solution was added and then incubated on ice for 10 mins. Cells were heat-shocked at 42°C for 2 mins, to allow the bacteria to take up the DNA constructs. Cells were then incubated on ice for 2 mins, after which 300 µl of antibiotic-free LB was added and the cells incubated at 37°C for 30 mins with gentle shaking. Following incubation, 50 µl of each transformation reaction was plated onto LB-Amp agar plates. Plates were incubated at 37°C with the agar side down for 20 mins, then inverted with the agar side up and left to grow overnight.

A negative control plate containing un-transformed competent cells was included. No growth should be seen on the negative control plate as only cells carrying the ampicillin resistance-containing vector DNA should survive.

2.3.3 Ampicillin agar plates

All plasmids used in this study carried the Ampicillin (Amp) resistance gene. Amp (50 µg/ml) was added to 1.5% bacterial agar (prepared in LB) which was poured into 10 cm dishes to a volume of 20 ml per dish. The agar was allowed to set at RT and then dried overnight at 37°C.

2.3.4 Large scale preparation of plasmid DNA

To screen for bacterial colonies carrying the WT or C>G DAF reporter construct, colonies were selected from the LB-Amp agar plates and used for the large-scale preparation of plasmid DNA. The bacterial colonies selected were removed with a sterile pipette tip, inoculated into 3 ml LB medium containing Amp and incubated overnight at 37°C with shaking. One millilitre of overnight culture was used to inoculate 250 ml LB-Amp broth. The PureYield Maxiprep System kit (Promega, USA), which makes use of a silica-membrane column filtration, was used following the manufacturers' instructions to isolate high quality plasmid DNA. Briefly, cells were collected by centrifugation at 5000 x g for 10 mins. The pelleted cells were resuspended in 12 mls of supplied Cell Resuspension Solution. To the above solution, 12 ml of supplied Cell Lysis Solution was added; the solution was mixed gently by inverting the tube 3-5 times and incubated at RT for 3 mins. This solution hydrolyses the bacteria and disrupts the cell membrane. The reaction was neutralised by adding Neutralisation Solution and inverted several times to completely precipitate the cell debris. Thereafter, the lysate was centrifuged at 7000 x g for 30 mins at RT in a fixed-angle rotor to pellet the bulk of the cellular debris. DNA was isolated from the lysate by being passed through a filtration column using a vacuum manifold. DNA becomes trapped on a binding membrane that is washed with an ethanol-containing Column Wash. Following this, the DNA was eluted in 200 µl of nuclease-free water. The DNA concentration was checked using the NanoDrop ND-1000 UV-visible spectrophotometer (NanoDrop Technologies, USA) together with the NanoDrop ND-1000 3.3 software (Coleman Technologies Inc., USA). The integrity of the DNA isolated was additionally checked by electrophoresis on a 2% agarose gel as previously described in section 2.2.4.

2.4 Maintenance of cells in culture

Lymphoblast (described in section 2.1), COS-7 (Green monkey epithelial kidney) and C2C12 (mouse myoblast) cells were cultured in DMEM supplemented with 10% heat inactivated FCS and 1% P/S antibiotics in a 37°C (95% air, 5% CO_2, 65% humidity). C2C12 cells were allowed to differentiate by switching from growth medium (DMEM + 10% FBS + 1% P/S) to differentiating medium (DMEM supplemented with 2% Horse serum and 1% P/S). All cultures were routinely subjected to mycoplasma tests and only mycoplasma free cells were used in experiments.

2.4.1 Mycoplasma test

Cells grown on a coverslip in antibiotic-free media for 2-3 days were fixed in a 1:3 mixture of glacial acetic acid and methanol for 5 seconds, washed briefly with water to remove the fixing solution and then air-dried at RT for 5 mins. Once dried, the DNA was stained with Hoechst 33258 (0.5 µg/ml) (Appendix C) for 6 mins, washed briefly with water to remove excess stain and then mounted on a slide with mounting fluid at pH 5.5 (Appendix A). The cells were viewed immediately by fluorescence microscopy under the DAPI filter. Mycoplasma negative cells stained positive with Hoechst 33258 only in the nucleus, while cells infected with mycoplasma showed staining in both the nucleus and cytoplasm.

2.5 Drug treatments

2.5.1 MG Drug treatments

Prednisone, methotrexate, cyclosporine A and azathioprine (all Sigma, USA) were the drugs used in this study. They were dissolved in specific reagents to stock concentrations (Appendix B). For western blotting, qRT-PCR analysis, luciferase and calcien assays cells were treated with presumed clinically relevant doses of the drugs for the indicated time durations. The doses for each drug used were: Prednisone = 0.1, 1, 3, 10, 30 and 60 µM (Ribichini et al., 2007; Wang et al., 2006); methotrexate = 10 and 22 µM (Fotoohi et al., 2004); cyclosporin A = 0.25 and 5 µM (Chen et al., 2004; Düfer et al., 2001); azathioprine = 5 µM (Bobbala et al., 2009; Petit et al., 2008).

2.5.2 Lipopolysaccharide treatment of cells

Powdered lipopolysaccharide (LPS) (Sigma, USA), was dissolved in 1x PBS to a final working concentration of 10 µg/ml (Koide and Steinman, 1987) (Appendix B). For promoter and calcien

assays, cells were treated with 10 µg/ml LPS for 12 hrs before processing of the respective assays. C2C12 cells were treated with 10 µg/ml LPS for 24 hrs while for qRT-PCR and western blot analysis performed in lymphoblasts, cells were treated with 10 µg/ml LPS for 19 hrs.

2.5.3 Serum treatment of cells

Serum was isolated from blood donated by normal healthy controls (not MG patients) or treatment-naïve MG patients (C.-198C>G SNP not confirmed in these individuals) with consent granted from the respective individuals. Briefly, coagulated blood was spun at 900 x g for 15 mins at RT using a bench top centrifuge. Under sterile conditions the supernatant sera was transferred to microcentrifuge tubes and stored at -20°C. For western blot analysis and calcein assays, cells were treated with appropriate dilutions of sera for the indicated durations.

2.6 Transient transfection

Prior to performing all transfections the quality and concentration of all DNA constructs was confirmed by agarose gel electrophoresis (data not shown).

COS-7 cells were transiently transfected with FuGENE HD (Roche, USA) according to the manufacturers' instructions. Briefly, cells were plated at 3 x 10^4/ml and grown to approximately 60% confluency in 12-well plates a day before transfection with 450 ng of either the WT or C>G DAF promoters cloned into a pGL3-basic firefly luciferase reporter vector. 45 ng of the pRL-TK vector (Promega, USA) was included in each transfection to serve as an internal control to monitor and standardize the transfection efficiency between samples. Exactly 1.5 µl of the transfection reagent diluted into 48.5 µl serum-free media (without antibiotic) was prepared at RT and allowed to stand for 5 mins. The diluted transfection reagent was added in a drop wise manner to the DNA, mixed and incubated at RT for 15 mins. Thereafter, the transfection reagent:DNA complex was added drop wise to the cells incubated for 6 hrs in a 37°C incubator. After incubation, transfection media was removed and 1 ml serum-free media (Appendix A) was added to each reaction well in the 12-well plate.

2.7 Luciferase assays

Luciferase assays were carried out using the Dual-Luciferase Reporter (DLR) Assay System (Promega, USA) according to the manufacturers' instructions. Firefly and *renilla* are two different enzymes acting on different substrates to produce a luminescent reaction. DLR enables the detection of the enzymatic activity of both enzymes within a single sample. Transfected cells were washed twice with cold 1x PBS and then lysed with 200 µl of 1x Passive Lysis Buffer by shaking at RT for 15 mins. Cell lysates were transferred into 1.5 ml centrifuge tubes and incubated at -80°C overnight. Luciferase assays were performed according to the instructions of the manufacturer and luciferase activity was measured using the Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA, USA). Briefly, the cell lysates were thawed at RT, vortexed and centrifuged (Heraeus Biofuge Pico, Germany) at 13 000 x rpm for 1 min, and 10 µl of each sample was assayed immediately for reporter gene activity. Results obtained were normalized for transfection efficiency by dividing firefly readings by the *renilla* readings. Promoter activity was calculated as a ratio of the luciferase activity generated by the internal pRL-Tk vector to that obtained in the presence of the appropriate WT or C>G DAF expression vector. The Microsoft Excel programme was used to calculate the standard deviation and statistically significant differences between samples using the paired 2-tailed Student *t* test. P values of ≤0.05 were considered statistically relevant.

2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

2.8.1. Total RNA extraction

Total RNA was extracted from lymphoblasts using the High Pure RNA Isolation Kit (Roche, USA) as per manufacturers' instructions. Diethyl pyrocarbonate (DEPC) treated microfuge tubes and pipette tips (Appendix A) were used to minimize RNA degradation during the extraction process as DEPC destroys RNases. The quality and concentration of RNA was determined by spectrophotometry and samples with an A260/A280 ratio equal to or above 1.8 were selected and stored at -80°C for further applications.

2.8.2 Reverse transcription

As RNA is easily degraded by RNAses, which are abundant in the environment, the RNA was quickly converted to complimentary DNA (cDNA) which is much more stable. The reverse transcriptase enzyme synthesizes double stranded cDNA from RNA using the reverse transcription process. The

mRNA was then transcribed into cDNA using the Improm-II Reverse Transcription System (Promega, USA) following the manufacturers' instructions. Briefly, 540 ng of RNA was combined with 1 µl of Oligo (dT) primer in a total volume of 5 µl, and then heat denatured at 70°C for 5 mins followed by incubation on ice for a further 5 mins. Thereafter, the reverse transcription mix was added (**Table 2.2**) and the mixture incubated for 5 mins at 25°C in a controlled-temperature heating block for the annealing process to take place and the cDNA strands allowed to extend at 42°C for an hour. The reverse transcriptase was then inactivated at 70°C for 15 mins and the cDNA stored at 4°C.

Table 2.2: Components of the Improm-II Reverse Transcription System Mix.

Reagent	Volume	Final Concentration
Reagent Buffer (5x)	4 µl	1x
$MgCl_2$	2.4 µl	3 mM
dNTP mix	1 µl	0.5 mM each dNTP
Recombinant RNasin Ribonuclease Inhibitor	0.5 µl	20 units
Reverse Transcriptase	1 µl	-
Nuclease free water	6.1 µl	-
Total volume	**15 µl**	**-**

2.8.3. Quantitative Real-time PCR

The Sensimix Lite kit (Quantace, UK) was used to quantify the mRNA expression levels of the target gene gDAF and the house-keeping gene, glucuronidase B (*GUS B*), following the manufacturers' instructions. The forward and reverse primers used were specifically designed by Dr Robea Ballo to amplify the membrane-bound gDAF. The forward and reverse primers were: (5'- GGATTCACCATGATTGGAGAGC- 3') and (5'- TGTGCCCAGATAGAAGAC- 3') respectively. The oligonucleotide primers for the house-keeping gene *GUS B* was obtained from Quantitect (QIAGEN, USA). Briefly, reagents were mixed in glass capillaries to a final volume of either 10 or 20 µl as shown in **Table 2.3**. The capillaries were sealed and then centrifuged at 3000 x rpm for 30 seconds. The procedure was performed in the Lightcycler machine (Roche, Switzerland) and the levels of fluorescence were quantified using the accompanying Light Cyler Software Version 3 (Roche, Germany). Cycling conditions were generally similar for the gDAF and the house-keeping gene but differed in the holding times in the cycling step (**Tables 2.4 and 2.5**). Each DNA sample was

quantified in duplicate and a negative control without cDNA template was run with every assay to assess the overall specificity.

Table 2.3: Real-time PCR parameters used for amplification of gDAF and *GUS B* genes.

Reagent	gDAF	*GUS B*
5x Sensimix Lite (dNTPs, 15 mM $MgCl_2$, stabilizers)	4 µl	2 µl
Enzyme mix (Taq DNA Polymerase)	1.5 µl	0.75 µl
50x SYBR green	0.4 µl	0.2 µl
Primers (10 pmol)	Forward: 0.4 µl Reverse: 0.4 µl	1.5 µl of combined forward and reverse primers
Sterile water	12.3 µl	4.55 µl
cDNA	1 µl	1 µl
Total volume	**20 µl**	**10 µl**

Table 2.4: Real-time PCR optimal cyclic conditions used for gDAF.

	Cycle number	Target °C	Holding time (mins)	Ramp/Slope (°C/sec)
Activation	1	95	10:00	20
Cycling **Denaturing** **Annealing** **Extension**	35	 95 55 72	 00:15 00:30 00:30	 20 20 20
Melting **Denaturing** **Annealing** **Extension**	1	 95 65 95	 00:00 00:15 00:00	 20 20 0.1
Cooling	1	40	00:30	20

Table 2.5: Real-time PCR optimal cyclic conditions used for *GUS B*.

	Cycle number	Target °C	Holding time (mins)	Ramp/Slope (°C/sec)
Activation	1	95	15:00	0
Cycling **Denaturing** **Annealing** **Extension**	35	 95 55 72	 00:05 00:03 00:05	 20 20 20
Melting **Denaturing** **Annealing** **Extension**	1	 95 65 95	 00:00 00:15 00:00	 20 20 20
Cooling	1	40	00:30	20

2.8.4 Quantification of mRNA

To quantitate differences in mRNA levels the relative quantification method was employed. The change in expression was calculated using the ΔΔCt method (Pfaffl, 2001). Relative mRNA expression levels were normalized to *GUS B* for each reaction. The Microsoft Excel programme was used to calculate the standard deviation and statistically significant differences between samples using the paired 2-tailed Student *t* test. P values of ≤0.05 were considered statistically relevant.

2.9 Western blotting

Approximately 2 x 10^6/ml lymphoblasts and 1.5 x 10^5/ml C2C12 cells cultured for protein analysis were plated in 6 cm dishes. C2C12 cells were harvested by trypsinisation (Appendix A) whilst lymphoblasts were collected directly in 12 ml tubes as they grow in suspension. The respective cell lines were pelleted by centrifugation for 5 mins at 4000 x rpm at 4°C. The cell pellets were then washed with 1 ml 1x PBS and then spun at 4°C in 1.5 ml microcentrifuge tubes for 4 mins at 12 000 x rpm. Whole cell extracts from C2C12 and lymphoblast cells were prepared under reducing and non-reducing conditions respectively using RIPA buffer (Appendix A) and stored on ice for 30 mins. After the incubation period, cell debris was pelleted by centrifugation at 4°C at 12 000 x rpm for 20 mins and the supernatant containing the cellular protein collected and stored at -80°C. Protein quantification for each cell extract was determined using the BCA Protein Assay Kit (Pierce, USA) with bovine serum albumin as the standard. Equal amounts of protein were loaded in each lane and

resolved on an 8% SDS-PAGE gel and then transferred electrophoretically to a Hybond ECL nitrocellulose membrane (Amersham, UK).

After transfer, the nitrocellulose membrane was rinsed in 1x PBS/0.1% Tween (Appendix A), and incubated in 5% milk in 1x PBS/ 0.1% Tween (Appendix A) for 1 hr at RT with shaking. The blot was incubated in the appropriate primary antibodies overnight at 4°C with shaking. The primary antibodies and appropriate dilutions were: mouse monoclonal BRIC 216 DAF (IBGRL 9404P) (NHS, UK) antibody diluted 1:100, rabbit polyclonal DAF (sc-9156) (Santa Cruz Biotechnologies, USA) antibody diluted 1:200, monoclonal mouse anti-α-tubulin (sc-51503) (Santa Cruz Biotechnologies, USA) antibody diluted 1:500. All antibodies were diluted in 5% milk in 1x PBS/0.1% Tween. Following overnight incubation in the primary antibody, the membrane was incubated with either peroxidase conjugated anti-rabbit or anti-mouse at a dilution of 1:5000 in 5% milk in 1x PBS/0.1% Tween. The proteins of interest were detected using either SuperSignal® West Dura Extended Duration substrate or West pico Chemiluminescent Substrate. For both detection solutions a total of 1 ml of the substrates A and B detection reagents (ratio 1:1) were added to the membrane for 1 min before the membrane was sandwiched between acetate sheets in a cassette and exposed to X-ray film. The film was immersed in developer until bands became visible, rinsed under running water and immersed in fixing solution for a minimum of 4 mins. The film was rinsed in running water and allowed to air-dry.

2.9.1 Stripping of nitrocellulose membrane

Membranes probed for more than one protein were immersed in pre-heated (at 50°C) stripping buffer (Appendix A) for 30 mins. This was followed by 2 × 10 mins and 2 × 5 mins washes in 1x PBS/0.1% Tween, after which standard western blotting procedure followed.

