

Potential mechanisms in MuSK-Myasthenia Gravis

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A thesis submitted for the degree of Doctor of Philosophy



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Abstract

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Autoimmunity is a failure to tolerate circulating or cell surface expressed self antigens, leading to activation of the immune system and attack of self tissues. Muscle-specific kinase (MuSK) myasthenia gravis (MG) is a disease caused by antibodies to MuSK and hallmarked by fatigable muscle weakness. MuSK is a tyrosine kinase that interacts with low-density lipoprotein receptor-related protein 4 (LRP4), resulting in maintenance of the high density of acetylcholine receptors (AChRs) at the neuromuscular junction; this high density is essential for efficient transmission of signals from nerve to muscle, and MuSK antibodies impair this transmission. MuSK antibodies are predominantly IgG4, a subclass that does not induce immunological damage. Thus how these antibodies cause neuromuscular junction dysfunction is not clear. Potential mechanisms of the MuSK antibodies were explored in *in vitro* experiments.

Plasmas from fourteen MuSK-MG patients were studied. IgG antibodies and IgG subclass profiles were measured with flow cytometry. Total IgG, Fabs, IgG4 and IgG1-3 subclass antibodies were prepared and purified; these were used to investigate the effects on MuSK surface expression, binding of LRP4 to MuSK, and agrin-LRP4-MuSK-induced AChR clustering in C2C12 mouse myotubes.

No evidence for MuSK endocytosis by MuSK IgG, IgG1-3 or IgG4 antibodies was found. The predominant IgG4 subclass, and the monovalent IgG Fabs, blocked binding between LRP4 and MuSK but both IgG4 and IgG1-3 subclass antibodies were equally able to disperse pre-formed and newly-induced AChR clusters in C2C12 myotubes.

The block of LRP4-MuSK interaction by IgG4 antibodies is likely to be a major pathogenic mechanism in MuSK-MG, which may lead to disrupted signal transduction, reduced AChR aggregation and neuromuscular transmission failure at the neuromuscular junction. In addition, MuSK IgG1-3, until now described as non-pathogenic, may also contribute to the reduced AChR density and neuromuscular dysfunction in MuSK-MG. These results provide new evidence concerning the pathogenic antibodies and their mechanisms in MuSK-MG.

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Chapter 1: Introduction

1.1 Autoimmune disease

Autoimmune diseases are individually rare, with the exception of rheumatoid arthritis and autoimmune thyroiditis, but taken together they affect approximately 5 percent of the population in Western countries (Sinha et al., 1990, Jacobson et al., 1997) and in women under 65 years of age, autoimmune diseases are among the ten leading causes of death (Rose and MacKay, 2006). At the end of the 19th Century, Paul Ehrlich first described the theory of “horror autotoxicus”. He hypothesized that anti-toxins in the blood serum could be directed against self-structures of the body, though he falsely concluded that these would not lead to disease (reviewed by (Silverstein, 2001)).

Autoimmune diseases arise due to lack of immune tolerance. In consequence, immune cells can be activated and cause an inappropriate immune response which may be directed to autoantigens. This produces antibodies and immune cells that can damage tissues that express the antigens. An example is autoimmune thyroiditis, first described in 1912 (Hashimoto, 1912), in which components of the thyroid gland are targeted by the immune system (Roitt et al., 1956). Autoimmune diseases are defined by the postulates first described by Ernst Witebsky. The updated “Witebsky postulates” define an autoimmune disease by three characteristics: 1. Direct evidence of pathogenic antibody or pathogenic T-cells, 2. Indirect evidence based on the reproduction of the autoimmune disease in experimental animals and 3. Circumstantial evidence from clinical cues (Witebsky et al., 1957, Rose and Bona, 1993).

One of the best known and most informative autoimmune diseases is myasthenia gravis (MG, from Greek: myos = muscle, asthenos = weakness, and

Latin: gravis = severe). MG is a disease of the neuromuscular junction characterised by fatigable muscle weakness. In 1960, John Simpson hypothesized that MG was an autoimmune disease (Simpson, 1960). This hypothesis was subsequently demonstrated to be true, and indeed MG was the first neurological disease identified to be antibody-mediated. MG fulfils the criteria of the Witebsky postulates, because pathogenic antibodies are present (Patrick and Lindstrom, 1973, Lindstrom et al., 1976), the disease is reproducible in experimental animals (Patrick and Lindstrom, 1973, Tokya et al., 1975), and most important, IgG antibodies are present at the neuromuscular junction (Engel et al., 1977), the patients improve clinically following plasma exchange (Pinching and Peters, 1976, Newsom-Davis et al., 1978) and the disease can be passively transferred to mice by injection of purified IgG antibodies (Tokya et al., 1975).

1.2 Myasthenia gravis

1.2.1 The history of Myasthenia gravis

In 1672, myasthenia gravis was described, probably for the first time, by Thomas Willis, an Oxford physician:

“...those labouring with a want of Spirits, who will exercise local motions, as well as they can, in the morning are able to walk firmly, to fling their Arms hither and thither, or to take up any heavy thing; before noon the stock of the Spirits being spent, which had flowed into the Muscles, they are scarce able to move Hand or Foot. At this time I have under my charge an Honest Woman, who for many years hath been obnoxious to this sort of spurious Palsie, not only in her Members, but also in her tongue; she for some time can speak freely and readily enough, but after she has spoke long, or hastily, or eagerly, she is not able to speak a word, but she becomes mute as a Fish, nor can

she recover the use of her voice under an hour or two.” ((Willis, 1672), translated by Pordage 1683)

As indicated above by Willis, the main symptom of myasthenia gravis is fatigable weakness of skeletal muscles that can also affect limb and respiratory muscles, which can be life-threatening. The weakness is exacerbated by effort and improved by rest. Samuel Wilks (Wilks, 1877), Wilhelm Erb (Erb, 1879) and Samuel Goldflam (Goldflam, 1893) described the clinical features of myasthenia gravis in detail, with ocular symptoms such as ptosis (weakness of the eyelid) as early symptoms, followed by weakness of muscles required for speaking, chewing and swallowing, that then spread to other muscles towards a more generalised weakness. They also emphasised the fluctuating weakness and the occurrence of relapses and remissions over time. The disease was thus referred to as Erb-Goldflam disease until in 1895 Friedrich Jolly (Jolly, 1895) renamed the disease “myasthenia gravis pseudoparalytica”. Six years later, sixty cases of Myasthenia gravis had already been reviewed (Campbell and Bramwell, 1900). By then, Jolly had demonstrated for the first time that neuromuscular transmission was affected in the patients, by showing that repetitive stimulation of a nerve results in decreased contraction of the innervated muscle (Jolly, 1895). Yet the exact mechanism how the neuromuscular transmission was affected was difficult to understand, because the mechanism by which nerve impulses are transmitted towards the muscle was yet to be discovered.

The chemical nature of neuromuscular transmission was first described in 1921 by Otto Loewi (Loewi, 1921). In 1934, Mary Walker found that an injection of pyridostigmine improved facial weakness in MG patients. It was already known that pyridostigmine prevented the break-down of acetylcholine (ACh), and taken together with Henry Dale’s findings that ACh release is crucial for neuromuscular

transmission (Dale and Feldberg, 1934, Dale et al., 1936), it was concluded that MG was thought to be a curare-like poisoning of the “motor end-organs”. Notably, pyridostigmine is still used today as a symptomatic treatment of MG.

In 1952, Fatt, Katz and Miledi described the quantal nature of ACh release; this is the release of neurotransmitter in the form of packets rather than as single molecules, which then evoke a depolarisation of the muscle membrane. The spontaneous release of one packet of ACh can be measured as a miniature end plate potential (MEPP) (Katz and Miledi, 1965, Fatt and Katz, 1952). The MEPPs were found to be reduced in amplitude in muscle biopsies from MG patients (Elmqvist et al., 1964), which suggests either a reduced amount of ACh in each packet or reduced sensitivity for ACh on the muscle membrane. In 1959, Coers and Desmedt described for the first time the characteristic histological malformations of the neuromuscular junction found in MG (Coers and Desmedt, 1959), and in the next year, Simpson and Nastuk independently suggested that MG may be an autoimmune disease (Simpson, 1960, Nastuk et al., 1960); Simpson’s hypothesis was based, among other aspects, on the occasional transfer of the disease from a mother to her child.

With the isolation of alpha-bungarotoxin (Chang and Lee, 1966, Changeux et al., 1970), a neurotoxin that binds to the AChR, from krait snakes, and the isolation of AChRs from the electric organs of animals such as the Torpedo fish (Miledi et al., 1971), it became possible to characterise the AChR in more detail. In 1973, Patrick and Lindstrom used AChR from the electric organ of eels to inject into rabbits, with the aim to produce antibodies. The rabbits did produce antibodies against AChR but these antibodies cross-reacted with the rabbits’ own AChRs and led to an autoimmune attack, with paralysis and death of the rabbits. Thus experimental autoimmune myasthenia gravis (EAMG) was first described (Patrick and Lindstrom, 1973). The

AChR antibodies were shown to block AChR function (Patrick et al., 1973). And using radioactive alpha-bungarotoxin, Fambrough and Drachman found that the number of AChR was markedly reduced in MG patient biopsies compared to biopsies from healthy individuals (Fambrough et al., 1973).

In 1975, the first reports of antibodies to the patients themselves began to emerge, mainly using a radioimmunoprecipitation assay (Lindstrom et al., 1976). Meanwhile Tokya and colleagues demonstrated that they could induce MG in mice by passive transfer of patient IgG (Tokya et al., 1975). The benefit of removing the pathogenic AChR antibodies from the circulation of the patients by plasma exchange was first demonstrated by John Newsom-Davis, Pinching and colleagues (Pinching and Peters, 1976, Newsom-Davis et al., 1978). Engel and colleagues found that there was IgG and complement deposited at the neuromuscular junctions – both in MG muscle biopsies and in EAMG mice (Engel et al., 1977, Sahashi et al., 1980).

Thus within a short time, MG was shown to be caused by antibodies towards the AChR, leading to complement-mediated damage to the neuromuscular junction, reduction of AChR and neurotransmission failure. However, MG was found to be a heterogeneous disease, with different forms of MG associated with different HLA, thymus pathology, age of onset (see below) (Aarli et al., 1990, Pirskanen, 1976, Compston et al., 1980) and subsequently the antibody target (Hoch et al., 2001, Higuchi et al., 2011, Pevzner et al., 2012). Despite this heterogeneity, all MG appears to be due to loss of AChRs and impairment of neuromuscular transmission.

1.2.2 Neuromuscular transmission at the neuromuscular junction

At the adult NMJ, the presynaptic motor axon terminal is directly opposite the postsynaptic membrane where the acetylcholine receptors (AChRs) are densely clustered at the top of postsynaptic membrane folds. The high concentration of

AChRs is crucial for efficient neuromuscular transmission. The motor nerve action potential leads to the opening of voltage-gated calcium channels in the presynaptic motor nerve membrane, and the influx of calcium induces the release of ACh from the synaptic vesicles, mediated by SNARE proteins, into the synaptic cleft (Sollner et al., 1993, Schiavo et al., 1997). ACh then rapidly crosses the synaptic space and binds to and opens the AChR, which is a ligand-gated cation channel, resulting in muscle contraction.

The influx of sodium through the AChR causes depolarisation of the postsynaptic membrane, which is also named the end-plate potential (EPP). If the EPP is higher than the threshold for activation of the muscle voltage-gated sodium channels, they open and propagate the muscle action potential throughout the muscle fibre. The muscle action potential is then translated into muscle contraction in a process called excitation-contraction coupling (Sandow, 1952). Communication from the motoneuron to muscle is also essential for correct formation, maintenance and function of the NMJ as will be discussed below (section 1.3.3).

1.2.3 Clinical aspects

Myasthenia gravis with antibodies to the AChR is the most common disorder of the NMJ, with a prevalence between 70 and 163 per million (Carr et al., 2010). The disease has a bimodal age of onset, with a peak in the second and third decade with a female predominance of 1:3 (male:female), and a more even gender distribution of 1.5:1 (male:female) in the second peak in the sixth and seventh decade (reviewed by (Vincent and Drachman, 2002)). Skeletal muscle weakness occurs in a fluctuating manner, which exacerbates with activity. Ocular muscles are usually involved early in the disease (reviewed by (Silvestri and Wolfe, 2012, Meriggioli and Sanders, 2009,

Vincent et al., 2001, Vincent, 2008)), usually followed by weakness of bulbar muscles that are involved in chewing, speaking and swallowing, and/or generalized muscle weakness with involvement of limb muscles (Grob et al., 2008). Respiratory muscle weakness occurs in up to 20% of the patients, and can be life threatening.

The diagnosis of MG involves the clinical picture, antibody tests and electrophysiological tests of neuromuscular transmission. Repetitive nerve stimulation at 3Hz typically shows a decrement of approximately 10% of the amplitude of compound muscle action potentials (Lange, 1997). A further test is single fibre electromyography, which measures the firing time between the nerve stimulus and the muscle contraction between two NMJs innervated by the same motor unit. In MG, occasional blocking and increased variability (jitter) can be found (Lange, 1997). *In vitro* study of muscle biopsies shows that the MEPP amplitudes are markedly reduced in the weak muscles of the patients (Elmqvist et al., 1964), which correlates with the loss of AChRs found by alpha-bungarotoxin binding (Fambrough et al., 1973).

There is also an increase in the quantal content of ACh, which appears to compensate for the reduced neuromuscular transmission caused by loss of AChR (Plomp et al., 1995). The quantal content can be assessed by electrophysiology, in which both EPPs and MEPPs are measured and compared. The amplitude of the EPPs indicates the total depolarisation that results from each nerve impulse in contrast to the MEPP which is caused by the release of a single packet of ACh. Measuring both allows one to establish the quantal content, which is the number of packets of ACh released from the motor nerve per impulse. While the MEPPs in AChR-MG patients are decreased, the EPPs are not as small as would be expected. This suggests that the motor nerve senses the lack of postsynaptic depolarisation and compensates by increasing the quantal content. Taken together, electrophysiological tests suggest a

reduction in the number and density of AChR, that cannot fully be compensated by increased quanta of ACh, as a cause for reduced neuromuscular transmission.

1.2.4 Acetylcholine receptor antibodies

As summarised above, the main defect in MG appears to be the loss of AChRs that result from binding of antibodies. The pathogenic mechanisms by which the antibodies lead to loss of AChRs has been established for some time.

1.2.5 Pathogenic mechanisms of acetylcholine receptor antibodies

There are three main mechanisms: (1) Fixation of complement via the classical complement pathway (Aharonov et al., 1975, Lennon and Lambert, 1981). AChR antibodies belong mainly, but not exclusively, to the complement fixing IgG1 and IgG3 subclass (Rodgaard et al., 1987, Vincent and Newsom-Davis, 1982, Lefvert et al., 1981); furthermore, C3, a component of the complement system, was found to be colocalised with patient IgG at the NMJ of patients (Engel et al., 1977). Activated C3 initiates the formation of the membrane attack complex which colocalises with AChR. This leads to complement-mediated damage of the endplate and lyses the postsynaptic membrane which leads to a loss of AChR at the NMJ (Nakano and Engel, 1993). (2) Antigenic modulation, cross-linking and endocytosis of AChR (the result of divalent binding of the antibodies) (Drachman et al., 1978, Heinemann et al., 1977). In these studies, patient IgG that bound to AChRs caused increased turnover and degradation of AChR in skeletal muscle cultures. Patient derived Fab fragments, although they bound to the AChR, did not show this effect. Only when the Fabs were cross-linked by the use of a secondary antibody could an increase in AChR turnover and degradation be observed. This suggests that patient IgG require divalent binding and

cross-linking of AChRs to induce endocytosis. (3) Direct block of function (Patrick et al., 1973) which, although probably not common, can occur in some patients (Vincent et al., 1993).

1.2.6 Heterogeneity of Myasthenia gravis

In most patients, myasthenia gravis is caused by antibodies directed against the AChR (AChR-MG). These patients can be divided in several subgroups.

Early onset MG (EOMG) is defined as presenting before the age of 40, and most patients are females. EOMG is genetically associated with HLA-B8 and DR3 (Compston et al., 1980), and the patients often have an enlarged thymus gland and respond well clinically to thymectomy.

Late onset MG (LOMG) is defined as MG with an age of onset of 40 years (or 50 years in some studies), with a bias towards men (Aarli, 2008). The genetic association is with B7 and DR2 (Compston et al., 1980).

Ten percent of MG patients are diagnosed with thymoma, usually identified when they present with MG (Grob et al., 2008). Thymoma-associated MG is most common in between 30 and 60 years of age, and has no clear gender predominance or genetic association (reviewed by (Vincent et al., 2001)). Antibodies to other, usually intracellular, muscle antigens such as titin and ryanodine receptor are common in these patients (Aarli et al., 1998, Marx et al., 1998).

Ocular myasthenia gravis is defined as MG that presents exclusively with symptoms and signs restricted to the eye muscles. There is no specific age of onset, but early onset is more common in females, whereas overall it is more common in males (Grob et al., 2008). The AChR antibody titres tend to be low (Lindstrom et al.,

1976), and undetectable in 40-60% of cases, but can become positive, if the disease progresses (reviewed by (Vincent et al., 2001)).

About 10-15% of MG patients have no detectable AChR antibodies and are defined as having "seronegative" MG (SNMG). In one study, up to 66% of SNMG cases were subsequently found to have antibodies that bound only to clustered AChRs and could be detected in cell based assays using AChRs clustered with rapsyn (Leite et al., 2008).

The AChR is not the exclusive antigenic target at the NMJ. In 2001, muscle-specific tyrosine kinase (MuSK) was discovered as a new neuromuscular antigen (Hoch et al., 2001), and it was found that variable numbers of SNMG patients had antibodies to MuSK rather than to AChR (Sanders et al., 2003, Scuderi et al., 2002). MuSK-MG is described in detail below (section 1.5). In more recent publications, antibodies against low density lipoprotein receptor-related protein 4 (LRP4) (Higuchi et al., 2011, Zhang et al., 2012, Richman, 2012, Pevzner et al., 2012, Motomura and Higuchi, 2012, Cossins et al., 2012a) were identified in a proportion of patients, but some of these have MuSK antibodies at higher levels (Higuchi et al., 2011). The number of patients and clinical characteristics are not yet well defined (Berrih-Aknin and Le Panse, 2013).

There are also genetic disorders of neuromuscular transmission, called congenital myasthenic syndromes, that are caused by mutations in some of the key functional molecules, particularly the AChR, rapsyn and DOK7, and much less frequently, MuSK (Finlayson et al., 2013).

1.3 MuSK myasthenia gravis – a partially different disease

1.3.1 Epidemiology

Formal epidemiology data for MuSK-MG are only available for two regions in Europe, with a prevalence of 2.9 per million in Greece and 1.9 per million in southern Holland (Carr et al., 2010, Niks et al., 2007), much lower than the prevalence for AChR-MG with 70-163 per million (Carr et al., 2010). The number of MuSK-MG patients reported from different myasthenia centres suggests a strong north to south increase in Europe and elsewhere (Vincent, unpublished data). The influence could be environmental, but also differs between different ethnic groups, suggesting a genetic influence.

1.3.2 Genetics

AChR-MG is associated with HLA-B8DR3 (Compston et al., 1980), HLA-A02 and HLA-A25 (Vandiedonck et al., 2009), whereas there is an association of MuSK-MG with HLA-DR14-DQ5 (Niks et al., 2006), suggesting (different) genetic factors in the development of each form of MG.

In addition, several reports suggest a higher frequency in the Afro-American population (Sanders et al., 2003, Stickler et al., 2005, Oh et al., 2006, Oh, 2009, Gupthill and Sanders, 2010). On the other hand, the incidence of MuSK-MG in populations other than the Afro-American (Motomura, 2011) is consistent with a role for other factors.

1.3.3 Clinical aspects

MuSK-MG has a strong female predominance (Evoli et al., 2003, Illa et al., 2005, Lavernic et al., 2005, Stickler et al., 2005) of up to 90% (Guptill and Sanders, 2010). The average age of onset is in the third decade (Zhou et al., 2004, Evoli et al., 2003, Stickler et al., 2005), but geriatric, paediatric and neonatal cases can also be found (Pasnoor et al., 2010, Niks et al., 2008b, Behin et al., 2008). In MuSK-MG patients (Scuderi et al., 2002, Sanders et al., 2003, Hoch et al., 2001) muscle weakness is often focused on cranial and bulbar muscles, and with a higher frequency of respiratory crises than in AChR-MG (Evoli, 2006), and less purely ocular disease (Berrih-Aknin and Le Panse, 2013, Guptill and Sanders, 2010). There appear to be three distinct clinical phenotypes in MuSK-MG:

- 1) a form indistinguishable from AChR-MG with more generalized muscle weakness,
- 2) a more focal form with neck, shoulder and respiratory muscle weakness, and
- 3) a severe form with prevalent bulbar weakness and frequent respiratory crises (Illa et al., 2005, Lee et al., 2006, Ohta et al., 2007, Evoli et al., 2008, Chang et al., 2009, Guptill and Sanders, 2010).

Thus there may be more than one pathogenic mechanism at work, but such distinctions are also found in myasthenia with AChR antibodies (e.g. ocular only MG and generalised MG), and no clear explanation has yet been provided. It is likely that patient-specific differences in the susceptibility of the muscles (for instance the extent of MuSK expression) are at least one reason for these distinctions, although differences in pathogenic mechanisms of the antibodies could certainly contribute.