2.10 Calcein assay

Calcein-AM is a non-fluorescent compound that easily penetrates intact cellular membranes. Once in the cytoplasm it is cleaved by cytosolic esterases into the strongly fluorescing compound calcein. The degree of fluorescence emitted by calcein can be quantified and used as a direct measure of cell viability. In this study, lymphoblasts were plated in triplicate in 96-well plates at a concentration of 1 x 10^3/200 µl cells per well and treated with control or treatment-naïve MG patient sera for 24 hrs with or without prednisone or LPS treatment for 24 or 12 hrs respectively. After treatments the assay was processed as per the manufacturers' instructions (Trevigen Inc, USA) following the suspension cell protocol. Briefly, lymphoblasts were pelleted for 5 mins at 250 x g using a bench top centrifuge with appropriate 96-well plate adaptors (Centrifuge 5810R, Eppendorf, Germany) and the supernatant removed. The cell pellets were washed twice with 1x calcein buffer (Appendix A) and then spun at 250 x g for 5 mins. After the last wash, cells were resuspended in 1x calcein buffer and 2 µM Calcein-AM (Appendix A) at a 1:1 ratio then transferred to black-walled 96-well plates as Calcein-AM is sensitive to light. The plates were covered in foil then placed in an incubator for 30 mins at 37°C to allow for uptake and cleavage of Calcein AM. Each assay was performed in triplicate and the following three controls were included; background control was subtracted from all measurements, a low lysis control was untreated normal cells and the high lysis control represented maximum calcein release after 40% triton X-100 (Sigma, USA). The results are presented as specific calcein release as measure of % cytotoxicity= (test sample-low control)/(high control–low control) x100.

Cell lysis was determined by inserting the 96 black-walled plates into the GloMax®- Multi Microplate Multimode Reader (Promega, USA) which was used to measure calcein fluorescence using a blue laser with a 490 nm excitation filter and a 520 nm emission filter. The fluorescence generated was used as a direct measure of cell viability.

Chapter 3: Results

We have previously identified myasthenia gravis (MG) patients of African or recent African ancestry that have an increased risk of developing a treatment-resistant severe extraocular muscle phenotype at Groote Schuur Hospital, Cape Town (South Africa). Furthermore, data generated in our laboratory showed (1) a significant association between the above myasthenics and a functional C>G SNP in the regulatory region of decay accelerating factor (DAF) and (2) that this C>G SNP prevented DAF upregulation in response to lipopolysaccharide (LPS)-induced immune stress. On the basis of these observations we hypothesized that those MG patients with the C>G SNP are treatment-resistant because they have an inadequate DAF response which may be aggravated by standard MG drug therapies. Therefore, the first aim of this study was to determine the effect of prednisone on wild-type (WT) and C>G DAF expression. Prednisone was selected because in clinical practice it is the first-line drug used for MG treatment. Due to the side effects of prednisone, additional immunosuppressive drugs are administered as steroid-sparing agents for long term control of MG. The second aim of this study was therefore to determine the effect(s) of these steroid-sparing drugs alone or in combination with prednisone on WT and C>G DAF expression.

3.1 Effect of prednisone on WT versus C>G DAF expression

3.1.1 Prednisone post-transcriptionally represses C>G DAF and not WT DAF

Wild-type (WT) and C>G DAF[1] lymphoblasts were treated with 0.1, 1, 3 or 60 µM doses of prednisone for 24 hrs or with LPS (10 µg/ml) for 19 hrs. The various doses of prednisone were chosen based on similar concentrations used in published studies (Ribichini et al., 2007; Wang et al., 2006). Total RNA was isolated, reverse transcribed into cDNA and DAF mRNA expression quantified by qRT-PCR. Levels of DAF mRNA were determined using the standard curve and relative quantification method (Wong and Medrano, 2005). **Table 3.1** shows the average fold activation, compared to untreated control cells, from three experiments each performed in duplicate. As shown previously by our group in response to LPS-induced immune stress, WT DAF mRNA levels increased by almost 2-fold, whereas C>G DAF mRNA levels were repressed by 1.4-fold compared to their untreated controls (Heckmann et al., 2010). Both WT and C>G DAF mRNA levels remained

[1] Nomenclature used to describe human decay-accelerating factor

basal in response to 0.1 or 3 µM prednisone and increased, although not significantly, after 1 or 60 µM prednisone treatment. Taken together these results suggest that at an mRNA level, in response to a range of prednisone doses, the C>G SNP does not induce a differential regulatory effect when compared to WT DAF mRNA expression. Furthermore, prednisone treatment, irrespective of WT or C>G DAF, does not appear to have a regulatory effect on DAF mRNA expression when compared to the respective untreated control cells as the results were not significantly different.

To confirm that prednisone does not regulate DAF mRNA levels at a transcriptional level, luciferase reporter assays were performed. Here, COS-7 cells were transfected with 900 ng of the firefly reporter driven by either the WT or C>G DAF promoters. COS-7 cells were used in these assays as they readily take up foreign DNA. The *renilla* luciferase construct was included in all experiments as an internal control for measuring transfection efficiency. Cells were cultured post-transfection for 24 hrs and treated with LPS (10 µg/ml) for 12 hrs or 0.1, 1, 3 or 60 µM prednisone for 24 hrs before extracts were assayed for firefly and *renilla* luciferase activity using the dual-luciferase assay system. The result of pooled data from two or three transfection experiments each performed in duplicate is shown in **Table 3.1**. The treatment of LPS had no effect on WT or C>G DAF promoter activity; a previous study also found that COS-7 cells do not respond to LPS treatment (Veal et al., 2004). Both the 0.1 and 1 µM doses of prednisone had no significant effect on either the WT or C>G DAF promoter activity compared to their untreated controls. However, unlike C>G DAF promoter activity which remained basal, WT DAF promoter activity significantly increased by approximately 1.3-fold in response to 3 or 60 µM doses of prednisone. The results do not match those achieved at an mRNA level and this could reflect differences in the regulation of the endogenous gene compared to the *in vitro* promoter system in response to the drug. Furthermore, there may be differences in the cell-type specific responses to the drug. Overall, these data suggest that the higher 3 and 60 µM doses of prednisone differentially regulates WT and C>G DAF promoter activity.

The lack of effect of prednisone on WT and C>G DAF mRNA levels seen above may not necessarily reflect protein levels which can also be regulated post-translationally. Therefore, we next investigated the effect of prednisone on endogenous DAF protein levels in both WT and C>G DAF lymphoblasts. Protein was extracted from the respective lymphoblasts 24 hrs post-treatment with 0.1, 1, 3 or 60 µM doses of prednisone, or with LPS (10 µg/ml) for 19 hrs, and subjected to western blot analysis using the BRIC 216 monoclonal antibody (IBGRL, UK) to human DAF as well as an antibody to tubulin which was used as a loading control. Unfortunately, due to technical difficulties

experienced with the human BRIC 216 antibody the results obtained were not always clear. Examples of two western blots displaying the technical difficulties experienced with the human DAF antibody can be found in Appendix D. As a result, for a few experiments only one result was obtained. The data in **Table 3.1** represents the average fold activation of two or three western blots based on densitometric readings, first normalized to tubulin expression and then to the respective untreated control cells and **Figure 3.1** is a representative blot. As expected, LPS treatment increases (2.6-fold) WT DAF protein levels while repressing (3.3-fold) C>G DAF protein expression. In response to a range of prednisone doses (0.1, 1 and 3 µM doses), WT DAF protein expression did not fluctuate significantly although it did increase by 1.5-fold at a 60 µM dose, albeit not significant. Conversely, C>G DAF protein levels were repressed by 2.5-fold in response to 0.1, 1 and 3 µM prednisone treatment while the 60 µM dose showed marginal repression (1.2-fold). Taken together, these data suggest prednisone doses of 3 µM and below greatly repress C>G DAF protein expression in lymphoblasts unlike that observed for WT DAF.

Table 3.1: The effect of prednisone on WT and C>G DAF mRNA and protein expression, and DAF promoter activity.

	LPS	Prednisone			
	10 µg/ml	**0.1 µM**	**1.0 µM**	**3 µM**	**60 µM**
A) Lymphoblast DAF mRNA levels					
WT	1.98 ± 0.07*	1.00 ± 0.04	1.33 ± 0.06	1.14 ± 0.13	1.70 ± 0.02
C>G	0.69 ± 0.04*	1.07 ± 0.03	1.72 ± 0.06	0.88 ± 0.04	1.24 ± 0.06
B) COS-7 DAF promoter activity					
WT	1.16 ± 0.01	0.90 ± 0.04	0.87 ± 0.02	1.30 ± 0.03*	1.35 ± 0.03*
C>G	1.07 ± 0.11	0.80 ± 0.33	0.90 ± 0.22	1.02 ± 0.07$^{\#}$	1.06 ± 0.07$^{\#}$
C) Lymphoblast DAF protein expression					
WT	2.6*†	1.2	0.8	1.2$^{\#}$	1.5
C>G	0.3*†	0.4	0.4	0.4$^{\#}$	0.8

(A) Total RNA was extracted from WT and C>G DAF lymphoblasts treated with 0.1, 1, 3 or 60 µM prednisone or LPS (10 µg/ml) for 24 and 19 hrs respectively. Relative fold expression was normalized to the house-keeping gene *GUS B.* The results shown are of data pooled from three independent experiments each performed in duplicate and represent average fold activation compared to untreated control cells ± standard error of the mean (SEM). **(B)** The WT and C>G DAF luciferase reporter constructs were transiently transfected into COS-7 cells and treated with 0.1, 1, 3 or 60 µM prednisone or LPS (10 µg/ml) for 24 and 12 hrs respectively thereafter promoter activity was determined. The result is representative of the average fold activation ±SEM of two ($^{\#}$) or three independent experiments in which each construct was tested in duplicate. **(C)** Total protein lysate was isolated from lymphoblasts 24 hrs post-treatment with 0.1, 1, 3 or 60 µM prednisone or 19 hrs post-treatment with LPS (10 µg/ml) and analysed on an 8% SDS-PAGE by western blotting using an antibody to DAF as well as an antibody to tubulin which was included as a loading control. The results are representative of the average densitometric readings of two ($^{\#}$) or three western blots. All densitometric reading results were first normalized to tubulin expression and then to the untreated control cells. (*) indicates significance at p≤0.05 between treated and untreated cells, (†) indicates significance between WT and C>G DAF at p≤0.05.

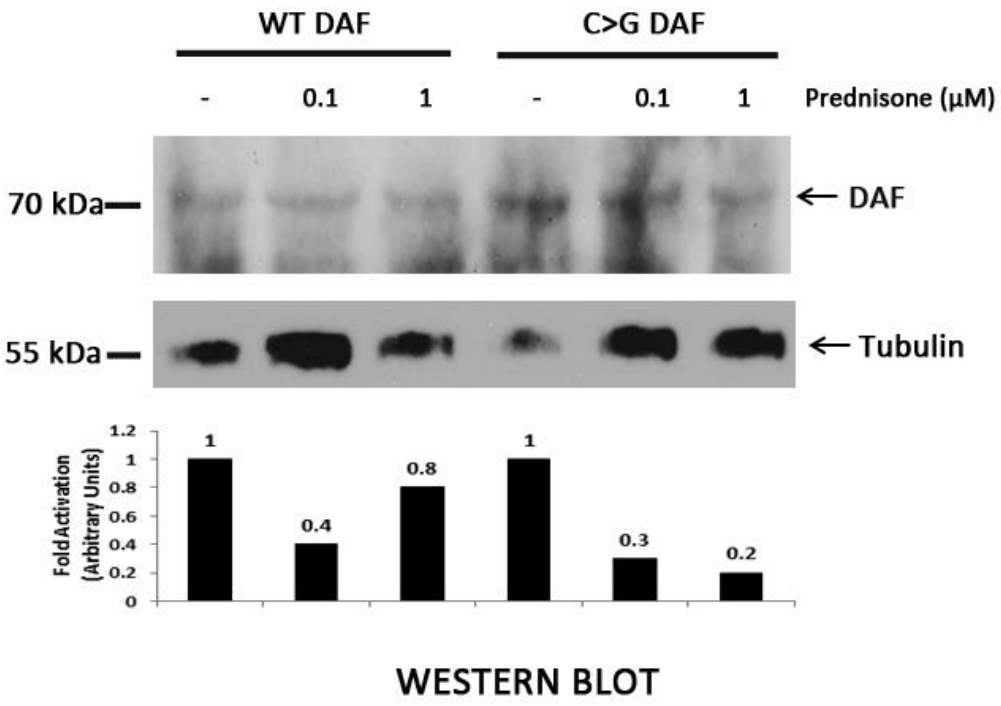


Figure 3.1: Prednisone is more repressive towards C>G DAF protein expression compared to WT DAF. Total protein lysate was isolated from lymphoblasts 24 hrs post-treatment with 0.1 and 1 μM of prednisone. Total protein from each sample was analysed on an 8% SDS-PAGE and assessed by western blotting using antibody to DAF as well as an antibody to tubulin which served as a loading control. Arrows indicate the position of the proteins of interest as determined by the protein marker banding pattern shown on the left in kilodaltons (kDa).

3.1.2 Cytotoxicity of LPS, sera and prednisone on WT and C>G DAF lymphoblasts

Based on our hypothesis, we speculated that MG patients with the C>G SNP were more susceptible to complement-mediated lysis due to an inadequate DAF response which may be exacerbated in the presence of MG drugs (e.g. prednisone) and an immune stress. Therefore, to investigate the effect of the SNP on DAF's ability to protect against complement-mediated lysis, calcein assays were performed in WT and C>G DAF lymphoblasts. The assay is based on the principle that the highly lipophilic non-fluorescent compound calcein-AM readily permeates live intact cell membranes. Once in the cell it is hydrolysed by esterases to form the strongly fluorescing molecule, calcein and hence fluorescence generated is used as a direct measure of cell viability.

We assessed the lytic response of lymphoblasts expressing WT and C>G DAF in response to treatment with either LPS (10 µg/ml), 0.1 or 1 µM prednisone, MG treatment-naïve patient sera (hereafter referred to as MG sera) or control sera (derived from healthy individuals) alone or combinations of sera and prednisone treatment. It is important to note that the DAF genotypes of the sera used from the MG patients and control individuals is unknown. The MG sera was taken from newly diagnosed patients who had the presence of the ocular phenotype, while the control sera was derived from healthy individuals who did not have MG. The cytotoxicity effect of the respective sera on WT and C>G DAF lymphoblasts was investigated because MG auto-antibodies and activated complement are essential for disease pathogenesis. It is important to note that in control sera no pathogenic auto-antibodies or significant levels of activated complement are expected to be present. The assay was performed as described in section 2.10 and three controls were included; background control was subtracted from all measurements, a low lysis control was untreated normal cells and the high lysis control represented maximum calcein release after 40% triton X-100. The results are presented as specific calcein release as measure of % cytotoxicity= (test sample-low control)/(high control–low control) x100. The results shown in **Figure 3.2** are representative of pooled data from two independent experiments each performed in triplicate. In response to 12 hrs of LPS treatment the C>G DAF lymphoblasts showed a trend to greater cell lysis (62%) compared to lymphoblasts with WT DAF (32%) ($p<0.1$), although this was not significant. This supports the hypothesis of this study that LPS results in lower DAF expression in the C>G SNP lymphoblasts thereby increasing the susceptibility of the lymphoblasts to complement-mediated lysis.

To determine the effect of control and MG sera on the lytic susceptibility of WT versus C>G DAF lymphoblasts, cells were treated with a range of dilutions (2.5, 5 and 10%) of either sera for 3 hrs. In response to the most diluted concentration of control sera (2.5%), WT DAF lymphoblasts exhibited a greater cytotoxic response (33%) compared to C>G DAF lymphoblasts (9%). This may be due to C>G DAF lymphoblasts expressing higher (up to 8-fold more) basal levels of DAF protein compared to WT (Heckmann et al., 2010) thereby providing the C>G DAF lymphoblasts with increased protection against "sublytic levels" of complement normally present in control sera (Lang et al., 1997). Various control sera at higher dilutions (5-10%) proved more cytotoxic compared to 2.5% sera, but equally lytic to both WT and C>G DAF lymphoblasts (approximately 39%). In contrast, MG sera even at the lowest dilution of 2.5% displayed similar cytotoxicity of approximately 60% for both cell lines. The observation that 2.5% MG sera showed similar cytotoxicity to both WT and C>G DAF lymphoblasts is probably due to the presence of factor(s), such as activated complement, in the serum that increase the susceptibility of the cells to complement-mediated lysis. Ideally, an even lower dose (1%) should have been used to assess whether it had a differential cytotoxic effect.