1.3.4 Response to treatment

Acetylcholinesterase (AChE) inhibitors that prolong the exposure of AChRs to ACh are used as symptomatic treatment in AChR-MG, but MuSK-MG patients often do not respond well and side effects are more evident (Evoli et al., 2008, Hatanaka et al., 2005). In AChR-MG, plasma exchange (PEX) (Pinching and Peters, 1976) and intravenous immunoglobulin (IVIG) treatments (Fateh-Moghadam et al., 1984) were established for use in acute exacerbation and myasthenic crisis and these are also used in MuSK-MG (reviewed by (Evoli et al., 2008). The thymus is less abnormal in MuSK-MG (Leite et al., 2005, Saka et al., 2005), and thymectomy is not usually beneficial in MuSK-MG (Evoli et al., 2003, Bartoccioni et al., 2006, Evoli et al., 2008). General immunosuppression with prednisone alone or in combination with azathioprine or cyclosporine are described to be beneficial for most patients (Evoli et al., 2008, Evoli et al., 2003). Rituximab, which is a therapeutic anti-CD20 antibody that depletes B-cells, is emerging as a promising new therapy in MuSK-MG (Hain et al., 2006, Thakre et al., 2007, Nowak et al., 2011, Blum et al., 2011, Diaz-Manera et al., 2012, Keung et al., 2013), where it is more effective than in AChR-MG (Illa et al., 2008, Diaz-Manera et al., 2012).

1.3.5 The importance of MuSK in the structure and function of the NMJ

For a review of potential mechanisms in MuSK-MG, it is important to understand the important role of MuSK in the development and maintenance of the NMJ. MuSK is a key organizer of NMJ development. Figure 1.1A shows the main proteins that are involved in NMJ development, maintenance and function. Targeted mutations in many of these proteins result in NMJ failure and often foetal or neonatal death in animal models (Weatherbee et al., 2006, DeChiara et al., 1996, Okada et al., 2006, Gautam et al., 1995, Gautam et al., 1996). During development, agrin, a heparin

sulphate proteoglycan, is released by the motoneuron (McMahan, 1990), and interacts with LRP4 (Kim et al., 2008, Zhang et al., 2008), which then associates with MuSK. This leads to activation of the MuSK tyrosine kinase, which stimulates a signalling pathway that culminates in AChR clustering (Glass et al., 1996, DeChiara et al., 1996). The details of this are still not known, but DOK7, a muscle-intrinsic activator of MuSK, binds to MuSK and is essential for down-stream signalling and NMJ formation (Okada et al., 2006). DOK7 is not only an activator of MuSK, but becomes phosphorylated and then serves as an adapter protein that recruits Crk and Crk-L (Hallock et al., 2010). Eventually, rapsyn, a cytoplasmic protein, associates with the AChR subunits (Burden et al., 1983, LaRochelle and Froehner, 1986), and leads to effective AChR clustering (Gautam et al., 1995).

In more detail, the neural isoform of agrin contains a small insert of 4 amino acids in the y-site and 8, 11 or 19 amino acids at the z-splicing site that are required for AChR clustering ((Ferns et al., 1993), reviewed by (Burgess et al., 2000)). Agrin binds to LRP4 on the post-synaptic membrane (Zhang et al., 2008, Kim et al., 2008), and a recent crystal structure showed that the 8 amino acid insert mediates this interaction (Zong et al., 2012). Binding of agrin to LRP4 then enhances the binding of LRP4 to the first Ig-like domain of MuSK (Zhang et al., 2011). This leads to MuSK activation by MuSK dimerisation and trans-autophosphorylation of Tyr553 (Herbst and Burden, 2000). MuSK pTyr553 binds to the phosphotyrosine-binding domain of adaptor protein DOK7. Dimerisation of MuSK-associated DOK7 further stimulates MuSK dimerisation and transphosphorylation of three tyrosine residues in the activation loop of MuSK, increasing its catalytic activity (Bergamin et al., 2010). One report indicated that after activation by agrin, MuSK is internalised, and that this

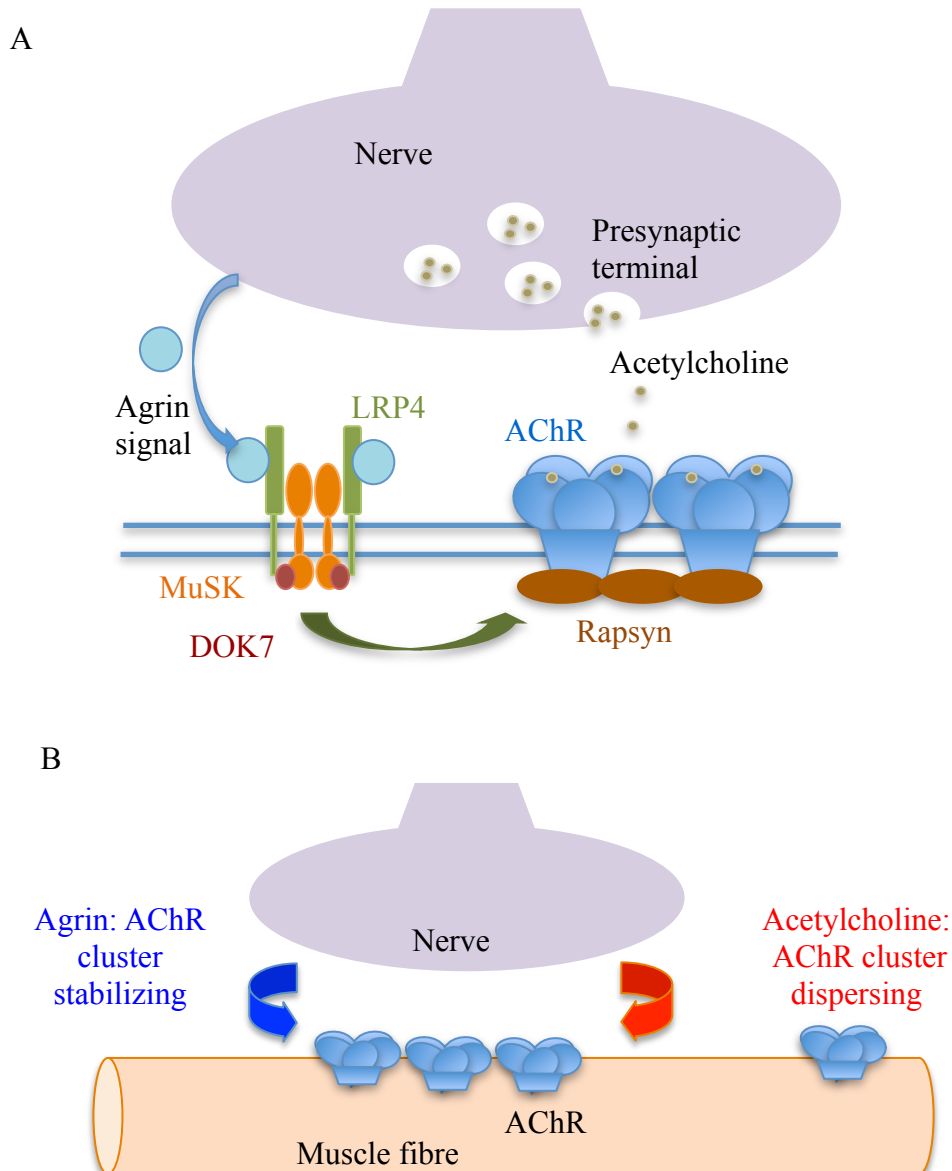


Figure 1.1: The neuromuscular synapse. (A) Development of the neuromuscular junction. Agrin is released by the motoneuron into the synaptic cleft and binds to LRP4 in the postsynaptic membrane. This induces close interactions with MuSK, which then leads to dimerisation and autophosphorylation of MuSK followed by binding of DOK7 to MuSK, cumulating via downstream signalling in dense aggregation of AChR by rapsyn adjacent to the motoneuron. The neurotransmitter acetylcholine is released by the motoneuron and binds to the AChR as a signal for muscle contraction, and thus mediate neuromuscular transmission. Adapted from Koneczny et al., 2013. (B) Positive and negative signals regulate AChR clustering. Agrin is a positive signal for AChR cluster stabilisation, and acetylcholine is a negative signal that leads to dispersal of AChR clusters. Adapted from Wu et al., 2010.

process is essential for MuSK downstream signalling and AChR clustering (Zhu et al., 2008), but this has not been confirmed.

There are aspects of neuromuscular transmission which modify the development of the NMJ. AChR subunit transcription is restricted to synaptic nuclei (Merlie and Sanes, 1985), and AChRs from aneural AChR clusters may be redistributed towards the synaptic area (Anderson and Cohen, 1977). ACh, released by the motoneuron, binds and activates AChR. The resulting muscle activation leads to a change in signal transduction (Fu et al., 2005, Lin et al., 2005, Tang et al., 2001) that inhibits AChR clustering, suppresses AChR expression (Salpeter et al., 1986, Tang et al., 2004) and promotes fragmentation and dissipation of AChR clusters (Lin et al., 2005, Misgeld et al., 2005, Wu et al., 2010, Brandon et al., 2003, Misgeld et al., 2002). These negative signals need to be counteracted by positive signals to ensure the high density of AChR at the NMJ. One of these signals is agrin, released by the motoneuron (figure 1.1B, (McMahan, 1990, Misgeld et al., 2005) also reviewed by (Wu et al., 2010)).

1.3.6 The structure of MuSK and LRP4

The interaction between MuSK and LRP4 is crucial in the agrin-LRP4-MuSK pathway (Kim et al., 2008, Zhang et al., 2008). MuSK is a type I single-pass transmembrane glycoprotein, that has three extracellular Immunoglobulin-like domains (Ig1-3) and a cysteine-rich domain that is similar to the CRD domain of Frizzled proteins. A transmembrane region, consisting of a single helix, connects the extracellular domain to the cytoplasmic region that consists of a juxtamembrane

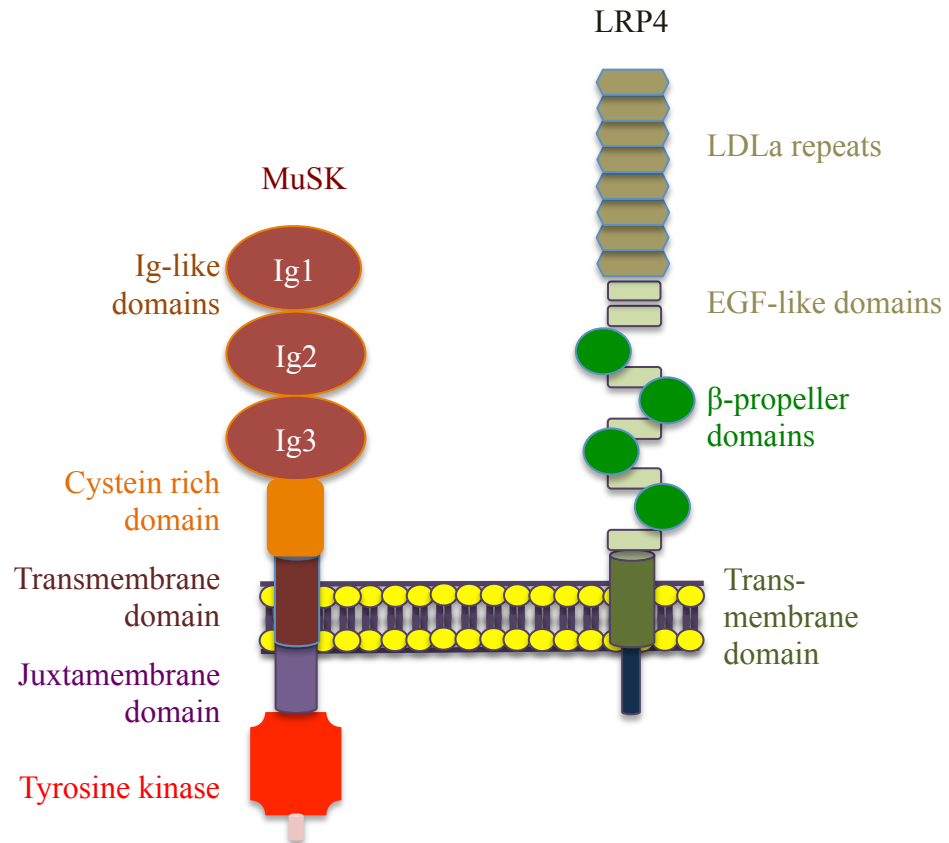


Figure 1.2: The structure of MuSK and LRP4. MuSK consists of three Ig-like domains (Ig1-3), a frizzled-like cystein rich domain, a transmembrane domain, a juxtamembrane domain and a tyrosine kinase domain followed by a short cytoplasmic tail. LRP4 is comprised of eight LDL (class a) repeats, followed by two EGF-like domains, four β -propeller domains intermitting with four further EGF-like domains, a transmembrane domain and a short cytoplasmic tail. Adapted from Burden et al., 2013.

domain, the tyrosine kinase domain and a short cytoplasmic tail (figure 1.2). The atomic structure has been studied with X-ray crystallography (Stiegler et al., 2006, Stiegler et al., 2009, Till et al., 2002).

The first Ig-like domain has a key role in MuSK activation. An isoleucine (Ile96) in this region was found to be part of the LRP4 binding site on MuSK (Zhang et al., 2011). Furthermore, the first Ig-like domain contains a hydrophobic patch, centred at Leu38, which has been suggested to mediate dimerisation of Ig1-2 and thus MuSK dimerisation (Stiegler et al., 2006). Thus, there are already two essential mechanisms involved in MuSK activation, namely LRP4-MuSK binding and MuSK dimerisation. Notably MuSK autoantibodies predominantly bind to the Ig-like domains (Hoch et al., 2001).

LRP4 is a single-spanning transmembrane protein that consists of a large extracellular region with eight LDL class A repeats, two epidermal growth factor (EGF)-like domains and four β -propeller domains with neighbouring EGF-like domains (figure 1.2). The extracellular region is anchored by a transmembrane region, and there is a short cytoplasmic region that is not essential for neuromuscular development (Gomez and Burden, 2011). In the absence of agrin, LRP4 and MuSK bind constitutively and keep MuSK partially activated (Zhang et al., 2008, Zhang et al., 2011). Agrin, via its z8 splice insert (Zong et al., 2012), binds to the first β -propeller domain of LRP4, which then induces a conformational change in LRP4 culminating in closer interaction with MuSK. The third β -propeller domain of LRP4 is required for this close association between LRP4 and MuSK (Zhang et al., 2011).

1.4 Potential pathogenic mechanisms of MuSK myasthenia gravis

1.4.1 Introduction

The pathogenic mechanisms of MuSK-MG are not known, but several potential pathogenic mechanisms have been investigated. Intriguingly, the MuSK antibodies belong predominantly to the IgG4 subclass (McConville et al., 2004), which has structural features leading to a role in the immune system that is not yet fully understood.

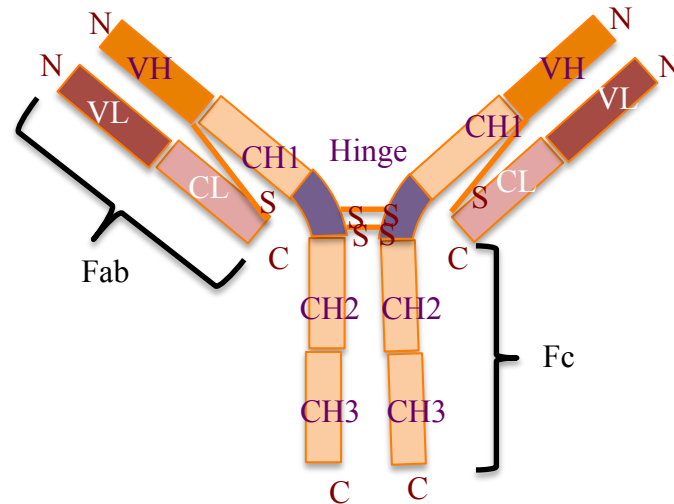
1.4.2 Immunoglobulin G and the IgG subclasses

Immunoglobulins differ in their distribution in the body and in their interaction with other components of the immune system. There are four subclasses of IgG: IgG1, 2, 3 and 4, numbered in the order of their serum concentration (table 1.1). IgG4 has plasma levels that are considerably lower than of other IgG subclasses (French, 1986), table 1.1). In contrast to IgG1, 2 and 3 subclasses, IgG4 subclass antibodies do not bind C1q, thus are not complement fixing, and are non-opsonising and have low affinity binding to Fc γ receptors (reviewed by (Nirula et al., 2011).

1.4.3 IgG structure

IgGs are heterodimeric proteins that consist of two heavy (H) and two light (L) chains (figure 1.3A). Each heavy or light chain consists of one N-terminal variable (V) domain responsible for antigen binding, and one or more C-terminal constant (C) domains, which together form a half-molecule. The L chain contains one C domain (C_L). The H chain of IgG contains three constant domains, C_H1, C_H2 and C_H3, of which the C_H1-domain is located in the Fab region, and the remaining C_H2 and C_H3

A



B

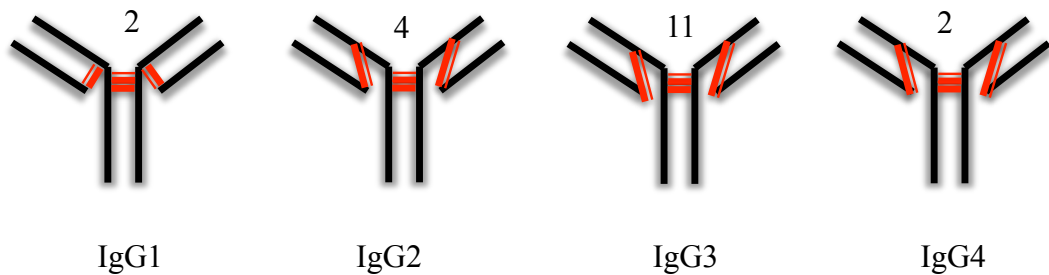


Figure 1.3: The structure of human IgG and the four IgG subclasses. (A) IgG consists of two heavy (H) chains (orange) and two light (L) chains (dark red). The two heavy chains are connected by disulphide bonds in the hinge region (violet). N-terminal variable (V) domains in the H- and L- chain mediate the recognition and binding of the antigen, and build up the Fab fragment together with the first constant (C) domain of the H chain and the sole C domain of the L chain. The Fc region is comprised of the CH2 and CH3 domains of the H chain. (B) The number of interchain-disulphide bonds (depicted as red lines) vary between the IgG subclasses. IgG1 and IgG4 have two interchain-disulphide bonds that connect the two H chains in the hinge region, while IgG2 has four and IgG3 eleven. The interchain-disulphide bonds that connect H and L chain also vary between subclasses, in IgG1 they connect the c-term of the CL domain with the C-term of the CH1 domain, while in IgG2-4 the c-term of the CL domain is connected to the n-term of the CH1 region. Adapted from Schroeder and Cavacini, 2010, Liu et al.,2012.

domains comprise the Fc region. The Fc region defines the isotype and subclass of the immunoglobulin, and hence the effector function. The CDR3 hypervariable region of each Fab, that recognizes and binds specific antigenic epitopes, is comprised of the variable domains of heavy (V_H) and light (V_L) chains, as well as the C_{H1} region of the heavy chain, and the C_L region of the light chain (reviewed by (Schroeder and Cavacini, 2010)).

There is a hinge region between C_{H1} and C_{H2} , which connects the two H chains by interchain disulphide bridges. The four IgG subclasses have very distinct disulphide bond structures (Pinck and Milstein, 1967, Frangione and Milstein, 1967, Frangione et al., 1969), with varying numbers of disulphide bridges between the heavy chains in the hinge region (figure 1.3B, interchain disulphide bridges are represented by red lines): two for IgG1 and IgG4, four for IgG2 and eleven for IgG3.

Table 1.1: Properties of human IgG subclasses (Nirula et al., 2011, Liu and May, 2012, Palmeira et al., 2012)

Subclass	Adult serum level (mg/ml)	% of total IgG	Complement activation, C1q binding	Opsonising	FcγR binding	Inter-chain disulphide bridges	Placental transfer (37 week of gestation)
IgG1	5-11	43-75	++	yes	I,II,III	2	++++
IgG2	1.5-6	16-48	+	yes	(II)	4	+
IgG3	0.2-1	1.7-7	+++	yes	I,II,III	11	++
IgG4	0.08-1.4	0.8-11.7	-	no	(I)	2/0	+++

1.4.4 IgG4 subclass has anti-inflammatory characteristics

While IgG4 shares approximately 95% sequence homology of the C regions with the other IgG subclasses, several small amino acid changes lead to important changes of function. Single amino acid changes in the C_H2 region are responsible for the loss of binding to C1q (P331S) and Fc receptor (L234F, P331S, (Tao et al., 1993, Brekke et al., 1994, Canfield and Morrison, 1991)).

IgG4 has been found to occur with two different structures of disulphide bridges, forming either intrachain or interchain disulphide bridges in the hinge region. The interchain disulphide bridges are in equilibrium with the intrachain disulphide bonds (figure 1.4, (Bloom et al., 1997, Angal et al., 1993, Schuurman et al., 2001)). This phenomenon is caused by a single amino acid in the middle of the IgG4 hinge region, which differs from IgG1; IgG1 has two proline residues in the hinge, IgG4 has a proline and a serine (P228S), which may allow for more chain flexibility. When the serine was exchanged for proline, stable interchain disulphide bonds were obtained (Bloom et al., 1997, Angal et al., 1993, Schuurman et al., 2001). Further experiments suggest that the two heavy chains of IgG4, that have intrachain disulphide bonds, are associated only by non-covalent interactions rather than covalent and non-covalent binding together, as in the other IgG subclasses (Petersen and Dorrington, 1974, Angal et al., 1993, Schuurman et al., 2001).