The cytotoxicity of prednisone treatment on WT versus C>G DAF lymphoblasts was next determined by treating the cells for 24 hrs with 0.1, 1 or 3 µM prednisone. A low dose (0.1 µM) of prednisone induced very little cell lysis in both the WT and C>G DAF lymphoblasts (approximately 8.5%). However, 50% of the C>G DAF lymphoblasts lysed in response to the higher doses of prednisone compared to 16% WT DAF control lymphoblasts (pooled data for 1-3 µM shown is representative of 1 and 3 µM independently). Although this difference was not statistically significant, these results suggest that the C>G SNP increases the risk of cell lysis in response to higher doses of prednisone.

Due to the increased cytotoxicity of MG sera on lymphoblasts, the combined effect of prednisone and MG sera was next investigated on the lytic response of WT versus C>G DAF lymphoblasts. In response to 0.1 µM prednisone and 2.5% MG sera, almost 50% of the WT DAF lymphoblasts lysed and up to 70% of the C>G DAF lymphoblasts underwent cell lysis. Strikingly, the combination of MG sera and a higher dose of 3 µM prednisone resulted in cell lysis of 82% of C>G DAF lymphoblasts compared to only 9% of WT DAF lymphoblasts ($p<0.05$). Taken together, these data suggest that the combination of MG sera and prednisone reduces DAF protein expression in C>G DAF lymphoblasts thereby increasing their susceptibility to complement-mediated lysis. In contrast, the WT DAF lymphoblasts exposed to MG sera and prednisone, showed significantly less cell lysis.

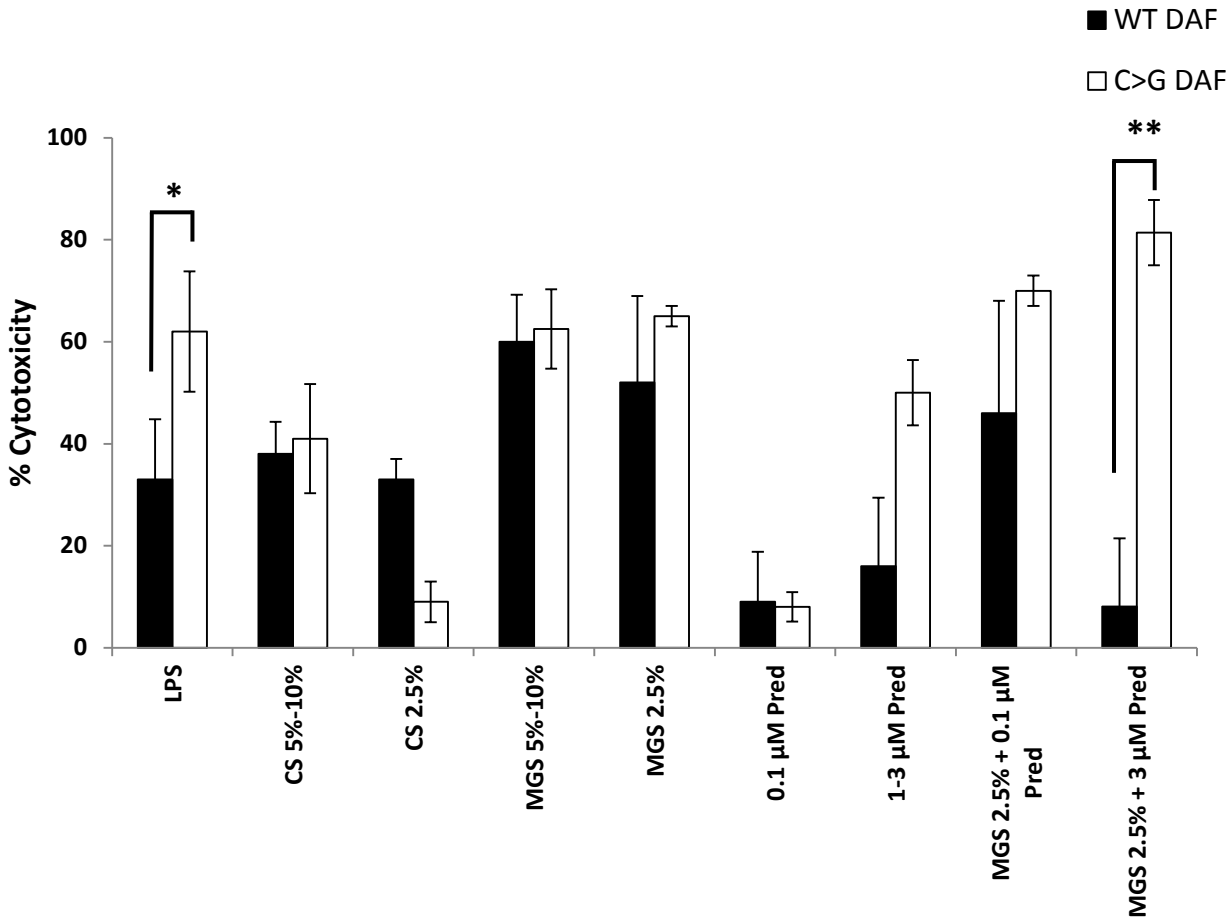


Figure 3.2: Cytotoxicity of prednisone on C>G DAF lymphoblasts is significantly enhanced in the presence of MG sera compared to lymphoblasts expressing WT DAF. Calcein assays were performed on WT or C>G DAF lymphoblasts treated alone or in combination with LPS (10 µg/ml) for 12 hrs, control sera (CS) (2.5%-10%) or MG sera (MGS) (2.5%-10%) for 3 hrs and prednisone (0.1, 1 or 3 µM) for 24 hrs. To obtain % cytotoxicity, fluorescence obtained after exposure of cells to Calcein-AM for 30 mins was normalized to the positive control, 40% Triton-X100 (20 mins treatment). The result shown is data pooled from two independent experiments performed in triplicate. Error bars represent standard error of the mean. (*) indicates a trend with $p<0.1$ and (**) indicates significance $p<0.05$.

3.1.3 Effect of prednisone and LPS on WT Daf in skeletal muscle cells

As mentioned previously, a subset of MG patients develop a severe paralysis affecting the extraocular muscles, which is treatment-resistant. A more relevant approach would thus have been to study the effect of prednisone on muscle cells. However, extraocular muscle cell lines expressing either WT or C>G DAF were not available and therefore we opted to test the effect of current MG drugs on C2C12 skeletal myoblasts expressing WT Daf[2]. These myoblasts were differentiated over a period of 9 days into myotubes by changing the growth medium to differentiation medium supplemented with 2% horse serum (**Figure 3.3**). Importantly, while we did not have access to a C>G DAF muscle cell line this study was considered relevant because to date there is no published data showing the effect of either prednisone or LPS on WT DAF expression in skeletal muscle. Six day differentiated C2C12 myotubes were treated for up to 72 hrs with a range of prednisone doses (0.1, 1, 3, 10 and 30 µM). Thereafter, protein was harvested and analysed by western blotting using an antibody to mouse polyclonal Daf (sc-9156) or an antibody to tubulin which was used as a loading control. The data in **Table 3.2** is representative of the average fold activation of two or three western blots based on densitometric readings first normalized to tubulin expression and then to the respective untreated cells. The results show that under all conditions tested WT Daf protein is repressed in response to prednisone in the differentiated C2C12 myotubes when compared to the untreated control cells. A representative blot is shown in **Figure 3.4** and includes prednisone treatments of 0.1, 1, 3 and 10 µM.

[2] Nomenclature used to describe mouse decay-accelerating factor

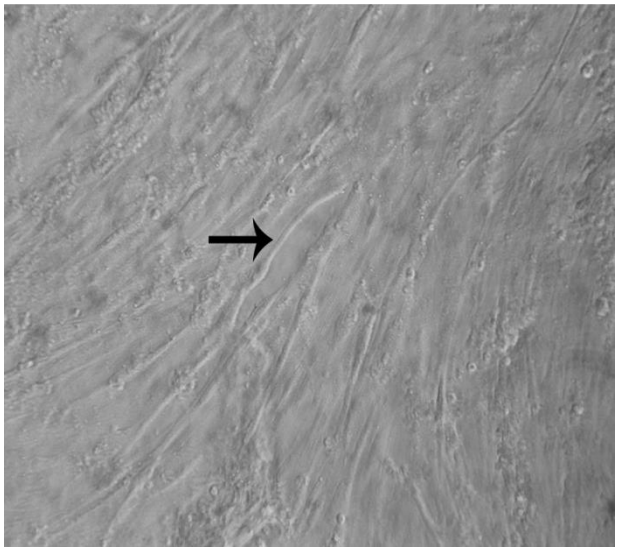


Figure 3.3: Image of myoblasts differentiated into myotubes (40X). C2C12 myoblasts were differentiated for six days into myotubes by changing the growth medium (DMEM + 10% FCS + 1% P/S) to differentiation medium (DMEM + 2% Horse Serum + 1% P/S) and incubating them at 37°C. After six days the spindle-shaped myoblast-like cells started to fuse together and form the long thin muscle fibres (shown by the arrow).

Table 3.2: Effect of prednisone, LPS and control or MG sera treatment on WT Daf protein levels in C2C12 muscle cells.

	No Prednisone	0.1 µM Prednisone	1 µM Prednisone	3 µM Prednisone	10-30 µM Prednisone
C2C12 cells	1.0	0.4	0.2	0.2	0.5[#]
+ LPS (10 µg/ml)	1.6	1.2	-	0.2	0.5[#]
+ 10% Control Sera	0.8	0.8	1.0	0.7*[#]	-
+ 10% MG sera	1.1	0.2	0.3	0.5*[#]	-

Six day differentiated C2C12 cells were treated with 0.1, 1, 3, 10 or 30 µM prednisone in the absence or presence of either LPS for 24 hours or 10% Control or 10% MG sera for 24 hrs. Thirty micrograms of total protein was separated on an 8% SDS-PAGE and analysed by western blotting using antibodies to Daf and tubulin. The results are representative of the mean of the densitometric readings of two ([#]) or three western blots. All densitometric reading results were first normalized for tubulin expression and then to the untreated control cells. (*) indicates significance at p≤0.05.

The effect of prednisone on WT Daf protein levels under conditions of immune stress was next investigated. Here, 6 day differentiated C2C12 cells were treated with prednisone for a total of 72 hrs in the presence or absence of LPS during the last 24 hrs of treatment, thereafter the protein was harvested and analysed by western blotting as described above. As shown in **Table 3.2**, LPS on its own increased WT Daf protein expression much like its effect in WT DAF lymphoblasts (**Table 3.1**). When LPS was combined with 3, 10 and 30 µM doses of prednisone the repressive effect seen for prednisone treatment-alone was observed. Interestingly, WT Daf levels remained stable (1.2-fold) after a low dose (0.1 µM) of prednisone treatment combined with LPS stimulation suggesting, at least in mouse muscle cells, lower doses of prednisone treatment maintain WT Daf at basal levels under conditions of immune stress.

Overall, prednisone doses ranging from 0.1 to 30 µM had a repressive effect on WT Daf protein expression in mouse muscle cells. Under conditions of immune stress such as those mimicked by LPS, higher prednisone doses of 3 µM to 30 µM also resulted in severe WT Daf repression.

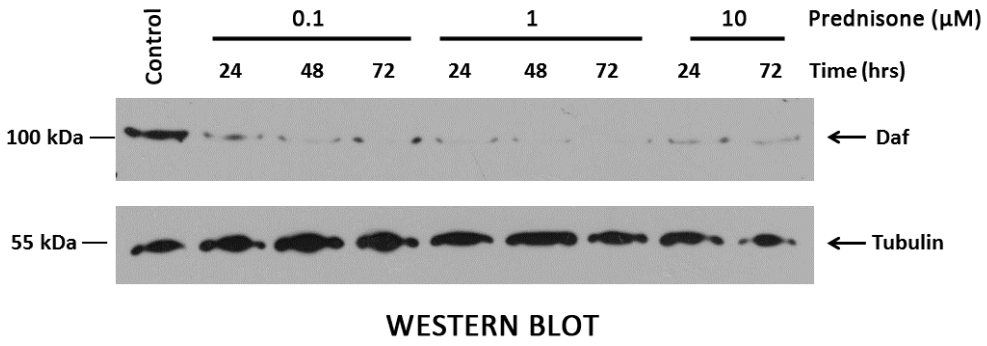


Figure 3.4: Prednisone downregulates WT Daf in mouse skeletal muscle cells. Six day differentiated C2C12 myotubes were treated with 0.1, 1 or 10 µM prednisone for up to 72 hrs after which cell lysates (30 µg) were separated on an 8% SDS-PAGE and analysed by western blotting using antibodies to Daf and tubulin. The arrows indicate the position of the proteins of interest as determined by the protein marker banding pattern shown on the left in kilodaltons (kDa).

3.1.4 MG treatment-naïve patient sera is unable to overcome the repressive effect of prednisone on WT Daf protein levels in muscle cells

Since auto-antibodies and activated complement are essential in the pathogenesis of MG, we next looked at the effect that active treatment-naïve MG patient sera (MG Sera) had on WT Daf protein expression in differentiated C2C12 myoblast cells. Briefly, C2C12 cells were differentiated for 6 days to form myotubes and then treated with prednisone (0.1, 1 or 3 µM) for a total of 72 hrs in the presence or absence of either 10% control sera (from healthy individuals) or 10% MG sera for 24 hrs. C2C12 cells cultured under standard differentiation conditions i.e. with differentiation medium were included as a control. The results in **Table 3.2** show the average fold change compared to untreated control cells from two or three western blots and a representative blot is shown in **Figure 3.5**. WT Daf protein levels were reduced by 1.3-fold in response to control sera alone while in combination with 3 µM prednisone a significant repression of 1.4-fold ($p \leq 0.05$) was observed. However, lower doses of prednisone combined with control sera either did not show significant repression or remained basal. In contrast, MG sera treatment alone had no effect on WT Daf protein expression but interestingly in combination with all doses of prednisone tested WT Daf expression was greatly repressed (3 µM prednisone, $p \leq 0.05$).

Taken together, the above data suggests that the composition of control and MG sera are different and that factor(s) in the control sera has/have the ability to reduce the repressive effect of prednisone on WT Daf expression in mouse skeletal muscle cells.

It is important to note that in these experiments the mouse polyclonal Daf antibody (sc-9156) gave clear and reproducible bands (**Figure 3.5**). Sometimes multiple banding patterns were achieved and in such cases all bands were analysed in the 70 to 100 kDa range, as specified in the data sheet accompanying the Daf antibody, and used as the signature for WT Daf protein expression in the C2C12 myoblasts. Unfortunately due to technical difficulties and inconsistent results experienced with the calcein assay in C2C12 cells, we were unable to assess the effect of prednisone and control or MG sera on the lytic response of these cells.

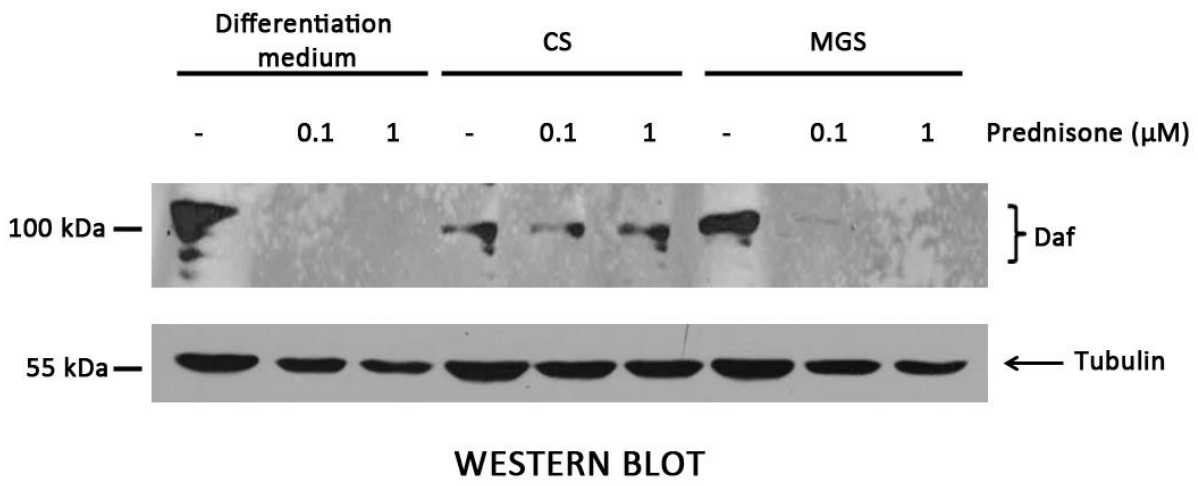


Figure 3.5: Control sera, unlike MG sera, overcomes the repressive effect of prednisone on WT Daf protein in mouse skeletal muscle cells. Six day differentiated C2C12 myotubes were treated with 0.1 or 1 µM prednisone for a total of 72 hrs in the absence or presence of 10% control sera (CS) or 10% MG sera (MGS) for 24 hrs. Cells cultured under standard differentiation conditions where included as a control. Thirty micrograms of cell lysate was separated on an 8% SDS-PAGE and analysed by western blotting using antibodies to Daf and tubulin. The arrow and bracket indicate the position of the proteins of interest as determined by the protein marker banding pattern shown on the left in kilodaltons (kDa).