This structural difference has important consequences. The reduced binding strength between the two heavy chains, caused by the loss of the disulphide bridges, enables IgG4 to split into half-molecules (figure 1.4B) (King et al., 1992, Margni and Binaghi, 1988, van der Zee et al., 1986) and recombine with other half-molecules, in

a process termed Fab arm exchange, to form bi-specific antibodies (figure 1.4C, (van der Neut Kolfshoten et al., 2007)).

This bi-specificity or functional monovalency has the consequence that IgG4 antibodies are unable to form immune complexes with antigen in the circulation or in tissues. Such immune complexes that are formed with IgG1-3 subclasses would lead to complement activation or antigen mediated phagocytosis. Thus, taken together, IgG4 has very little potential to induce such an immune reaction. This immunological inertness is the reason that the IgG4 constant region was selected to create many recombinant therapeutic antibodies, such as anti- α -integrin, anti-TNF- α or anti-C5 (Feldmann et al., 1998, Miller et al., 2003, Chan and Carter, 2010).

Furthermore, it has been suggested that IgG4 binding may displace binding of IgG1 or IgE antibodies, thus counteracting their pro-inflammatory action (reviewed by (Nirula et al., 2011)). This may explain the increase of the IgG4 subclass after long-time exposure to antigen, as observed with bee-venom specific antibodies in bee-keepers (Rieben and Blaser, 1989, Kemeny et al., 1983a, Kemeny et al., 1983b); the antibodies have a protective role if passively transferred to patients with bee venom anaphylaxis (Lessof et al., 1978).

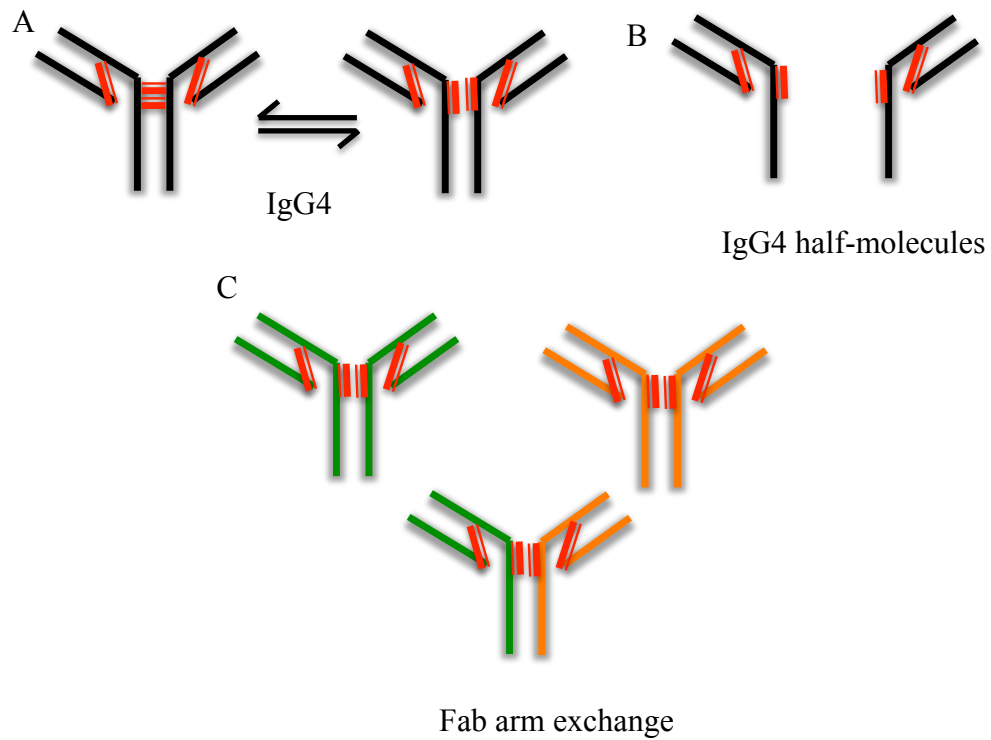


Figure 1.4: IgG4 can split into half-molecules and exchange Fab arms. (A) The interchain-disulphide bonds in IgG4 can change to intrachain-disulphide bonds, thus severing the disulphide connection between the two H chains. (B) Next, the two half-molecules of IgG4 can detach from each other, and (C) recombine with half-molecules from other IgG4 molecules with different antigen specificity. This process is named Fab arm exchange. Adapted from Liu et al., 2012, Aalberse et al., 2009.

1.4.5 IgG4 subclass antibodies in MuSK-MG and other diseases

MuSK antibodies are predominantly of the IgG4 subclass (McConville et al., 2004), and IgG4 subclass correlates broadly with disease severity (Niks et al., 2008a). It is unknown how IgG4 subclass antibodies to MuSK could cause myasthenia gravis.

Diseases with IgG4 subclass antibodies have been described, for example pemphigus (Futei et al., 2001, Kricheli et al., 2000, Ayatollahi et al., 2004, Sitaru et al., 2007). The antibody target is desmoglein, which is expressed in the cell membrane of keratinocytes, and the antibodies appear to be the primary cause of the disease, with absent complement- or otherwise immune-involvement. A group of diseases termed IgG4-related disease (IgG4-RLD) shows elevated levels of IgG4 in the circulation, though whether IgG4 is a cause of disease or simply a bystander product is not known (reviewed by (Stone, 2012)).

1.4.6 Complement is not involved in MuSK-MG

IgG4 subclass antibodies do not activate complement, and thus, contrary to AChR-MG, complement-activated damage is thought to be an unlikely mechanism in MuSK-MG. Therefore it is not surprising that muscle biopsies and electrophysiological studies on intercostal or upper limb muscles from patients did not show any complement deposition, despite exhibiting reduced miniature endplate potentials (MEPPs) or reduced decrement in repetitive nerve stimulation (Selcen et al., 2004, Shiraishi et al., 2005b). Although the intercostal muscles used by Selcen et al. were severely involved, facial muscles are often more involved in MuSK-MG, and *in vivo* neuromuscular transmission studies reveal more defects in facial muscles than in limb muscles (Shiraishi et al., 2005b, Nemoto et al., 2005, Farrugia et al., 2006). For obvious reasons, though, facial muscles are not used for muscle biopsies

and thus the nature of the difference in muscle weakness and the potential involvement of complement in facial muscles cannot be explored directly in the patients.

1.4.7 MuSK antibodies cause MuSK-MG

The absence of complement deposition or loss of AChR in patient biopsies led some to question whether MuSK antibodies are the main cause of MuSK-MG (Selcen et al., 2004). However, the pathogenicity of MuSK antibodies has been demonstrated by passive transfer of patient IgG to experimental animals (Mossman et al., 1988, Burges et al., 1994); in these experiments the majority of samples were shown subsequently to be MuSK antibody positive (Vincent et al., unpublished data); more recent results have confirmed this (Cole et al., 2008, Viegas et al., 2012).

Furthermore passive transfer of MuSK-MG patient derived IgG4 but not IgG1-3 antibodies to mice caused disease (Klooster et al., 2012). In addition, active immunisation of mice (Jha et al., 2006, Viegas et al., 2012, Mori et al., 2012a) and rabbits (Shigemoto et al., 2006, Shigemoto et al., 2008) produced weakness and electrophysiological evidence of neuromuscular junction defects.

In both active and passive experiments, complement activation was not observed, and complement was shown to be unnecessary for development of MuSK-MG in a complement-deficient animal model (Mori et al., 2012a). The evidence therefore suggests that MuSK antibodies are pathogenic, but complement activation is not a pathogenic mechanism of MuSK antibodies in MG.

1.4.8 MuSK antibodies affect AChR clustering

One study showed that patient IgG injected into foot muscles in mice decreased endplate size only in regenerating mouse muscles, and not in normal muscles (Ter Beek et al., 2009). However, a reduction of AChR cluster size and branching, and disruption of the NMJ, or reductions in AChR numbers, were found in MuSK immunised mice and mice injected with patient serum (Jha et al., 2006, Cole et al., 2008, Viegas et al., 2012, Morsch et al., 2012). Thus it seems likely that the action of the antibodies is to reduce the density and number of AChRs at the NMJ.

AChR clustering can be studied *in vitro* by using the C2C12 mouse muscle cell line. The C2C12 cells were originally derived in 1977 from the leg muscle of an adult dystrophic mouse (C3H mice) 70 hours after crush injury in order to study the cause of muscular dystrophies (Yaffe and Saxel, 1977). The cell line is maintained as undifferentiated myoblast cells in medium that is high in growth factors. The myoblasts can be differentiated by cell fusion, to form multinucleated, contractile myotubes, by incubation in growth factor-reduced medium (figure 1.5A). During the differentiation to myotubes, protein expression changes. MuSK, the key regulator of postsynaptic development, is not expressed in myoblasts but is upregulated and well expressed in myotubes (DeChiara et al., 1996, Ip et al., 2000, Valenzuela et al., 1995), and similarly upregulated is LRP4 (Zhang et al., 2008). Myotubes can be stimulated to form AChR clusters by adding soluble agrin to the differentiation medium (figure 1.5B, (Wallace, 1986)).

Experiments on C2C12 mouse myotubes showed that MuSK-MG patient serum impaired AChR clustering evoked by agrin stimulation (Hoch et al., 2001), but in similar experiments antibodies did not affect the total number of AChR (Farrugia et al., 2007), suggesting that they may merely lead to a loss of the visibly clustered AChR. Nevertheless, the *in vitro* findings suggest that MuSK antibodies

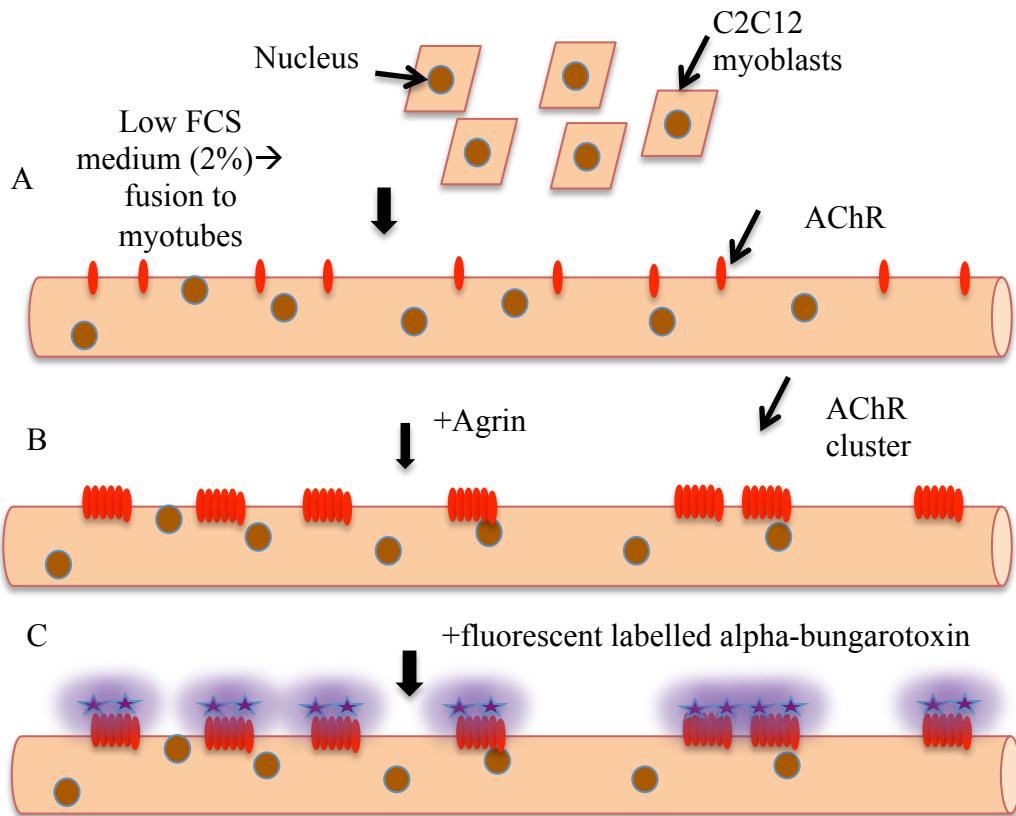


Figure 1.5: The principle of the C2C12 AChR clustering assay. (A) C2C12 myoblasts fuse and differentiate to myotubes when growth factors are depleted using 2% FCS medium. The resulting myotubes have multiple nuclei (brown circles) due to the fusion. (B) Incubation with agrin over night will induce the dense aggregation of AChR (red ovals). (C) AChR clusters can be visualized by addition of fluorescent-labelled alpha-bungarotoxin (purple stars). The number, length and shape of the AChR clusters can then be analyzed by fluorescence microscopy.

may alter MuSK function and thereby interfere with the development or maintenance of the high density of AChRs that are required for efficient function.

1.4.9 MuSK antibodies affect MuSK function

Does binding of MuSK antibodies interfere with MuSK function as a receptor tyrosine kinase? On the contrary, when applied acutely, MuSK antibodies induce phosphorylation of MuSK and the AChR beta subunit, which is a downstream target of MuSK (Shigemoto et al., 2006, Cole et al., 2010). Since it has previously been found that artificial dimerisation of MuSK by rabbit divalent antibodies leads to MuSK phosphorylation (Hopf and Hoch, 1998a), it seems likely that the patient antibodies divalently bind MuSK and thus lead to autophosphorylation of MuSK; this could be responsible for the agrin independent AChR clustering observed in C2C12 myotubes in the presence of MuSK antibodies (Hoch et al., 2001) and would tend to consolidate NMJ structure rather than disrupt it.

Nevertheless, this adds another complication because MuSK antibodies are mainly IgG4 and thus functionally monovalent (van der Neut Kolfshoten et al., 2007) and hence not capable of divalent binding. However, there are a minority of MuSK antibodies of the IgG1-3 subclasses that can bind divalently and could be responsible for the spontaneous clustering *in vitro*. Whether divalent binding of patient antibodies leads to desensitisation of the MuSK pathway or induce loss (internalisation) of MuSK (as occurs when divalent antibodies bind, for instance, to AChR (Drachman et al., 1978)) is not yet clear.

In fact, the successful passive transfer of MuSK-MG to mice with IgG4, but not IgG1-3 (Klooster et al., 2012) suggested that IgG4 MuSK antibodies are pathogenic and not IgG1-3. Apparently both whole IgG and Fab fragments (which

are monovalent) from a rabbit model of MuSK-MG reduced agrin-induced AChR clustering *in vitro* (Mori et al., 2012b), suggesting that monovalent binding may be sufficient to impair AChR clustering. On the other hand, Fab fragments, in contrast to whole IgG, were not capable of inducing MuSK autophosphorylation (Mori et al., 2012b), suggesting that the activation of MuSK requires divalent antibody binding.

Taking all of this into account, the most rational hypothesis is that antibodies of the IgG4 subclass induce a pathogenic mechanism that does not require divalent binding, and those of IgG1-3 subclasses can cross-link MuSK, stimulate MuSK phosphorylation and induce agrin-independent AChR clustering *in vitro*, but may lead to loss of MuSK activity *in vivo*.

1.5 Aims

The aim of this project was to identify pathogenic mechanisms of MuSK autoantibodies in MuSK-MG.

1. To see whether MuSK antibodies induce MuSK endocytosis and if so which subclass of antibodies was responsible. In AChR-MG, antigenic modulation, cross-linking and endocytosis of AChR as a result of divalent binding of the antibodies are a key pathogenic mechanism (Drachman et al., 1978, Heinemann et al., 1977). More recent studies demonstrated endocytosis as key mechanism for other autoantibodies, by using cell-based assays (Hinson et al., 2007, Dalmau et al., 2008, Hughes et al., 2010). Endocytosis will thus be studied using similar cell-based assays, in which cell surface antigen bound patient IgG will be assessed over time.
2. To test whether the patient antibodies disrupt the interaction between LRP4 and MuSK. The close interaction between LRP4 and MuSK, demonstrated among other experiments by co-immunoprecipitation, is crucial for the dense

aggregation of AChRs at the NMJ (Kim et al., 2008, Zhang et al., 2008), and impaired AChR clustering was described *in vitro* (Hoch et al., 2001). Thus the interaction between LRP4 and MuSK will be studied with co-immunoprecipitation experiments in the presence of healthy control and patient antibodies.

3. To see whether divalent binding is necessary for pathogenicity. IgG4 is the predominant IgG subclass in MuSK-MG (McConville et al., 2004), a subclass that was described to be functionally monovalent (reviewed by Nirula et al., 2011). Thus the relevance of divalent binding for pathogenicity will be studied by production of Fab fragments from patient and healthy control IgG, and by investigating their effect in comparison with whole patient or healthy control IgG on AChR clustering *in vitro*.
4. Finally to dissociate carefully the effect of IgG1-3 and IgG4 and how they contribute individually or together. IgG4 is the main pathogenic subclass in MuSK-MG (McConville et al., 2004). To study patient derived IgG4 subclass antibodies in comparison to IgG1-3 subclasses, IgG4 will be purified from patient plasma using an IgG4 affinity matrix, and IgG4 depleted IgG1-3 subclasses will be isolated with protein G sepharose. Patient derived IgG4 and IgG1-3 will be characterised by studying protein concentration, MuSK antibody concentration, binding affinity and their effect on AChR clustering *in vitro*, MuSK endocytosis and LRP4-MuSK interaction.

Chapter 2: Materials and Methods

2.1 Materials

See table 2.1 and table 2.2.

2.2 Methods

2.2.1 Tissue culture

HEK293 cells were purchased from ECACC. C2C12 and Phoenix Eco cells were purchased from ATCC.

Human embryonic kidney 293 (HEK293) cells and Phoenix-Eco cells were maintained in HEK cell growth medium (DMEM supplemented with 10% foetal calf serum and 500 units/ml of penicillin, 500 µg/ml of streptomycin, and 1.25 µg/ml of Fungizone®(antibiotic-antimycotic)) at 37°C, 5.5% CO₂ at 30-80% confluency.

C2C12 mouse myoblasts were maintained in growth medium (DMEM supplemented with 15% Foetal calf serum and 500 units/ml of penicillin, 500 µg/ml of streptomycin, and 1.25 µg/ml of Fungizone® (antibiotic-antimycotic)) and differentiated to form myotubes for 4-7 days in differentiation medium (DMEM supplemented with 2% Foetal calf serum and 500 units/ml of penicillin, 500 µg/ml of streptomycin, and 1.25 µg/ml of Fungizone® (antibiotic-antimycotic)) at 37°C, 5.5% CO₂.

DOK7 infected C2C12 myoblasts were maintained in growth medium supplemented with 1µg/ml puromycin at 37°C, 5.5% CO₂ at 30-80% confluency and differentiated to form myotubes for 4-7 days in differentiation medium (DMEM supplemented with 2% Foetal calf serum and 500 units/ml of penicillin, 500 µg/ml of streptomycin, and 1.25 µg/ml of Fungizone® (antibiotic-antimycotic)) at 37°C, 5.5% CO₂.

Table 2.1: Materials

Product	Company	Product number
¹²⁵ I labelled AChR	RSR Ltd	RBA/25
¹²⁵ I labelled MuSK	RSR Ltd	MSK/TR
0.20µm syringe filters	Sartorius	16534
Alkaline phosphatase	Promega	M182A
Alexa Fluor-594 conjugated bungarotoxin	Invitrogen	B13423
Amersham ECL Western blotting system	GE healthcare	RPN2108
Ampicillin	Sigma	A1593
Fungizone® (antibiotic- antimycotic)	Gibco	15240
BSA	Sigma-Aldrich	A7906
Confocal-Matrix for fluorescence and confocal microscopy	Micro Tech Lab	CONFO30
DMEM	Sigma	D6429-24X500ML
<i>E. coli</i> JM109	Promega	P9751
EDTA pH 8.0	Gibco	15575
Fetal calf serum	PAA	A15-151
Goat serum	Sigma-Aldrich	G9023
Hartmann's solution (compound sodium lactate)	Baxter healthcare	FKE2323
HEPES	Sigma	H3375
HiMark Pre-stained High molecular weight protein standard	Invitrogen	LC5699
IgG4 affinity matrix	Captureselect	2900.05
Kanamycin	Sigma	60615
Miniprep kit	Qiagen	27104

Neon Transfection System 10µl Kit	Invitrogen	MPK1025
NheI	New England Biolabs	R0131S
NuPage LDS sample buffer	Invitrogen	NP0008
NuPage Novex Tris-acetate mini gels	Invitrogen	EA03755BOX
NuPage reducing agent	Invitrogen	NP0004
PageBlue Protein Staining solution	Thermo scientific	24620
Papain beads	Thermo scientific	20341
Paraformaldehyde	TAAB Laboratories Equipment Ltd.	F003
PBS	Oxoid	BK0014G
Pierce Fab preparation kit	Thermo scientific	44985
pIRES2-AcGFP1	Clontech	632515
Polybrene	Insight Biotechnology Ltd	sc-134220
Poly L-Lysine	Sigma	P4832
Protease inhibitor cocktail	Sigma	P8340
Protein A sepharose	Sigma	P9424
Protein G sepharose	Sigma	P3296
Protein G coupled magnetic beads, Dynabeads	Invitrogen	100.03D
Puromycin	Sigma	P9620
QIAquick Gel Extraction Kit	Qiagen	28704
SnakeSkin Dialysis Tubing, 10K MWCO, 22mm	Thermo scientific	68100
VisionWorks®LS Image Acquisition Software	UVP	97-0186-03
XhoI	New England Biolabs	R0146s