3.2 Effect of immunosuppressive steroid-sparing drugs alone or in combination with prednisone on DAF expression

As previously mentioned, prednisone often results in side effects which become intolerable in the long-term. Immunosuppressive steroid-sparing drugs are thus commonly administered to patients to allow for a gradual decrease in this prednisone induced toxicity while still maintaining MG immunosuppressive treatment. The second aim of this study was therefore to determine the effect(s) of current MG immunosuppressive steroid-sparing drugs, cyclosporin A, azathioprine and methotrexate on WT and C>G DAF expression alone or in combination with prednisone.

3.2.1 Cyclosporin A post-transcriptionally represses both WT and C>G DAF expression

To determine the effect of cyclosporin A (CsA) on WT and C>G DAF mRNA levels, lymphoblasts were treated with 0.25 or 5 µM CsA for a total of 48 hrs with or without LPS (10 µg/ml) for 19 hrs. Total RNA was isolated, reverse transcribed into cDNA and DAF mRNA levels quantified by qRT-PCR. The doses of CsA were selected based on similar concentrations used in published studies (Chen et al., 2004; Düfer et al., 2001). **Table 3.3** shows the average fold change, compared to untreated control cells, from three independent experiments each performed in duplicate. In response to 0.25 and 5 µM CsA, WT DAF mRNA levels were slightly increased by approximately 1.3-fold, while C>G DAF mRNA levels showed no significant change and remained basal. While not statistically significant, the co-treatment of 0.25 µM CsA and LPS increased WT DAF mRNA expression (1.4-fold) but repressed C>G DAF mRNA levels (1.3-fold). The higher, 5 µM CsA dose in combination with LPS further repressed C>G DAF mRNA expression to 1.5-fold while WT DAF mRNA levels remained basal. Overall, the results suggest that the treatment of WT and C>G DAF lymphoblasts with either 0.25 or 5 µM CsA, in the presence or absence of LPS, does not significantly influence DAF mRNA activity.

To determine whether the effect of CsA on DAF mRNA levels is not transcriptional, luciferase reporter assays were performed. COS-7 cells transiently transfected with either the WT or C>G DAF promoter constructs were treated with 0.25 or 5 µM CsA for 24 hrs and extracts harvested 48 hrs post-transfection for firefly and *renilla* luciferase activity. The results of pooled data representing the average fold change from three independent experiments each performed in duplicate are shown in **Table 3.4.** The lower 0.25 µM CsA dose had no effect on WT or C>G promoter activity.

Although not significant, WT DAF promoter levels increased by 1.2-fold in response to 5 µM CsA treatment while C>G DAF promoter activity remained basal. The mRNA data above matched the trend seen at the promoter level as no significance was observed after CsA treatment. Therefore, CsA appears to have little regulatory effect on WT and C>G DAF mRNA levels and promoter activity.

The effect of CsA on endogenous DAF protein levels was next investigated. Here, lymphoblasts expressing either WT or C>G DAF were treated with 0.25 or 5 µM CsA for 48 hrs in the absence or presence of LPS (10 µg/ml) for 19 hrs. Protein was extracted from the lymphoblasts and subjected to western blot analysis using antibodies to either DAF or tubulin (loading control). The data in **Table 3.3** represents the average fold change of two independent western blots (unless specified) based on densitometric readings, first normalized to tubulin expression and then to the respective untreated control cells. **Figure 3.6 (A)** shows a representative western blot. The results suggest that WT DAF protein levels are repressed by 1.7-fold and 1.3-fold in response to both 0.25 and 5 µM CsA respectively, while C>G DAF protein levels are significantly repressed by 1.4-fold ($p \leq 0.05$) in response to either dose of CsA. In the presence of LPS, 0.25 µM CsA continues to repress both WT and C>G DAF protein expression by 1.4-fold and 2-fold respectively. Similarly, the co-treatment of 5 µM CsA and LPS repressed WT DAF protein levels by 1.4-fold, whereas the same treatment combination increased C>G DAF protein expression 1.5-fold. However, only one western blot showing the effect of the co-treatment of CsA and LPS on WT and C>G DAF protein expression was obtained; therefore, this experiment will need to be repeated to confirm the above observations.

Taken together, the data shows that in response to CsA the DAF mRNA and protein levels do not match suggesting that CsA has a post-transcriptional effect on DAF expression. Apart from the co-treatment with 5 µM CsA and LPS which increased C>G DAF protein levels, WT and C>G DAF protein expression was consistently repressed in response to CSA, with or without LPS. This further suggests that, compared to WT DAF, the C>G SNP does not result in a differential regulatory effect on DAF expression in response to CsA treatment.

Table 3.3: The effect of MG drugs on lymphoblast (WT and C>G) DAF mRNA and protein expression.

			(A) mRNA		(B) Protein	
Drug	**Dose (µM)**	**Time (hrs)**	**WT**	**C>G**	**WT**	**C>G**
Cyclosporin A	0.25	48	1.32 ± 0.20	0.92 ± 0.01	0.6	0.7*
	0.25 + LPS	48	1.43 ± 0.05	0.79 ± 0.09	0.7[a]	0.5[a]
	5	48	1.25 ± 0.26	0.90 ± 0.04	0.8	0.7*
	5 + LPS	48	1.04 ± 0.07	0.66 ± 0.02	0.7[a]	1.5[a]
Azathioprine	5	24	1.60 ± 0.01*	1.02 ± 0.01	3.3†	0.4†
	5 + LPS	24	1.40 ± 0.03*	0.92 ± 0.01	1.7	0.8
Methotrexate	10	24	0.89 ± 0.09	0.87 ± 0.10	0.9	1.4
	10 + LPS	24	1.37 ± 0.02	0.89 ± 0.04	2.4	1.0
	22	24	0.94 ± 0.06	0.84 ± 0.04	0.9	1.8*
	22 + LPS	24	0.90 ± 0.03	0.80 ± 0.10	1.6	1.2

Total RNA or protein was extracted from WT and C>G DAF lymphoblasts treated with cyclosporin A, azathioprine or methotrexate for the specified hrs in the absence or presence of LPS (10 µg/ml) for 19 hrs. **(A)** WT and C>G DAF mRNA levels. All results were normalized to the house-keeping gene *GUS B* and the data shown is the average fold change, compared to the untreated control cells, of three independent experiments each performed in duplicate. Error bars represent standard error of the mean. **(B)** WT and C>G DAF protein levels. Total protein lysate (40 µg) was analysed on an 8% SDS-PAGE by western blotting using an antibody to DAF as well as an antibody to tubulin which was included as a loading control. Unless indicated, the data shown is the average fold change obtained from densitometric readings from at least two independent western blot experiments. Fold change was calculated by first normalizing densitometric readings obtained for DAF levels to tubulin levels and then to their respective untreated control cells. (*) indicates significance at p≤0.05 between treated and untreated control cells, (†) indicates significance between WT and C>G DAF at p≤0.05, ([a]) indicates the results are representative of one experiment.

Table 3.4: The effect of steroid-sparing drugs on WT and C>G DAF promoter activity in COS-7 cells.

DAF promoter activity			
	Dose	**WT**	**C>G**
Cyclosporin A	**0.25 µM**	0.94 ± 0.03	0.90 ± 0.05
	5 µM	1.24 ± 0.04	1.01 ± 0.05
Azathioprine	**5 µM**	0.82 ± 0.02	0.87 ± 0.05
Methotrexate	**10 µM**	1.99 ± 0.17*	2.16 ± 0.10*
	22 µM	2.03 ± 0.15*	2.16 ± 0.06*

The WT and C>G DAF luciferase reporter constructs were transiently transfected into COS-7 cells and treated 24 hrs post-transfection with the indicated doses of either cyclosporin A, azathioprine or methotrexate 24 hrs after which promoter activity was determined. The result shown is the average fold change, compared to untreated control cells, from three independent experiments each performed in duplicate. Error bars represent standard error of the mean. (*) indicates significance at $p \leq 0.05$ between treated and untreated control cells.

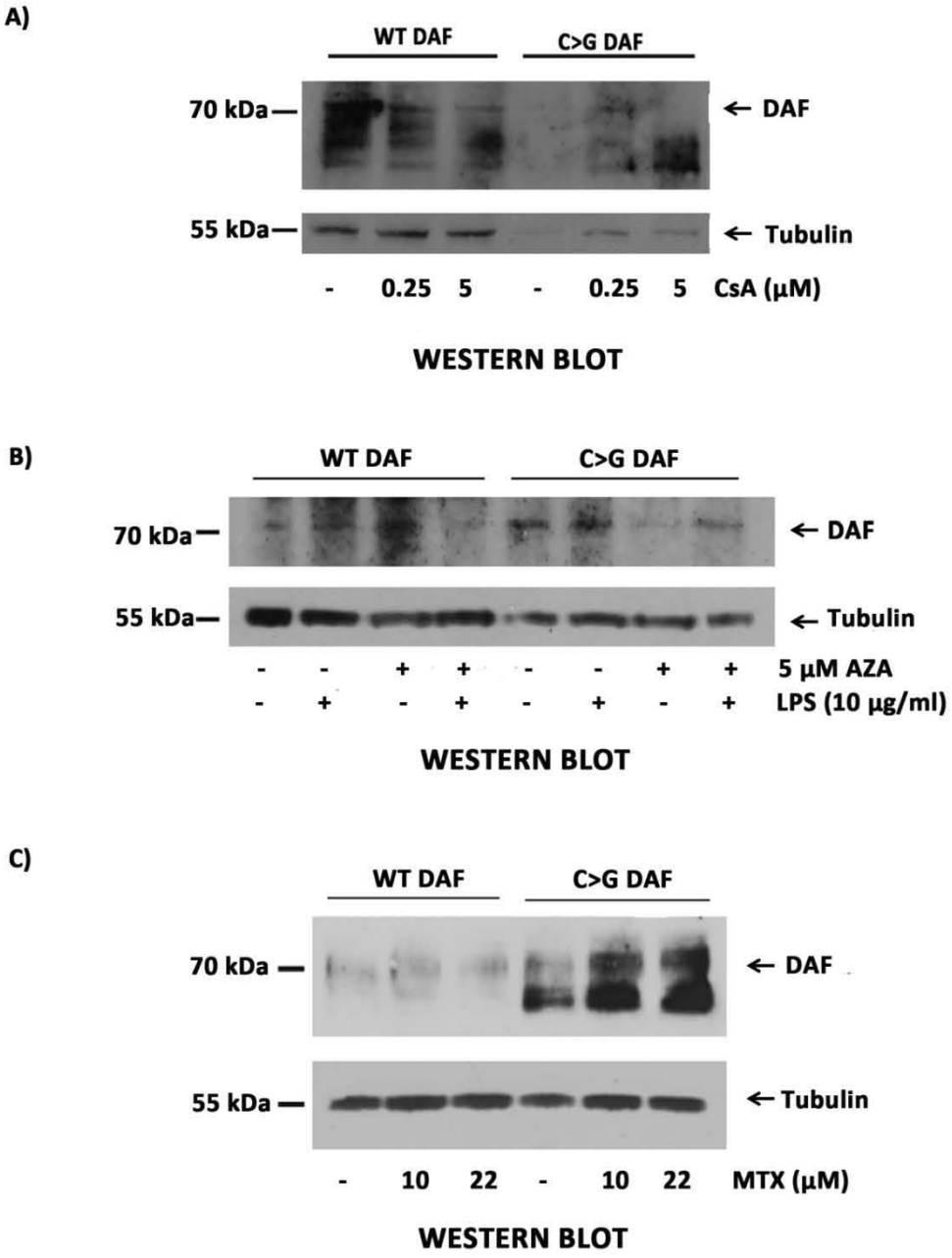


Figure 3.6: Effect of steroid-sparing agents on WT and C>G DAF protein expression. Total protein lysate was isolated from lymphoblasts 24 hrs post-treatment with either A) 0.25 and 5 µM cyclosporine A (CsA), B) 5 µM azathiorprine (AZA) or C) 10 and 22 µM methotrexate (MTX). In B) LPS (10 µg/ml) was included in the last 19 hrs of treatment prior to harvesting. Total protein from each sample was analysed on an 8% SDS-PAGE and assessed by western blotting using an antibody to DAF as well as an antibody to tubulin which served as a loading control. Arrows indicate the position of the proteins of interest as determined by the protein marker banding pattern shown on the left in kilodaltons (kDa).

3.2.2 Azathioprine differentially regulates WT and C>G DAF at both an mRNA and protein level

To determine the effect of azathioprine (AZA) on WT versus C>G DAF mRNA expression, lymphoblasts were treated with 5 µM AZA for 24 hrs with or without LPS as described before. The dose of AZA selected for this study was based on similar concentrations used in a published study (Bobbala et al., 2009). Total RNA was isolated post-treatment, reverse transcribed into cDNA and analysed by qRT-PCR. The results in **Table 3.3** show the average fold change, compared to untreated control cells, of three independent experiments each performed in duplicate. In the presence or absence of LPS, AZA significantly upregulated WT DAF mRNA levels by 1.6-fold (p=0.008) and 1.4-fold (p=0.002) respectively, while C>G DAF mRNA expression showed no change in response to treatments.

As for CsA above, the effect of AZA on WT and C>G DAF promoter activity was next determined. Transfected COS-7 cells were treated with 5 µM AZA for 24 hrs and extracts assayed 48 hrs post-transfection for firefly and *renilla* luciferase activity. **Table 3.4** shows the average fold activation, compared to untreated control cells, from three independent experiments each performed in duplicate. Both WT and C>G DAF promoter activity showed no significant change compared to their respective untreated control cells in response to 5 µM AZA treatment. Overall, unlike results obtained for WT DAF, the C>G DAF promoter activity matched the trend seen for the mRNA results above. A possible explanation as to why AZA increased WT DAF mRNA levels while WT DAF promoter activity remained basal may reflect differences in the regulation of the endogenous gene compared to the *in vitro* promoter system in response to AZA. Furthermore, there may be differences in the cell-type specific responses to the drug.

To determine the effect of AZA on endogenous DAF protein levels, WT and C>G DAF lymphoblasts were treated as described previously for qRT-PCR. Protein was extracted from lymphoblasts and subjected to western blot analysis using antibodies to either DAF or tubulin which was included as a loading control. The data shown in **Table 3.3** represents the average fold change of two independent western blots based on densitometric readings, first normalized to tubulin expression and then to the respective untreated control cells. Wild-type DAF protein levels significantly (p=0.018) increased by 3.3-fold in response to AZA when compared to C>G DAF protein levels, that in turn were repressed by 2.5-fold. Although not significant, co-treatment with LPS and AZA increased WT DAF protein levels by 1.7-fold while it led to a 1.2-fold repression of C>G DAF protein levels. The lower WT DAF protein increase after AZA+LPS treatment is likely due to a complex cross-

talk between AZA and LPS cell signalling. The western blot in **Figure 3.6 (B)** is representative of the results in **Table 3.3** above. Taken together, these results suggest that AZA in the presence or absence of LPS upregulates WT DAF protein expression while LPS alleviates the repressive effect seen for AZA-only treatment on C>G DAF protein levels.

Overall, AZA appears to have a post-transcriptional repressive effect on C>G DAF but a transcriptional activation effect on WT DAF.