Table 2.2: Antibodies

Antibody name	Company	Product Number	Species	Application	Dilution
anti-human IgG	RSR Ltd	RBA/25 (part of a kit)	sheep	RIA	1:3
anti-human IgG1, 2, 3 or 4	The Binding Site	MC003,11,6,5)	mouse	FACS	1:50
APC-conjugated anti-mouse antibody	Invitrogen	A21235	goat	FACS	1:200
anti rMuSK antibody	R&D system	AF562	goat	IP, WB, FACS	variable
anti-mCherry antibody	BioVision	5993-100	rabbit	WB	1:1000
anti-EGFP antibody	Abcam	ab6556	rabbit	WB	1:2000
Anti-human IgG Alexa Fluor 568	Invitrogen	A21090	goat	CBA, FACS	1:750, 1:200
Anti-human IgG Alexa Fluor 488	Invitrogen	A11013	goat	CBA, FACS	1:750, 1:200
Anti-human IgG Alexa Fluor 647	Invitrogen	A21445	goat	CBA, FACS	1:750, 1:200
HRP-conjugated anti-rabbit	Dako	P044801-2	goat	WB	1:2500
HRP-conjugated anti-mouse	Dako	P044701-2	goat	WB	1:2500
HRP-conjugated anti-goat	Dako	P044901-2	rabbit	WB	1:2500
Anti-DOK7 (H-284)	Santa Cruz Biotechnology, Inc.	sc-50463	rabbit	WB	1:1000
Anti-human IgG (Fab specific)	Sigma	I5260	goat	IP, WB	variable

2.2.2 Transfection protocols

2.2.2.a. Polyethylenimine

HEK293 or Phoenix Eco cells were plated at densities of 3×10^5 cells/well in a 6-well plate. After 24 hours, 3 μ g plasmid DNA was mixed with 1.5 μ l polyethylenimine (PEI), 1.25 μ l 20% glucose and 1 ml HEK cell growth medium per well and the transfection mix was added to the cells. For larger quantities of cells the materials were upscaled accordingly. All materials in contact with the Phoenix Eco cells or the supernatant were decontaminated for at least 15 minutes with vircon before disposal.

2.2.2.b. Electroporation

C2C12 mouse myotubes were trypsinised and resuspended at a concentration of 1×10^8 cells/ml in resuspension buffer from the Neon 10 μ l transfection kit. 30 μ l cell suspension was mixed with 6.5 μ g MuSK-mCherry plasmid DNA and transferred into a 10 μ l transfection tip. The cells were subjected to one pulse of 1400V for 30ms and immediately transferred into a well of a 6-well plate with antibiotic-free C2C12 growth medium.

2.2.2.c. Infection with retrovirus

The vector pBABE-IRES-DOK7-EGFP was transfected with PEI into the Phoenix Eco cell line, a retrovirus producing cell line based on HEK293T cells that expresses viral packaging proteins to produce replication incompetent virus that only infects rodent cells. After 24 hours, the medium was changed, and after a further 48 hours, cell supernatant containing replication incompetent virus carrying the viral DOK7 vector was harvested and stored at -80°C .

6×10^4 C2C12 myoblasts were seeded into a T25 flask, and 24 hours later, 1ml of virus supernatant as well as 8ng polybrene were added to the cells. After 24 hours, the cells were trypsinised and transferred into a T175 flask in C2C12 growth medium. After 24 hours, the medium was switched to medium containing $1 \mu\text{g/ml}$ puromycin to select for infected cells. EGFP expression in the cells was a marker of infection.

2.2.3 Cell lysis and protein concentration

Cells were washed 3 times in growth medium, resuspended in $220 \mu\text{l}$ ice-cold lysis buffer (10mM Tris-HCl, pH 7.4, 100mM NaCl, 1mM EDTA, 1% Triton-X supplemented with 1:100 protease inhibitor cocktail) and placed on ice for 15 minutes. The lysate was centrifuged 15 min $12000g$ at 4°C to remove membrane fragments, and the supernatant contained the cell lysate.

OD₂₈₀ of protein preparations was measured using a nanodrop. Protein concentration was then calculated from OD₂₈₀ using an equation derived from the Beer-Lambert law:

$$c = \frac{A}{\epsilon * b}$$

With c = protein concentration (mg/ml), A = OD₂₈₀, ϵ = molar extinction (or absorption) coefficient (molar extinction coefficient of IgG was 13.7, of Fab fragments was 13.5), and b = cuvette length in cm, here 0.1cm.

2.2.4 Radioimmunoassay (RIA)

MuSK and AChR antibody concentrations were determined by RIA as previously described (McConville et al., 2004). Plasmas were serially diluted 5-fold in PTX (0.02M PBS, 0.1% Triton X) to a total volume of $50 \mu\text{l}$ and incubated with $50 \mu\text{l}$ of

¹²⁵I labelled AChR or MuSK and 5µl carrier serum (healthy control serum) over night at 4°C. 50µl anti-human IgG was added and incubated at room temperature until precipitation was visible (approximately 60 minutes). After addition of 500µl PTX for washing, the samples were centrifuged 5 min 13000g at room temperature, and then cpm were measured with a Wallac Wizard counter.

MuSK antibody concentrations were calculated using the following equation:

$$x \text{ nM} = \frac{(\text{cpm sample} - \text{cpm control}) \times \text{decay factor}}{(\text{Vol. sample } (\mu\text{l}) \times \text{specific activity } (\frac{\text{Ci}}{\text{mmol}})) \times \text{counter efficiency} \times 2.22}$$

Assuming a counter efficiency of 0.815.

2.2.5 IgG purification

IgG was purified from patient plasma using protein G sepharose (figure 2.1A). 10 ml plasma was diluted 1:2 in Hartmann's solution and incubated with 2.5 ml protein G sepharose over night at 4°C with gentle shaking (figure 2.1B). Bead slurry was packed into a column, washed with 50ml Hartmann's solution, and bound IgG was eluted with 0.1M glycine, pH 2.3 (figure 2.1C). 20 x 1ml fractions were collected into eppendorf tubes containing 150µl Tris-HCl pH 8.0 to raise the pH, and analysed for protein content by measuring the OD₂₈₀. Fractions with OD₂₈₀ > 1 were pooled and dialysed over night against 200 volumes of Hartmann's solution.

2.2.6 Cloning

Full length human MuSK was inserted into the vector pIRES2-AcGFP1 by subcloning. First full length human MuSK was excised from mammalian expression vector pcDNA3.1 (McConville et al., 2004), and pIRES2-AcGFP1 was digested

within the multiple cloning site with XhoI and NheI restriction enzymes. 6µg pcDNA-MuSK or 0.5µg pIRES2-AcGFP1 were mixed with XhoI, NheI, buffer 4, 1mg/ml BSA and made up to 20µl with dH₂O, and incubated at 37°C. To prevent religation of the pIRES2-AcGFP1 vector, the 5' overhang was dephosphorylated with alkaline phosphatase for 1h in a 37°C water bath. To purify digested products, the digests were subjected to gel electrophoresis using a 0.7% agarose gel, and digested vector (approximately 5.3kbp) and digested MuSK (2.7kbp) bands were excised and purified using a gel extraction kit. DNA concentration was determined with a nanodrop, and a molar ratio of 3:1 (insert:vector) was calculated using the following equation:

1:1 molar ratio:

$$\frac{a \text{ ng vector} \times b \text{ bp insert}}{c \text{ bp vector}} = d \text{ ng insert.}$$

50ng vector were used, the insert was 2.7kbp and the vector 5.3kbp, which was used to calculate the ng insert required for a 1:1 molar ratio:

$$\frac{50 \text{ ng vector} \times 2700 \text{ bp insert}}{5300 \text{ bp vector}} = 25\text{ng insert}$$

For a 3:1 ratio, 3x 25ng = 75ng insert and 50ng vector were ligated by adding ligase, ligase buffer and dH₂O, and incubating over night at RT. The ligation of vector pIRES2-AcGFP1 and the MuSK insert was then transformed into *E. coli* JM109 bacteria by gentle mixing, incubation for 10 minutes on ice, 45 seconds heat shock (42°C water bath) followed by 5 minutes incubation on ice. 1ml LB medium was added to the bacteria, followed by 30 minutes shaking at 37°C. Bacteria were centrifuged for 3 minutes 1200g and the pellet resuspended in 100µl supernatant and

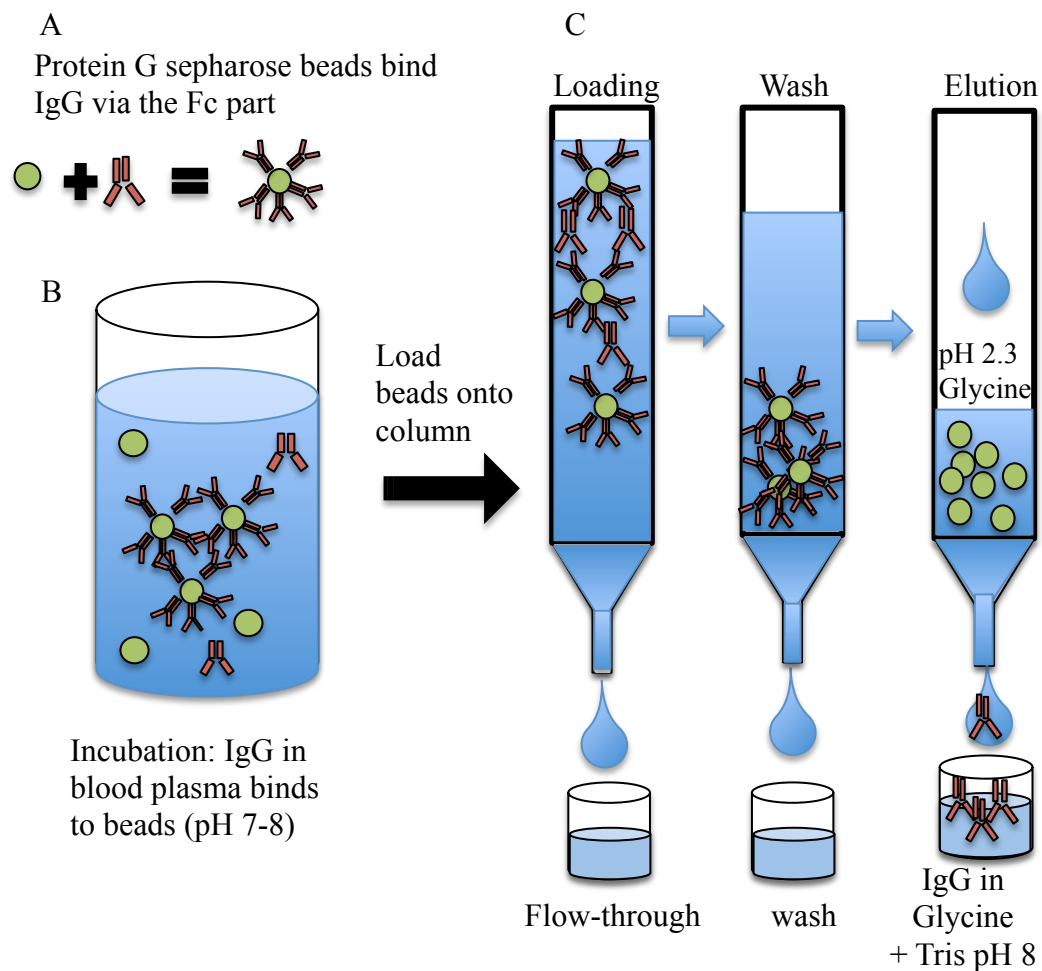


Figure 2.1: The principle of IgG purification. (A) Protein A or G sepharose beads bind to the Fc region of IgG. (B) Human plasma is incubated with protein A or G sepharose beads over night. The pH must be neutral to ensure proper binding. (C) The bead slurry is packed into a column, the flow-through is being collected, and the beads are washed with PBS. Then bound IgG is eluted from the matrix by reducing the pH, using 0.1M glycine at pH 2.3. The eluate is collected in tubes containing Tris-HCl pH 8 to raise the pH.

plated on LB-agar plates containing 40µg/ml kanamycin. Colonies were picked and grown in 5ml LB containing 40µg/ml kanamycin. Bacteria were centrifuged and DNA was purified from the pellet with a miniprep kit. 5µl DNA per colony was then digested with XhoI and NheI for 4h at 37°C, and the digest was subjected to gel electrophoresis. When plasmids contained a 2.7kbp insert, they were sequenced using primers for pIRES2-AcGFP1 vector or MuSK (table 2.3) to verify insertion of MuSK and absence of mutations.