3.2.3 Methotrexate does not repress WT or C>G DAF expression

To investigate the effect of methotrexate (MTX) on WT versus C>G DAF mRNA expression, lymphoblasts were treated with a low (10 µM) and a high (22 µM) dose of MTX for 24 hrs with or without LPS as described before. Total RNA was isolated, reverse transcribed into cDNA and DAF mRNA levels quantified by qRT-PCR. The doses of MTX were selected because they were considered clinically relevant (Fotoohi et al., 2004). **Table 3.3** shows the average fold change, compared to untreated control cells, from three independent experiments each performed in duplicate. In response to both 10 and 22 µM MTX no significant change was observed on either WT or C>G DAF mRNA levels. Although not significant, WT DAF mRNA levels increased by 1.4-fold in response to 10 µM MTX in the presence of LPS unlike C>G DAF mRNA levels which remained basal. Furthermore 22 µM MTX with LPS treatment had no significant effect on either WT or C>G DAF mRNA expression. These results therefore suggest that MTX has little regulatory effect on WT and C>G DAF mRNA in lymphoblasts.

We next investigated the effect of MTX on WT and C>G DAF promoter activity. Here, COS-7 cells transiently transfected for 24 hrs were treated with 10 or 22 µM MTX for 24 hrs and extracts assayed for firefly and *renilla* luciferase activity. The results of the average fold change, compared to untreated control cells, from three independent experiments each performed in duplicate are shown in **Table 3.4.** In response to both doses of MTX, WT and C>G DAF promoter activity significantly ($p \leq 0.05$) increased by approximately 2-fold compared to untreated control cells. These data do not reflect those generated at an mRNA level above and may reflect differences in the regulation of endogenous gene expression and *in vitro* promoters.

To determine the effect of MTX on endogenous DAF protein levels, WT and C>G DAF lymphoblasts were treated as described for qRT-PCR above. Protein was extracted from lymphoblasts and

subjected to western blot analysis using antibodies to either DAF or tubulin which was included as a loading control. The data in **Table 3.3** represents the average fold change of two independent western blots based on densitometric readings, first normalized to tubulin expression and then to the respective untreated control cells. A representative western blot is shown in **Figure 3.6 (C)**. Whereas WT DAF protein levels were unchanged in response to both concentrations of MTX treatment, C>G DAF protein levels showed a 1.4-fold and 1.8-fold (p=0.004) increase in response to the 10 and 22 µM MTX doses respectively. The co-treatment of both MTX doses with LPS increased WT DAF protein expression by 2.4-fold and 1.6-fold but only the higher 22 µM dose of MTX in combination with LPS increased C>G DAF protein levels by 1.2-fold. Overall, MTX in the presence or absence of LPS did not inhibit WT or C>G DAF protein expression. These results have important implications for MG patients harbouring the C>G SNP because they suggest that MTX treatment does not repress DAF expression.

3.2.4 Effect of steroid-sparing drugs on WT Daf in skeletal muscle cells

As for prednisone, we next assessed the effect of the steroid-sparing drugs CsA, AZA and MTX on WT Daf expression in skeletal muscle cells. Here, six day differentiated C2C12 myoblasts were treated with the indicated doses of each drug alone for a total of 72 hrs with or without LPS (10 µg/ml) for 24 hrs. Thereafter, protein was harvested and analysed by western blotting using an antibody to mouse polyclonal Daf or an antibody to tubulin which was included as a loading control. The results shown in **Table 3.5** represents the average fold change of two independent western blots based on densitometric readings, first normalized to tubulin expression and then to the respective untreated control cells. **Figure 3.7** shows representative western blots of data depicted in **Table 3.5**. In response to 0.25 and 5 µM CsA, WT Daf protein levels increased by 2.2-fold and 1.7-fold respectively and this activation response was maintained in the presence of LPS (1.4-fold and 1.6-fold respectively). AZA (5 µM) treatment increased WT Daf protein expression by 2.1-fold while the co-treatment of AZA and LPS increased WT Daf levels by 1.5-fold ($p \leq 0.05$). In the presence of 10 µM and 22 µM MTX, WT Daf protein levels increased by 2-fold and 1.8-fold respectively while co-treatment of MTX with LPS reduced the observed activation response to basal (10 µM MTX+LPS) or below basal (22 µM MTX+LPS) levels.

Overall, the results for AZA and MTX, but not CsA, match the trend seen in WT DAF lymphoblasts i.e. treatment with these steroid-sparing agents alone do not appear to repress WT Daf protein levels in the differentiated C2C12 myotubes. This was also true for the co-treatment of LPS with CsA, AZA or a lower dose of MTX.

Table 3.5: The effect of MG drugs on WT Daf protein expression in differentiated C2C12 mouse myotubes.

C2C12 Daf protein expression					
		-	+ LPS (10 µg/ml)	Prednisone	
				0.1 µM	1 µM
Cyclosporin A	**0.25 µM**	2.2	1.4	1.0	0.9
	5.0 µM	1.7	1.6	0.3	0.2
Azathioprine	**5.0 µM**	2.1	1.5*	0.9	1.0
Methotrexate	**10 µM**	2.0	0.9	0.8	0.9
	22 µM	1.8	0.7	0.7	0.6

Total protein was isolated from six day differentiated C2C12 myotubes treated with cyclosporine A, azathioprine or methotrexate for 24 hrs in the absence or presence of LPS for 24 hrs. Cell lysates (30 µg) were separated on an 8% SDS-PAGE and analysed by western blotting using antibodies to Daf and tubulin. The results represent the average fold change of two independent western blots based on densitometric readings, first normalized to tubulin expression and then to the respective untreated control cells. (*) indicates significance at $p \leq 0.05$ between treated and untreated control cells.

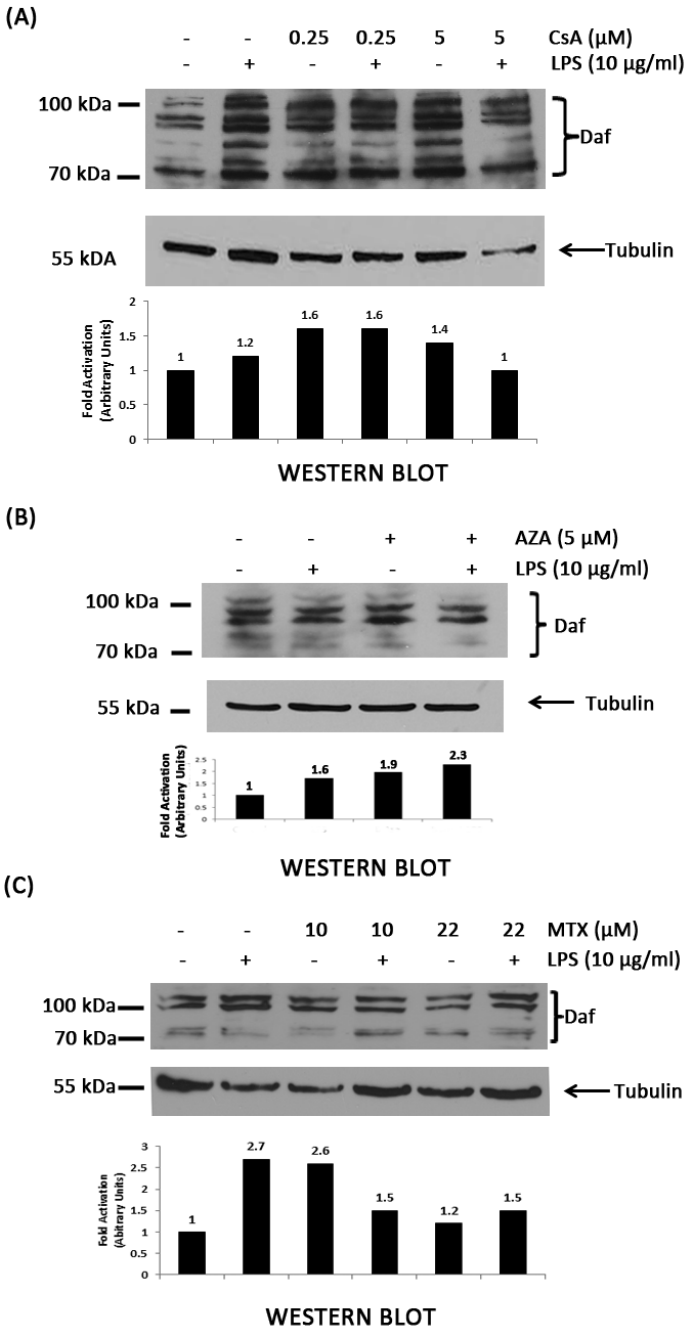


Figure 3.7: Steroid-sparing immunosuppressive drugs used in the treatment of MG do not repress WT Daf protein levels in mouse skeletal muscle cells. Six day differentiated C2C12 myotubes were treated with either **(A)** 0.25 and 5µM cyclosporine A (CsA) or **(B)** 5 µM azathioprine (AZA) or **(C)** 10 and 22 µM methotrexate (MTX) all for a total of 72 hrs with or without LPS (10 µg/ml) for 24 hrs. In **(A)**, **(B)** and **(C)** cell lysates (30 µg) were separated on an 8% SDS-PAGE and analysed by western blotting using antibodies to Daf and tubulin. The bar graph represents the densitometric readings comparing the intensity of the DAF protein normalized to the tubulin loading control. The arrows and brackets indicate the position of the proteins of interest as determined by the protein marker banding pattern shown on the left in kilodaltons (kDa).

3.2.5 Effect of prednisone in combination with steroid-sparing drugs on WT Daf in a skeletal muscle model

To further investigate the effect of prednisone in combination with the steroid-sparing drugs on WT DAF expression, mouse skeletal myoblasts differentiated for 6 days into myotubes were treated with either 0.1 or 1 µM prednisone for a total of 72 hrs. Protein was harvested and analysed by western blotting as described above in section 3.2.4. The data in **Table 3.5** shows the average fold change of two independent western blots based on densitometric readings, first normalized to tubulin expression and then to the respective untreated control cells. The 0.25 µM dose of CsA in combination with both doses of prednisone maintained WT Daf protein levels at a basal level; however, although not statistically significant, 5 µM CsA combined with 0.1 or 1 µM prednisone greatly repressed WT Daf (3.3-fold and 5-fold respectively). Interestingly, 5 µM AZA was able to overcome the repressive effect seen for 0.1 and 1 µM prednisone in **Table 3.2** and also shown in **Figure 3.8**. This was also seen for 10 µM MTX while a repressive effect was observed for the co-treatment of 22 µM MTX with 0.1 or 1 µM prednisone (1.4-fold and 1.7-fold respectively). Taken together, these results suggest that AZA and the lower doses of CsA and MTX in combination with prednisone prevent the repression of WT Daf protein levels by prednisone in the skeletal C2C12 muscle cells.

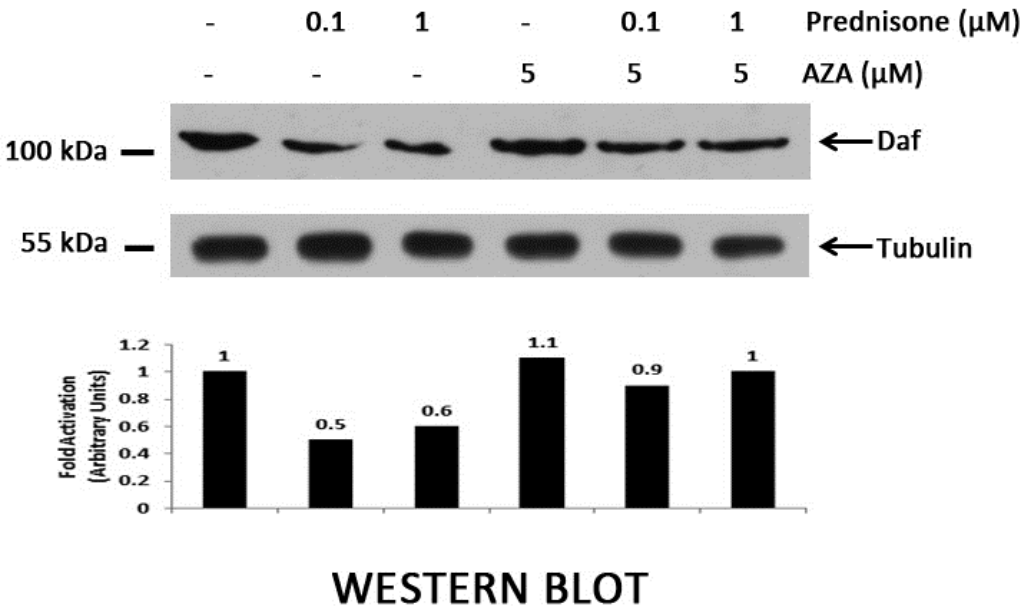


Figure 3.8: Azathioprine in combination with prednisone maintains basal WT Daf protein levels in C2C12 mouse myotubes. Six day differentiated C2C12 myotubes were treated with 5 µM azathioprine (AZA) with or without 0.1 and 1 µM prednisone for 72 hrs. Cell lysates (30 µg) were separated on an 8% SDS-PAGE and analysed by western blotting using antibodies to Daf and tubulin. The bar graph represents the densitometric readings comparing the intensity of the DAF protein normalized to the tubulin loading control. The arrows indicate the position of the proteins of interest as determined by the protein marker banding pattern shown on the left in kilodaltons (kDa).

Chapter 4: Discussion

Myasthenia gravis (MG) is an antibody-mediated autoimmune disease in which autoantibodies target the acetylcholine receptors (AChRs) of the neuromuscular junction. Recently our laboratory identified a functional c.-198C>G SNP (C>G SNP) in the promoter of decay accelerating factor (DAF). This SNP was shown to be significantly associated with the development of a severe treatment-resistant extraocular phenotype in MG patients. As MG patients with this C>G SNP developed ophthalmoplegia while on standard treatment this thesis focused on the effects of drugs used in the management of MG on wild-type (WT) and C>G DAF expression levels. The data obtained increases our understanding of DAF expression in response to these drugs at the molecular level and have implications for the treatment of MG patients carrying the C>G SNP.

4.1 The effect(s) of prednisone on wild-type versus C>G DAF expression

Repression of C>G DAF protein levels by prednisone increases the risk of cell lysis

This is the first study to investigate the effect of prednisone, the first-line immunosuppressive drug used in MG, on DAF expression in human lymphoblasts. Lymphoblast cell lines were first established from healthy control individuals expressing WT DAF or from MG patients harbouring the C>G DAF SNP. Subsequently, DAF mRNA and protein levels were determined after the exposure of these lymphoblast cell lines to different doses of prednisone. Overall, prednisone doses between 0.1 and 60 µM did not appear to have a regulatory effect on WT or C>G DAF mRNA expression or promoter activity as levels remained close to basal or no statistical significance was achieved. However, western blotting performed on total protein revealed that a range of prednisone doses has a repressive effect on C>G DAF but not WT DAF protein levels. Indeed, in response to the 0.1, 1 and 3 µM doses of prednisone, C>G DAF protein levels were repressed by 2.5-fold compared to WT DAF.

To determine the functional significance of C>G DAF protein repression by prednisone, calcein assays were performed. Although not significant, prednisone doses of 1-3 µM, but not 0.1 µM, were shown to increase the degree of cell lysis in C>G DAF lymphoblasts (50%) when compared to WT DAF (16%). Therefore, the results generated from the calcein assay indicate that WT DAF protein levels after exposure to prednisone treatment are sufficient to protect against cell lysis. Conversely, the repressive effect of the higher 1 and 3 µM prednisone doses on C>G DAF protein

levels appears to lead to an inadequate DAF response, that consequently results in cell lysis. Although western blot analysis indicated 0.1 µM prednisone was equally repressive towards C>G DAF (as seen for 1 and 3 µM prednisone), calcein assays suggested that C>G DAF lymphoblast have a low risk of undergoing cell lysis after exposure to this dose of prednisone. These results would suggest that the repression of C>G DAF at 0.1 µM prednisone is not sufficient to lead to cell lysis. It is however important to note that DAF is not the only complement regulatory protein expressed on lymphoblasts and it is possible that at higher doses of prednisone one or more of these are inactivated which together with decreased levels of C>G DAF results in cell lysis. Furthermore, this study also shows that while LPS, which mimics immune stress and which has previously been shown to repress C>G DAF protein levels (Heckmann et al., 2010), induced approximately 60% of the C>G DAF lymphoblasts to lyse it was only able to cause 33% of WT DAF lymphoblasts to undergo cell lysis (**Figure 3.1**). Together these results confirm that LPS or prednisone treatment reduced levels of C>G DAF expression which correlated directly with susceptibility to cell lysis. In addition they suggest that in our MG patients with the C>G SNP, prednisone treatment may be lowering their EOM DAF levels to below the threshold needed for optimal protection against complement-mediated lysis. These observations may impact on treatment approaches for our MG patients harbouring the C>G SNP, as currently MG treatment protocols frequently use prednisone.

Interestingly, when WT and C>G DAF lymphoblasts were treated with high concentrations (5%-10%) of either MG patient or control sera, there was a trend to greater cell lysis for the MG sera and the effect was comparable in WT and C>G DAF lymphoblasts. This was also the case for 2.5% MG sera which suggests that compared to control sera, MG sera is more cytotoxic irrespective of the presence or absence of the C>G SNP. For future experiments, the concentration of the active MG sera, which is expected to downregulate DAF much like LPS, should be further reduced for example to 1%, to establish whether there is a difference in cytotoxicity between WT and C>G DAF lymphoblasts. However, if after the above treatment the level of cytotoxicity remains similar, this could imply that the additional complement receptor proteins, but not DAF, are protecting against complement-mediated lysis. Results shown in **Figure 3.1** also reveal that co-treatment with 3 µM prednisone and MG sera resulted in the death of 82% of C>G DAF lymphoblasts compared to 9% WT DAF lymphoblasts. This data suggests, that under conditions similar to the MG environment, albeit *in vitro*, the C>G SNP appears to significantly increase the risk of cell lysis after prednisone treatment.

Evidence in the literature suggests that, in response to prednisone treatment, the putative NFκB binding site introduced by the C>G SNP could play a significant role in the repression of C>G DAF protein levels. As mentioned previously, NFκB is critical for immune homeostasis and glucocorticoids, such as prednisone, are known to repress NFκB-driven pro-inflammatory genes. The mechanism by which glucocorticoids repress NFκB-driven genes is thought to be by direct protein-protein interaction of glucocorticoid receptors (GRs) with NFκB. For example, an *in vitro* study by Ray et al. (1994) showed that GRs associated with the NFκB subunit p65 which is required for binding essential components of the basal transcriptional machinery such as TFIIB and TBP (Schmitz et al., 1995). The binding of GRs to p65 thus prevents its interaction with the basal transcriptional machinery and hence NFκB target genes are repressed. Additionally, GR binding to p65 may affect the interaction of p65 with other transcription factors and therefore influence the complex cross-talk that occurs amongst transcription factors involved in the immune regulatory pathways. It would be important for future studies to explore whether this is the mechanism by which prednisone represses C>G DAF an anti-inflammatory pathway.

Prednisone represses WT Daf in a muscle model

The effect of prednisone on DAF expression in muscle was of great interest because it is the extraocular muscles (EOM) that are affected in the MG phenotype under investigation. Unfortunately, at the time that this study was initiated neither an EOM cell line nor patient-derived muscle cell lines expressing WT or C>G DAF were available. Furthermore, there were no reports documenting the effect of prednisone on muscle DAF expression. Therefore, to gain an insight into the effect of prednisone on DAF expression in muscle, the experiments performed in lymphoblasts were repeated in a C2C12 mouse myoblast cell line which expresses WT Daf. Overall, the data consistently showed that prednisone repressed WT Daf at various doses ranging from 0.1 to 30 µM in the differentiated mouse myotubes (**Table 3.2**). Furthermore, in the presence of LPS, prednisone doses of 3-30 µM continued to repress WT Daf protein levels. Interestingly, this data did not correspond with the results obtained in human lymphoblasts expressing WT DAF. While this suggests that the effects of prednisone may be species and/or cell-type specific the possibility cannot be excluded that prednisone may also repress DAF levels in human EOM. Interestingly, western blot analysis performed in the differentiated mouse myotubes showed that co-treatment of MG sera with prednisone, greatly repressed WT Daf protein expression, while control sera was able to overcome the repressive effect of prednisone (**Table 3.2**). This suggests that under

conditions mimicking MG *in vitro*, prednisone treatment represses WT Daf protein expression. Since corticosteroids, such as prednisone, can inhibit both the JNK pathway and affect the p65 NFκB pathway (De Bosscher et al., 2000), the combination of prednisone and a critical level of complement factors may thus contribute to a breach of muscle defences in cells susceptible to complement-mediated damage, such as EOMs in MG patients harbouring the C>G SNP.

As mentioned previously in section 1.8.1, post-transcriptional modification is one mechanism by which glucocorticoids are thought to influence gene expression. The prednisone-induced lymphoblast DAF repression (and probably Daf myotube repression) observed in this thesis is highly suggestive of a post-transcriptional mechanism in these cell types as C>G DAF promoter activity and mRNA levels remained basal in response to prednisone. Therefore, to establish whether Daf protein repression by prednisone is transcriptional or translational in the C2C12 myotubes future experiments should include qRT-PCR analysis where Daf mRNA levels are quantitated.

Based on the results of this study, the following model is proposed to explain the effects prednisone may have on DAF protein levels in MG patients expressing either WT or C>G DAF. Those MG patients with the C>G SNP who are administered prednisone and are exposed to an immune stress,may have critically repressed DAF protein expression in their EOMs that is below the threshold necessary to protect against complement-mediated damage (**Figure 4.1**). As mentioned earlier, it is speculated that DAF expression is already low in the EOMs and is the dominant CRP required for complement-mediated lysis protection (Kaminski et al., 2004); therefore MG patients harbouring the C>G SNP would be more susceptible to EOM damage and hence development of the extreme EOM phenotype. Conversely, WT DAF protein expression is less repressed and levels are still sufficient to prevent autologous complement-mediated lysis in their EOMs. The validity of this model should be tested by examining both WT and C>G DAF protein expression levels in a human EOM cell line in response to prednisone treatment.

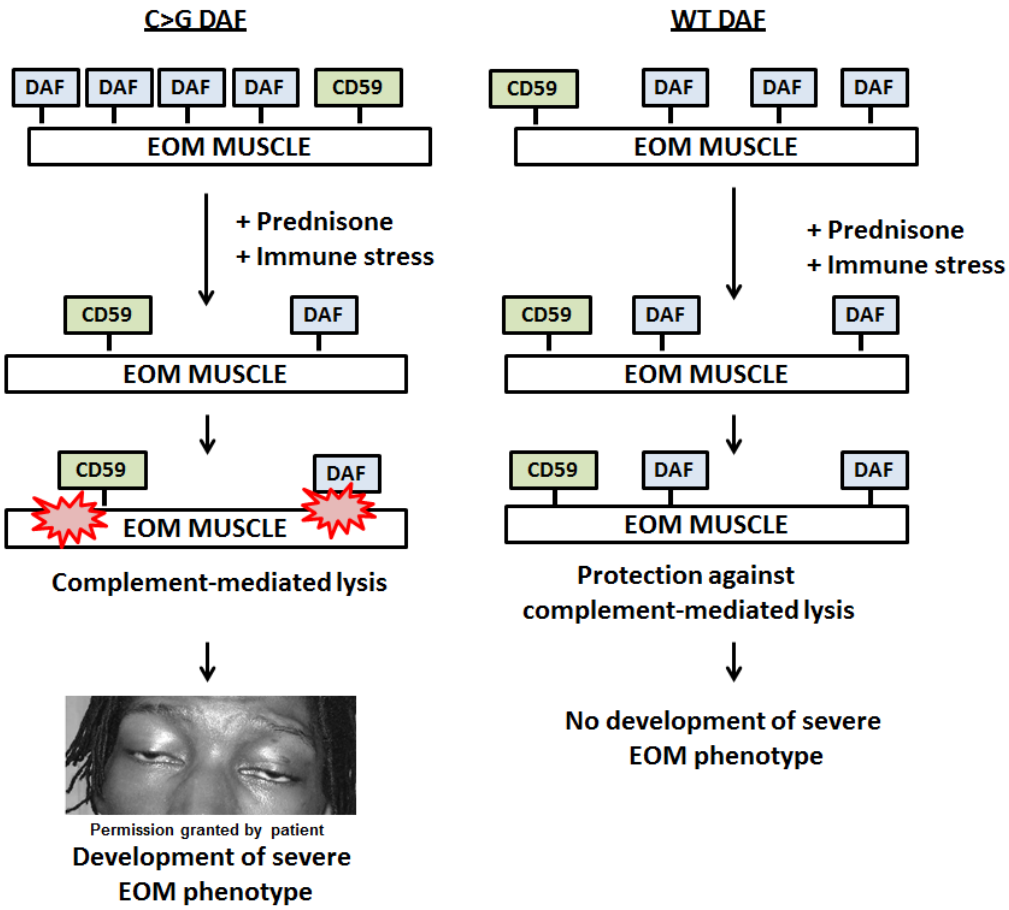


Figure 4.1: Schematic diagram of the proposed model showing the influences of prednisone treatment under conditions of immune stress on WT or C>G DAF protein expression in extraocular muscles.

4.2 The regulation of DAF/Daf expression in response to cyclosporin A, azathioprine and methotrexate

The second aim of this study was to determine the effects of current MG immunosuppressive steroid sparing drugs, cycosporin A (CsA), azathioprine (AZA) and methotrexate (MTX), on WT and C>G DAF expression alone or in combination with prednisone. Overall, the studies in lymphoblasts as well as luciferase promoter reporter assays show that CsA has no statistically significant effect on the transcription of both WT and C>G DAF (**Tables 3.3 and 3.4**). CsA, however, under most conditions tested repressed WT and C>G DAF protein levels in lymphoblasts even in the presence of LPS which suggests that its regulation of DAF levels is post-transcriptional. It should however be pointed out that only one western blot was achieved for DAF protein analysis investing the co-treatment of CsA with LPS, so these results should be interpreted with caution. Interestingly, the opposite results were obtained in the C2C12 muscle cells where CsA treatment alone or in combination with LPS was shown to increase WT Daf protein expression (**Table 3.5**). The discrepancy between results obtained in WT DAF lymphoblasts and mouse skeletal muscle C2C12 cells suggests that CsA may exert cell-type and/or species-specific effects. Indeed, a cell type specific effect may explain the conflicting reports for the effect of CsA on DAF in the literature. For example, whereas Mason et al (2004) showed that CsA has no effect on basal levels of DAF in human endothelial cells, CsA was reported to increase both DAF and CD49 expression in a kidney endothelial cell model (Kim et al., 2007). To date, the present study is the first to investigate combinations of drugs relevant to the clinical MG scenario. Importantly the co-treatment of skeletal muscle cells with a low 0.25 µM CsA dose together with prednisone showed that CsA was able to overcome the repressive effect as seen for prednisone-only treated cells. This therefore suggests these co-treatments, albeit *in vitro*, may be beneficial in a patient with complement-mediated extraocular muscle disease.

To date, no reports exist on the effect(s) of AZA treatment on DAF expression and the results in this study show that WT DAF and C>G DAF are differentially regulated at both an mRNA and protein level. Whereas AZA increased WT DAF mRNA and protein levels, it did not affect C>G DAF mRNA levels but significantly repressed its protein levels (**Table 3.3**). Studies in the C2C12 mouse muscle model showed that, similar to results generated in WT DAF lymphoblasts, AZA upregulated WT Daf protein expression in the absence or presence of LPS. Importantly, the combination of AZA and

prednisone treatment of muscle cells overcame the repressive effect seen for prednisone-alone treatment on WT Daf. This has relevance to MG patients, as co-treatment of AZA and prednisone may therefore have a positive effect on EOM DAF levels although, to date, we do not know its effect in C>G DAF muscle.

This thesis is the first to give an insight to the effect that MTX has on DAF expression. Of particular importance to the current thesis are the results from western blot analysis which showed that MTX treatment alone increased C>G DAF protein levels and that co-treatment of MTX with LPS had no effect on C>G DAF protein levels (**Table 3.3**). Interestingly, promoter studies indicated that MTX transcriptionally activates both WT and C>G DAF promoter activity (**Table 3.4**), while having no significant effect on WT and C>G DAF mRNA levels. Together these data suggest that the mechanism(s) by which MTX regulates DAF may be complex and an explanation for how MTX regulates C>G DAF protein levels is currently unknown. However the results are promising as MTX was the only drug studied in this thesis that did not repress C>G DAF protein expression which suggests that it may be an effective treatment for MG patients harbouring the C>G SNP. Indeed, a recent report has shown that MTX is a cost-effective alternative to AZA due to a similar efficacy and safety profile (Heckmann et al., 2011).

4.3 Future considerations and limitations of this study

As previously mentioned, the complement regulatory proteins (CRPs) including DAF, MCP, CD59 and CR1 are expressed on the surface of most cells and serve critical roles in the body by preventing complement-mediated lysis in autologous cells. Indeed, DAF and CD59 are two complement regulatory proteins shown to be particularly important in the defence against EAMG-associated complement-mediated muscle damage (Kaminski et al., 2006; Morgan et al., 2006). Furthermore, experimental autoimmune MG studies found that DAF or CD59 knock-out mice showed activation of complement, AChR loss at the muscle endplate as well as detectable weakness compared with wild-type littermates (Kaminski et al., 2006; Morgan et al., 2006). Interestingly, Mason et al (2002) and Kusama et al (2003) demonstrated that DAF is the main CRP required for protection against complement-mediated lysis in human umbilical endothelium cells and swine endothelial cells respectively. Similarly, Kaminski et al (2004) found that in an experimental mouse autoimmune MG model that DAF expression in EOM was less than in other skeletal muscle but was more important then CD59 for the protection of EOM against complement-mediated damage. Conversely, in human

skeletal myoblasts CD59 was shown to be the main CRP, as its silencing, but not DAF's, led to cell lysis (Gasque et al., 1996). It is therefore necessary to investigate the effects of the drugs studied in this thesis in an EOM cell line derived from control WT DAF individuals and C>G DAF expressing MG patients. These cell lines would be as close to the MG environment as possible, and the results would be more relevant than patient-derived lymphoblasts.

Another drug which could be assessed in the efficacy of treatment for C>G SNP DAF MG patients is Eculizumab, a compliment inhibitor drug. It would be interesting to assess the response of MG patients with the extreme ocular phenotype to this drug alone or in combination with methotrexate.

Furthermore, an obvious additional limitation of the current study is the use of cell culture models as they are *in vitro* and therefore the results obtained cannot reliably inform the treatment regimens in MG patients. In particular, the *in vitro* environment including the doses of drugs and treatment duration might not represent the *in vivo* situation and thus the results from the current study should be interpreted with caution.

4.4 Conclusion

This project focused on investigating current immunosuppressive drug therapies on DAF expression as well as to elucidate if any regulatory differences were present between WT and C>G DAF expression. The experimental findings in this thesis suggested that prednisone at specific doses, is repressive to lymphoblast C>G DAF protein expression more so than to WT DAF, and that the repression appears to significantly increase susceptibility to complement-mediated injury. Furthermore, prednisone showed a substantial repressive effect on the expression of myotube WT Daf. This may be of importance to MG patients who are susceptible to complement-mediated extraocular muscle damage. The postulate would be that EOMs with increased susceptibility to complement-mediated damage, such as a MG patient expressing C>G DAF, have an inadequate DAF response to prednisone treatment and an immune stress, which renders EOMs vulnerable.

The steroid-sparing agents tested in this thesis showed that only MTX was able to increase or maintain basal expression of C>G DAF protein levels, suggesting that MTX may be an appropriate treatment for MG patients harbouring the C>G SNP. This thesis is the first to study the co-treatment

of prednisone with CsA, AZA or MTX on DAF expression and results would suggest that AZA and low doses of CsA and MTX are able to overcome the prednisone-induced Daf repression.

References

- **Ahmad SR, Lidington EA, Ohta R, Okada N, Robson MG, Davies KA, Leitges M, Harris CL, Haskard DO, Mason JC.** Decay-accelerating factor induction by tumour necrosis factor-alpha, through a phosphatidylinositol-3 kinase and protein kinase C-dependent pathway, protects murine vascular endothelial cells against complement deposition. *Immunology* 110: 258-268, 2003.
- **Amano Y, Lee SW, Allison AC.** Inhibition by glucocorticoids of the formation of interleukin-1 alpha, interleukin-1 beta, and interleukin-6: mediation by decreased mRNA stability. *Mol Pharmacol* 43: 176-182, 1993.
- **Anderson P, Phillips K, Stoecklin G, Kedersha N.** Post-transcriptional regulation of proinflammatory proteins. *J Leukoc Biol* 76: 42-47, 2004.
- **Andoh A, Kinoshita K, Rosenberg I, Podolsky DK.** Intestinal trefoil factor induces decay-accelerating factor expression and enhances the protective activities against complement activation in intestinal epithelial cells. *J Immunol* 167: 3887-3893, 2001.
- **Atreya I and Neurath MF.** Understanding the delayed onset of action of azathioprine in IBD: are we there yet? *Gut* 58: 325-326, 2009.
- **Barankiewicz J, Ronlov G, Jimenez R, Gruber HE.** Selective adenosine release from human B but not T lymphoid cell line. *J Biol Chem* 265: 15738-15743, 1990.
- **Barnes PJ.** Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci* 94: 557-572, 1998.
- **Bateman KJ, Schinkel M, Little F, Liebenberg L, Vincent A, Heckmann JM**. Incidence of seropositive myasthenia gravis in Cape Town and South Africa. *S Afr Med* 97: 630-634, 2005.
- **Belgi G and Friedmann PS.** Traditional therapies: glucocorticoids, azathioprine, methotrexate, hydroxyurea. *Clin Exp Dermatol* 27: 546-554, 2002.
- **Ben-Horin S, Goldstein I, Fudim E, Picard O, Yerushalmi Z, Barshack I, Bank I, Goldschmid Y, Meir SB, Mayer L, Chowers Y.** Early preservation of effector functions followed by eventual T cell memory depletion: a model for the delayed onset of the effect of thiopurines. *Gut* 58: 396-403, 2009.
- **Bobbala D, Koka S, Geiger C, Föller M, Huber SM, Lang F.** Azathioprine favourably influences the course of malaria. *Malar J* 8: 1-7, 2009.

- **Borel JF, Feurer C, Gubler HU, Stähelin H**. Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions* 6: 468-475, 1976.
- **Brivanlou AH and Darnell JE Jr.** Signal transduction and the control of gene expression. *Science* 295: 813-818, 2002.
- **Caelles C, González-Sancho JM, Muñoz A.** Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev* 11: 3351-3364, 1997.
- **Cauvi DM, Cauvi G, Pollard KM.** Constitutive expression of murine decay-accelerating factor 1 is controlled by the transcription factor Sp1. *J Immunol* 177: 3837-3847, 2006.
- **Chen T, Guo J, Yang M, Han C, Zhang M, Chen W, Liu Q, Wang J, Cao X.** Cyclosporin A impairs dendritic cell migration by regulating chemokine receptor expression and inhibiting cyclooxygenase-2 expression. *Blood* 103: 413-421, 2004.
- **Christadoss P.** C5 gene influences the development of murine myasthenia gravis. *J Immunol* 140: 2589-2592, 1988.
- **Cocuzzi ET, Bardenstein DS, Stavitsky A, Sundarraj N, Medof ME.** Upregulation of DAF (CD55) on orbital fibroblasts by cytokines. Differential effects of TNF-beta and TNF-alpha. *Curr Eye Res* 23: 86-92, 2001.
- **Cronstein BN.** Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis. *Pharmacol Rev* 57: 163-172, 2005.
- **Cuffari C, Seidman EG, Latour S, Théorêt Y.** Quantitation of 6-thioguanine in peripheral blood leukocyte DNA in Crohn's disease patients on maintenance 6-mercaptopurine therapy. *Can J Physiol Pharmacol* 74: 580-585, 1996.
- **De Bosscher K, Vanden Berghe W, Vermeulen L, Plaisance S, Boone E, Haegeman G.** Glucocorticoids repress NF-κB-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proc Natl Acad Sci U S A* 97: 3919-3924, 2000.
- **Dean JL, Sully G, Clark AR, Saklatvala J.** The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. *Cell Signal* 16: 1113-1121, 2004.
- **Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI.** Differential activation of transcription factors induced by $Ca2^{+}$ response amplitude and duration. *Nature* 386: 855-858, 1997.

- **Dostert A and Heinzel T.** Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* 10: 2807-2816, 2004.
- **Düfer M, Krippeit-Drews P, Lembert N, Idahl LA, Drews G.** Diabetogenic effect of cyclosporin A is mediated by interference with mitochondrial function of pancreatic B-cells. *Mol Pharmacol* 60: 873-879, 2001.
- **Emmel EA, Verweij CL, Durand DB, Higgins KM, Lacy E, Crabtree GR.** Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science* 246: 1617-1620, 1989.
- **Engel AG, Lambert EH, Howard FM.** Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations. *Mayo Clin Proc* 52: 267-280, 1977.
- **Finberg RW, White W, Nicholson-Weller A.** Decay-accelerating factor expression on either effector or target cells inhibits cytotoxicity by human natural killer cells. *J Immunol* 149: 2055-2060, 1992.
- **Fotoohi K, Jansen G, Assaraf YG, Rothem L, Stark M, Kathmann I, Gregorczyk J, Peters GJ, Albertioni F.** Disparate mechanisms of antifolate resistance provoked by methotrexate and its metabolite 7-hydroxymethotrexate in leukemia cells: implications for efficacy of methotrexate therapy. *Blood* 104: 4194-4201, 2004.
- **Gasque P, Thomas A, Fontaine M, Morgan BP.** Complement activation on human neuroblastoma cell lines in vitro: route of activation and expression of functional complement regulatory proteins. *J Neuroimmunol* 66: 29-40, 1996.
- **Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP.** Mechanisms of action of methotrexate. *Immunopharmacology* 47: 247-257, 2000.
- **Gilbert ME, De Sousa EA, Savino PJ.** Ocular Myasthenia Gravis treatment: the case against prednisone therapy and thymectomy. *Arch Neurol* 64: 1790-1792, 2007.
- **Gray LC, Hughes TR, van den Berg CW.** Binding of human antigen R (HuR) to an AU-rich element (ARE) in the 3'untranslated region (3'UTR) reduces the expression of decay accelerating factor (DAF). *Mol Immunol* 47: 2545-2551, 2010.
- **Gruber HE, Hoffer ME, McAllister DR, Laikind PK, Lane TA, Schmid-Schoenbein GW, Engler RL.** Increased adenosine concentration in blood from ischemic myocardium by AICA riboside. Effects on flow, granulocytes, and injury. *Circulation* 80: 1400-1411, 1989.

- **Hamann J, Vogel B, van Schijndel GM, van Lier RA.** The seven-span transmembrane receptor CD97 has a cellular ligand (CD55, DAF). *J Exp Med* 184: 1185-1189, 1996.
- **Hart L, Lim S, Adcock I, Barnes PJ, Chung KF.** Effects of inhaled corticosteroid therapy on expression and DNA-binding activity of nuclear factor kappaB in asthma. *Am J Respir Crit Care Med* 161: 224-231, 2000.
- **Hayden MS and Ghosh S.** Signalling to NF-kB. *Genes Dev* 18: 2195-2224, 2004.
- **Hayden MS, West AP, Ghosh S.** NF-kappaB and the immune response. *Oncogene* 25: 6758-6780, 2006.
- **He Y, Lin F, Chipman P, Bator C, Baker T, Shoham M, Kuhn R, Medof M, Rossman M.** Structure of decay-accelerating factor bound to echovirus 7: A virus-receptor complex. *Proc Natl Acad Sci U S A* 99: 10325-10329, 2002.
- **Heckmann JM, Owen EP, Little F.** Myasthenia gravis in South Africans: Racial differences in clinical manifestations. *Neuromusc Dis* 17: 929-934, 2007.
- **Heckmann JM, Uwimpuhwe H, Ballo R, Kaur M, Bajic VB, Prince S.** A functional SNP in the regulatory region of the decay-accelerating factor gene associates with extraocular muscle pareses in myasthenia gravis. *Genes Immun* 11: 1-10, 2010.
- **Heckmann JM, Rawoot A, Bateman K, Renison R, Badri M.** A single-blinded trial of methotrexate versus azathioprine as steroid-sparing agents in generalized myasthenia gravis. *BMC Neurol* 11: 97, 2011.
- **Heckmann JM, Hansen P, Van Toorn R, Lubbe E, Janse van Rensburg E, Wilmshurst JM.** The characteristics of juvenile myasthenia gravis among South Africans. *S Afr Med J* 102: 532-536, 2012.
- **Hirano F, Tanaka H, Hirano Y, Hiramoto M, Handa H, Makino I, Scheidereit C.** Functional interference of Sp1 and NF-kappaB through the same DNA binding site. *Mol Cell Biol* 18: 1266-1274, 1998.
- **Ho S, Clipstone N, Timmermann L, Northrop J, Graef I, Fiorentino D, Nourse J, Crabtree GR.** The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol* 80(3 Pt 2): S40-5, 1996.
- **Hoffman EM.** Inhibition of complement by a substance isolated from human erythrocytes. I. Extraction from human erythrocyte stromata. *Immunochemistry* 6: 391-403, 1969.

- **Hojo M, Morimoto T, Maluccio M, Asano T, Morimoto K, Lagman M, Shimbo T, Suthanthiran M.** Cyclosporine induces cancer progression by a cell-autonomous mechanism. *Nature* 397: 530-534, 1999.
- **Holla VR, Wang D, Brown JR, Mann JR, Katkuri S, DuBois RN.** Prostagladin E2 regulates the complement inhibitor CD55/decay-accelerating factor in colorectal cancer. *J Biol Chem* 280: 476-483, 2005.
- **Ito K, Barnes PJ, Adcock IM.** Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. *Mol Cell Biol* 20: 6891-6903, 2000.
- **Kahan BD.** Cyclosporine. *N Engl J Med* 321: 1725-1738, 1989.
- **Kaminski HJ, Kusner LL, Block CH.** Expression of acetylcholine receptor isoforms at extraocular muscle endplates. *Invest Ophthalmol Vis Sci* 37: 345-351, 1996.
- **Kaminski HJ, Li Z, Richmonds C, Lin F, Medof ME.** Complement regulators in extraocular muscle and experimental autoimmune myasthenia gravis. *Exp Neurol* 189: 333-342, 2004.
- **Keesey JC**. "Crisis" in myasthenia gravis: an historical perspective. *Muscle Nerve* 26: 1-3, 2002.
- **Kim YO, Lim SW, Li C, Kang HJ, Ahn KO, Yang HJ, Ghee JY, Kim SH, Kim JY, Choi BS, Kim J, Yang CW.** Activation of intrarenal complement system in mouse model for chronic cyclosporine nephrotoxicity. *Yonsei Med J* 48: 517-525, 2007.
- **Khanna A, Li B, Stenzel KH, Suthanthiran M.** Regulation of new DNA synthesis in mammalian cells by cyclosporine. Demonstration of a transforming growth factor beta-dependent mechanism of inhibition of cell growth. *Transplantation* 57: 577-582, 1994.
- **Klaus GG, Hawrylowicz CM.** Activation and proliferation signals in mouse B cells. II. Evidence for activation (G0 to G1) signals differing in sensitivity to cyclosporine. *Eur J Immunol* 14: 250-254, 1984.
- **Koide S and Steinman RM.** Induction of murine interleukin 1: stimuli and responsive primary cells. *Proc Natl Acad Sci U S A* 84: 3802-3806, 1987.
- **Kusama T, Miyagawa S, Moritan T, Kubo T, Yamada M, Sata H, Fukuta D, Matsunami K, Shirakura R.** Downregulation of NK cell-mediated swine endothelial cell lysis by DAF (CD55). *Transplant Proc* 35: 529-530, 2003.

- **Kusner LL, Kaminski HJ, Soltys J.** Effect of complement and its regulation on myasthenia gravis pathogenesis. *Expert Rev Clin Immunol* 4: 43-52, 2008.
- **Lang T, Badea T, Wade R, Shin M.** Sublytic terminal complement attack on myotubes decreases the expression of mRNAs encoding muscle-specific proteins. *J Neurochem* 68: 1581-1589, 1997.
- **Lasa M, Brook M, Saklatvala J, Clark AR.** Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38. *Mol Cell Biol* 21: 771-780, 2001.
- **Lennard L.** The clinical pharmacology of 6-mercaptopurine. *Eur J Clin Pharmacol* 43: 329-339, 1992.
- **Li B, Sehajpal PK, Khanna A, Vlassara H, Cerami A, Stenzel KH, Suthanthiran M.** Differential regulation of transforming growth factor beta and interleukin 2 genes in human T cells: demonstration by usage of novel competitor DNA constructs in the quantitative polymerase chain reaction. *J Exp Med* 174: 1259-1262, 1991.
- **Lin F, Fukuoka Y, Spicer A, Ohta R, Okada N, Harris CL, Emancipator SN, Medof ME.** Tissue distribution of products of the mouse decay accelerating factor (DAF) genes: exploitation of a Daf1 knock-out mouse and site-specific monoclonal antibodies. *Immunology* 108: 215-225, 2001.
- **Lin F, Kaminski HJ, Conti-Fine BM, Wang W, Richmonds C, Medof ME.** Markedly enhanced susceptibility to experimental autoimmune myasthenia gravis in the absence of decay-accelerating factor protection. *J Clin Inves* 110: 1269-1274, 2002.
- **Liu J, Miwa T, Hilliard B, Chen Y, Lambris JD, Wells AD, Song WC.** The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. *J Exp Med* 201: 567-577, 2005.
- **Lu NZ and Cidlowski JA.** The origin and functions of multiple human glucocorticoid receptor isoforms. *Ann N Y Acad Sci* 1024: 102-123, 2004.
- **Lublin DM and Atkinson JP.** Decay-accelerating factor: biochemistry, molecular biology, and function. *Annu Rev Immunol* 7: 35-58, 1989.
- **Majumdar S and Aggarwal BB.** Methotrexate suppresses NF-kappaB activation through inhibition of IkappaBalpha phosphorylation and degradation. *J Immunol* 167: 2911-2920, 2001.

- **Mantegazza R, Antozzi C, Peluchetti D, Sghirlanzoni A, Cornelio F.** Azathioprine as a single drug or in combination with steroids in the treatment of myasthenia gravis. *J Neurol* 235: 449-453, 1988.
- **Marieb EN.** Essentials of human anatomy & physiology 8th Edition. San Francisco: Benjamin-Cummings/Pearson, 2006.
- **Mason JC, Ahmed Z, Mankoff R, Lidington EA, Ahmad S, Bhatia V, Kinderlerer A, Randi AM, Haskard DO.** Statin-induced expression of decay-accelerating factor protects vascular endothelium against complement-mediated injury. *Circ Res* 91: 696-703, 2002.
- **Mason JC, Steinberg R, Lidington EA, Kinderlerer AR, Ohba M, Haskard DO.** Decay-accelerating factor induction on vascular endothelium by vascular endothelial growth factor (VEGF) is mediated via a VEGF receptor-2 (VEGF-R2) and protein kinase C-alpha/epsilon (PKCalpha/epsilon)-dependent cytoprotective signaling pathway and is inhibited by cyclosporin A. *J Biol Chem* 279: 41611-41618, 2004.
- **Matsuda S, Moriguchi T, Koyasu S, Nishida E.** T lymphocyte activation signals for interleukin-2 production involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. *J Biol Chem* 273: 12378-12382, 1998.
- **McFarlin DE, Engel WK, Strauss AJ.** Does myasthenic serum bind to the neuromuscular junction? *Ann N Y Acad Sci* 135: 656-663, 1966.
- **Medof ME, Kinoshita T, Nussenzweig V.** Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. *J Exp Med* 160: 1558-1578, 1984.
- **Medof ME, Walter EI, Rutgers JL, Knowles DM, Nussenzweig V.** Identification of decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. *J Exp Med* 165: 848-864, 1987.
- **Meri S, Morgan BP, Davies A, Daniels RH, Olavesen MG, Waldmann H, Lachmann PJ.** Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology* 71: 1-9, 1990.
- **Montesinos MC, Takedachi M, Thompson LF, Wilder TF, Fernández P, Cronstein BN.** The antiinflammatory mechanism of methotrexate depends on extracellular conversion of adenine nucleotides to adenosine by ecto-5'-nucleotidase: findings in a study of ecto-5'-nucleotidase gene-deficient mice. *Arthritis Rheum* 56: 1440-1445, 2007.

- **Mukaida N, Morita M, Ishikawa Y, Rice N, Okamoto S, Kasahara T, Matsushima K.** Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* 269: 13289-13295, 1994.
- **Nakano S and Engel AG.** Myasthenia gravis: quantitative immunocytochemical analysis of inflammatory cells and detection of complement membrane attack complex at the end-plate of 30 patients. *Neurology* 43: 1167-1172, 1993.
- **Nastuk WL, Strauss AJ, Osserman KE.** Search for a neuromuscular blocking agent in the blood of patients with myasthenia gravis. *Am J Med* 26: 394-409, 1959.
- **Nicholson-Weller A, Burge J, Fearon DT, Weller PF, Austen KF.** Isolation of a human erythrocyte membrane glycoprotein with decay accelerating activity for C3 convertases of the complement system. *J Immunol* 129: 184-189, 1982.
- **Petit E, Langouet S, Akhdar H, Nicolas-Nicolaz C, Guillouzo A, Morel F.** Differential toxic effects of azathioprine, 6-mercaptopurine and 6-thioguanine on human hepatocytes. *Toxicol In Vitro* 22: 632-642, 2008.
- **Pfaffl MW.** A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids Res* 29: e45, 2001.
- **Pham T, Kaul A, Hart A, Goluszko P, Moulds J, Nowicki S, Lublin DM, Nowicki BJ.** dra-related X adhesins of gestational pyelonephritis-associated Escherichia coli recognize SCR-3 and SCR-4 domains of recombinant decay-accelerating factor. *Infect Immun* 63: 1663-1668, 1995.
- **Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR, Leahy P, Li J, Andrade FH.** Extraocular muscle is defined by a fundamentally distinct gene expression profile. *Proc Natl Acad Sci U S A* 98: 12062-12067, 2001.
- **Rao A, Luo C, Hogan PG.** Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15: 707-747, 1997.
- **Ray A, Prefontaine KE, Ray P.** Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor. *J Biol Chem* 269: 12940-12946, 1994.
- **Ribichini F, Joner M, Ferrero V, Finn AV, Crimins J, Nakazawa G, Acampado E, Kolodgie FD, Vassanelli C, Virmani R.** Effects of oral prednisone after stenting in a rabbit model of established atherosclerosis. *J Am Coll Cardiol* 50: 176-185, 2007.

- **Riley-Vargas RC, Gill DB, Kemper C, Liszewski MK, Atkinson JP.** CD46: expanding beyond complement regulation. *Trends Immunol* 25: 496-503, 2004.
- **Rincón M and Flavell RA.** AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J* 13: 4370-4381, 1994.
- **Sahashi K, Engel AG, Lambert EH, Howard FM Jr.** Ultrastructural localization of the terminal and lytic ninth complement component (C9) at the motor end-plate in myasthenia gravis. *J Neuropathol Exp Neurol* 39: 160-172, 1980.
- **Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS Jr.** Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Mol Cell Biol* 15: 943-953, 1995.
- **Schmitz ML, Stelzer G, Altmann H, Meisterernst M, Baeuerle PA.** Interaction of the COOH-terminal transactivation domain of p65 NF-kappa B with TATA-binding protein, transcription factor IIB, and coactivators. *J Biol Chem* 270: 7219-7226, 1995.
- **Schreiber SL and Crabtree GR.** The mechanism of action of cyclosporin A and FK506. *Immunol Today* 13: 136-142, 1992.
- **Seitz M.** Molecular and cellular effects of methotrexate. *Curr Opin Rheumatol* 11: 226-232, 1999.
- **Simpson JA.** Myasthenia Gravis, a new hypothesis. *Scot Med J* 5: 419-436, 1960.
- **Smoak K and Cidlowski JA.** Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. *Mol cell Biol* 26: 9126-9135, 2006.
- **Soltys J, Gong B, Kaminski JH, Zhou Y, Kusner LL.** Extraocular muscle susceptibility to Myasthenia gravis. Unique immunological Environment? *Ann N Y Acad Sci* 1132: 220-224, 2008.
- **Soltys J, Kusner LL, Young A, Richmonds C, Hatala D, Gong B, Shanmugavel V, Kaminski HJ.** Novel complement inhibitor limits severity of experimentally myasthenia gravis. *Ann Neurol* 65: 67-75, 2009.
- **Spencer RF and Porter JD.** Structural organization of the extraocular muscles. *Rev Oculomot Res* 2: 33-79, 1988.

- **Spiller OB, Criado-García O, Rodríguez De Córdoba S, Morgan BP.** Cytokine-mediated up-regulation of CD55 and CD59 protects human hepatoma cells from complement attack. *Clin Exp Immunol* 121: 234-241, 2000.
- **Stankunas K, Graef IA, Neilson JR, Park SH, Crabtree GR.** Signaling through calcium, calcineurin, and NF-AT in lymphocyte activation and development. *Cold Spring Harb Symp Quant Biol* 64: 505-516, 1999.
- **Su B, Jacinto E, Hibi M, Kallunki T, Karin M, Ben-Neriah Y**. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 77: 727-736, 1994.
- **Thomas DJ and Lublin DM.** Identification of the 5'-flanking regions affecting the expression of the human decay accelerating factor gene and their role in tissue-specific expression. *J Immunol* 150: 151-160, 1993.
- **Tian H and Cronstein BN.** Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis. *Bull NYU Hosp Jt Dis* 65: 168-173, 2007.
- **Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, Lehr HA, Wirtz S, Becker C, Atreya R, Mudter J, Hildner K, Bartsch B, Holtmann M, Blumberg R, Walczak H, Iven H, Galle PR, Ahmadian MR, Neurath MF.** CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J Clin Invest* 111: 1133-1145, 2003.
- **Tobler A, Meier R, Seitz M, Dewald B, Baggiolini M, Fey MF.** Glucocorticoids downregulate gene expression of GM-CSF, NAP-1/IL-8, and IL-6, but not of M-CSF in human fibroblasts. *Blood* 79: 45-51, 1992.
- **Tüzün E, Scott BG, Goluszko E, Higgs S, Christadoss P.** Genetic evidence for involvement of classical complement pathway in induction of experimental autoimmune myasthenia gravis. *J Immunol* 171: 3847-3854, 2003.
- **Veal N, Hsieh C-L, Xiong S, Mato JM, Lu S, and Tsukamoto H.** Inhibition of lipopolysaccharide stimulated TNF-α promoter activity by S-adenosylmethionine and 5'-methylthioadenosine. *Am J Physiol Gastrointest Liver Physiol* 287: 352-362, 2004.
- **Walker MB.** Case showing the Effect of Prostigmin on Myasthenia Gravis. *Proc R Soc Med* 28: 759-761, 1935.

- **Wang G, Wang YJ, Luo FM, Wang L, Jiang LL, Wang L, Mao B.** Effective use of corticosteroids in treatment of plastic bronchitis with hemoptysis in Chinese adults. *Acta Pharmacol Sin* 27: 1206-1212, 2006.
- **Wasicky R, Ziya-Ghazvini F, Blumer R, Lukas JR, Mayr R.** Muscle fiber types of human extraocular muscles: a histochemical and immunohistochemical study. *Invest Ophthalmol Vis Sci* 41: 980-990, 2000.
- **Wray D and Porter V.** Calcium Channel Types at the neuromuscular Junction. *Ann N Y Acad Sci* 681: 356-367, 1993.
- **Zorzetto M, Ferrarotti I, Trisolini R, Lazzari Agli L, Scabini R, Novo M, De Silvestri A, Patelli M, Martinetti M, Cuccia M, Poletti V, Pozzi E, Luisetti M.** Complement receptor 1 gene polymorphisms are associated with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 168: 330-334, 2003.

Appendix A: General recipes and reagents

Tissue culture

Dulbucco's Modified Eagles Medium
13.55g DMEM
3.7g sodium hydrogen carbonate
Make to 1 litre with autoclaved ddH_2O
pH to 7.2
Filter through 0.22 µM filter.
Store at 4°C. Medium older than 3 weeks was re-supplemented with L-glutamine because of its instability.

Penicillin/Streptomycin (P/S)
900mg penicillin
1500mg streptomycin
Make up to 150 mls in sterile 1x PBS
Filter through 0.22µM filter
Store at -20°C.

Trypsin-EDTA
8g NaCl
1.26g Na_2HPO_4
0.2g KCl
0.2g KH_2PO_4
0.5g Trypsin
0.2g EDTA
Make up to 1 litre with dH_2O
pH to 7.4
Filter sterilize through 0.22 µM filter
Store at 4°C.

Hoechst Stain Working Solution
1ml Hoechst 33258
100 mls Hanks balanced salt solution
Wrap in tin foil and store at 4°C.

Mounting Fluid
22.2 mls 0.1M Citric acid
27.8 mls 0.2M $Na_2HPO_4.2H_2O$
50 mls Glycerol
Make up to 100 mls, pH to 5.5
Store at 4°C.

Gel electrophoresis

2% Agarose Gel
1g agarose powder
50 mls 1xTBE (see below)
Heat solution to dissolve agarose
Cool to approximately 50°C
Pour and allow to solidify.

10x Tris-Borate-EDTA (TBE) Electrophoresis Buffer
108g Tris
55g Boric acid
40 mls 0.5M EDTA (pH 8.0)
Make up to 1 litre with dH_2O
Store at room temperature
For use, dilute to 1x.

Amplification of plasmids

Luria Broth (LB)
10g Bacto-tryptone
5g Yeast extract
10g NaCl
Make up to 1 litre with ddH_20
pH to 7.0
Autoclave and store at room temperature.

LB agar
15g agar to 1 litre of LB
Autoclave and store at room temperature.

Luciferase assays

Serum-free medium
49.25 mls DMEM
250 µl FBS
250 µl P/S
Store at 4°C.

RNA extraction

DEPC Treatment
0.1% DEPC in distilled water
Stir for 30 mins
Soak pipette tips and micro-centrifuge tubes in DEPC-treated water overnight.
Remove as much of the DEPC-treated water then autoclave.

Western blotting

RIPA buffer with SDS
150 mM NaCl
1% Triton X-100
0.1% SDS
20 mM Tris pH 7.5
1% deoxycholate
Make up to 50 mls with dH_2O
Store at 4°C.

Supplement with 1 mg/ml aprotinin, 1 mg/ml pepstatin A, 2 mM phenylmethanesulfonyl fluoride protease inhibitors (Sigma, USA) and 25x Proteinase Inhibitor Cocktail (Roche, Germany) just before use.

RIPA Buffer without SDS
150 mM NaCl
1% Triton X-100
50 mM Tris pH 8.0
Make up to 10 mls with ddH_2O.
Filter sterilize with 0.45µm filter. Store at 4°C.
Store at 4°C.

Supplement with 1 mg/ml aprotinin, 1 mg/ml pepstatin A, 2 mM phenylmethanesulfonyl fluoride protease inhibitors (Sigma, USA) and 25x Proteinase Inhibitor Cocktail (Roche, Germany) just before use.

10x Phosphate Buffered Saline (PBS)
160g NaCl
4g KCl
25.2g anhydrous $Na_2HPO_4.12H_2O$
4.8g KH_2PO_4
Make up to 2 litres with dH_2O
pH to 7.4 then autoclave.
Store at room temperature.

1x PBS/0.1% Tween
1x PBS made up to 1 litre
1 ml Tween 20
Mix well with stirrer bar
Store at 4°C

5% Skim-milk Blocking Buffer
5% powder skim milk (w/v)
Make up to 50 mls with 1x PBS/0.1% Tween
Store at 4°C.

Membrane Stripping Buffer
100 nM β-Mercaptoethanol
2% SDS
62.5 mM Tris-Cl pH 6.7
Fill up to final volume with dH_2O.
Cover in foil and store at room temperature.

Calcein Assay

1x Calcein Buffer
Dilute 10x Calcein AM DW Buffer to 1x with ddH_2O.

2 µM Calcein-AM
Suspend 50 µg Calcein-AM in 25 µl of anhydrous DMSO to make a 2 mM Calcein AM Stock Solution.
For 2 µM Calcein-AM working solution dilute in 1x calcein buffer (above).

Appendix B: Drug preparations

1. Prednisone (Sigma P6254-1g)

For 60 µM stock and treatment:

Dissolve 0.022g prednisone in 1 ml pure ethanol:chloroform (1:1 ratio). Cover stock and working solutions with foil and store at -20°C.

2. Cyclosporin A (Sigma C1832-5mg)

For 10 µM stock:

Dissolve 5 mg cyclosporin A in 0.416 µl pure ethanol. Cover stock and working solutions with foil and store at -20°C.

3. Methotrexate (Sigma M8407-100mg)

For 22 µM stock and treatment:

Dissolve 9.9968 mg methotrexate in 1 ml 0.25M Na_2CO_3. Cover stock and working solutions with foil and store at -20°C.

4. Azathioprine (Sigma A4638-1g)

For 10 µM stock:

Dissolve 2.77 mg of azathioprine in 1 ml NH_4OH. Cover stock and working solutions with foil and store at -20°C.

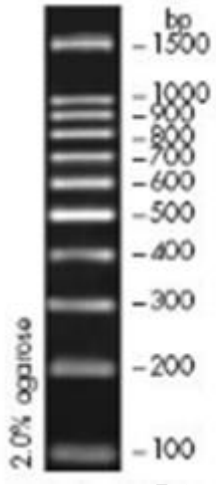
5. Lipopolysaccharide (Sigma L4391-1mg)

For 10 µg/ml working solution:

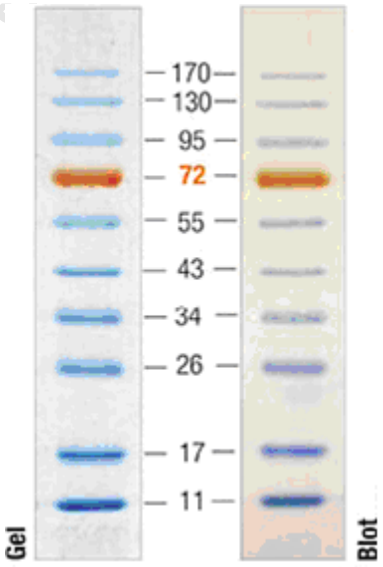
Dissolve 1 mg in 2 mls autoclaved 1 x PBS. Cover with foil and store at -20°C.

Appendix C: Molecular weight markers

GelPilot 100bp Plus (QIAGEN, USA)



peqGold Prestained Protein Marker IV
(PeqLab Biotechnologie, Germany)



Appendix D: Supplementary data

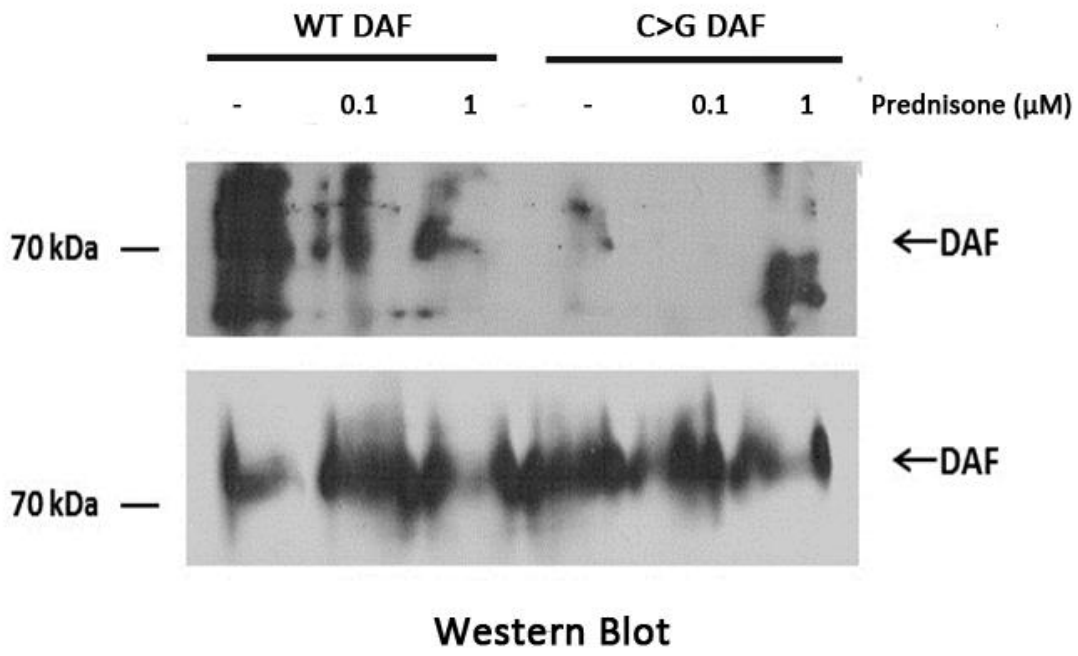


Figure 5.1: Two examples showing the technical difficulties experienced with the human DAF monoclonal BRIC 216 antibody (IBGRL, UK). Total protein lysate was isolated from lymphoblasts 24 hrs post-treatment with 0.1 and 1 μM prednisone. Protein from each sample was analysed on an 8% SDS-PAGE and analysed by western blotting using an antibody to DAF. Arrows indicate the position of DAF as determined by the protein marker banding pattern shown on the left in kilodaltons (kDa).