A full list of all vectors that were used in this project can be found in figure 2.2.

Table 2.3: Sequencing primers

Primer	Sequence 5'-3'
Reverse pIRES-EGFP	CCGGGCCCCGCGGTACCGTCGACTGCAGAAT
Forward pIRES-EGFP	GTCTATATAAGCAGAGCTGGTTTAGTGAAC
MuSKseq F1	AATCCCAAACCATCAGTGTC
MuSKseq F2	GGGAGGTGTGTAATGCAGTC
MuSKseq F3	TCACCTACATACTCCATGAC
MuSKseq F4	TGCAGCTCAGTCACAGTGA
MuSKseq R1	AATGCCTGTTGCTGTACAGT
MuSKseq R2	GCAGTACTCTCTGCAAATGG
MuSKseq R3	TTGGATACTCCAGGCTGAGC
MuSKseq R4	AGCTTTGTAGTAGTCTGCTG

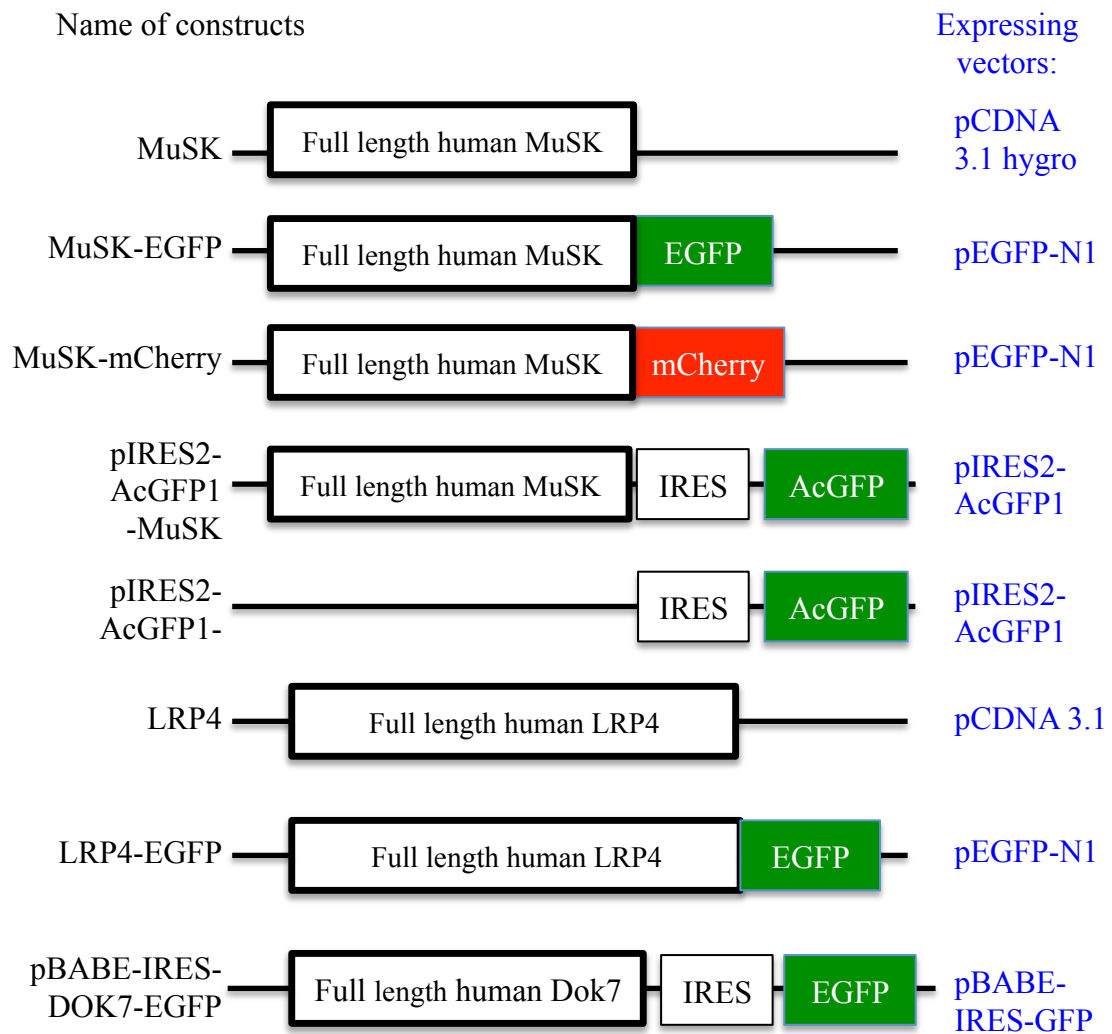


Figure 2.2: List of plasmids used in the project.

2.2.7 Flow cytometry

Binding of patient IgG to MuSK expressed on MuSK transfected HEK293 cells was quantified using fluorescent-labelled anti-human IgG (figure 2.3). HEK293 cells were transfected with DNA encoding either full length human MuSK inserted into the pIRES2-AcGFP1 vector (pIRES2-AcGFP1-MuSK), the empty vector (pIRES2-AcGFP1-) or no DNA using PEI. 1×10^5 cells were incubated with a 1:50 dilution of patient plasma or healthy control serum or goat anti-rMuSK (R&D systems) in incubation medium (DMEM supplemented with 1500 units/ml of penicillin, 500 $\mu\text{g/ml}$ of streptomycin, and 1.25 $\mu\text{g/ml}$ of Fungizone[®] (antibiotic-antimycotic), 1% BSA, 20mM HEPES) containing 10% goat serum for 20 minutes at 4°C with gentle rocking. After washing the cells in 500 μl incubation medium, cells were fixed for 5 minutes in 1% formaldehyde in incubation medium at 4°C, washed, and incubated with either anti-human IgG AlexaFluor 647 at 1:100 dilution or anti-goat APC at 1:200 dilution for 30 minutes at 4°C in the dark. For IgG subclass profiling, the cells were incubated with a 1:50 dilution of mouse anti-human IgG1, 2, 3 or 4 in incubation medium (DMEM supplemented with 500 units/ml of penicillin, 500 $\mu\text{g/ml}$ of streptomycin, and 1.25 $\mu\text{g/ml}$ of Fungizone[®] (antibiotic-antimycotic), 1% BSA, 20mM HEPES) for 30 minutes at 4°C with gentle rocking. After washing the cells with incubation medium, the cells used for IgG subclass profiling were incubated with 1:200 APC-conjugated anti-mouse antibody in incubation medium for 30 minutes at 4°C in the dark. Cells were washed and resuspended in PBS supplemented with 2.5mM EDTA, pH8.0 before analysis with a CyanADP flow cytometer. AcGFP (excitation maximum 484nm, emission maximum 510nm) was excited with a 25mW 488 blue laser and emission was measured between 510 and 550nm using a 530/40 band pass filter. Allophycocyanin (APC, excitation maximum 650nm,

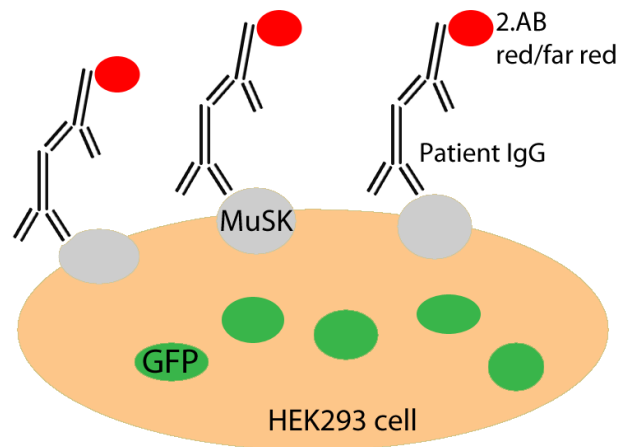


Figure 2.3: Analysis of antibodies to MuSK by cell-based assays. HEK293 cells were transfected with a plasmid expressing GFP (green) in the cytoplasm and human full length MuSK at the cell surface. Patient IgG is incubated with the cells and binds to MuSK. Bound human IgG is visualized by fluorescent-labelled secondary antibody (red for cell based assays or far red for flow cytometry).

emission maximum 660nm) and Alexa Fluor 647 (excitation maximum 652nm, emission maximum 668nm) fluorescence were measured between 655 and 675 using a 665/20 band pass filter following excitation with a 50mW 640nm red laser. AcGFP positive cells were gated and analysed for median far-red fluorescence intensity.

2.2.8 IgG4 and IgG1-3 purification

IgG4 affinity matrix was used in a similar manner as protein G sepharose (figure 2.1). 10ml patient plasma was filtered using 0.2µm syringe filters and diluted 1:2 with PBS, then incubated for 1h at 4°C with 1ml IgG4 affinity matrix and packed into a column. After washing with 3 x 10ml PBS, IgG4 was eluted with 0.1M glycine pH 2.3 in 20 x 1ml fractions into eppendorf tubes containing 150µl Tris-HCl pH 8 to increase the pH. The 4 fractions with the highest OD₂₈₀ were pooled and dialysed over night against 200 volumes of Hartmann's solution. The flow-through containing IgG1-3 was loaded a second time onto the IgG4 affinity matrix to ensure complete depletion of IgG4, and the flow-through was collected and incubated over night with 2.5ml protein G sepharose and eluted as described for IgG purification. Purity of the subclasses was verified using subclass-specific flow cytometry, and MuSK antibody concentrations were measured with the RIA. All preparations were applied at defined MuSK-antibody concentrations based on these results.

2.2.9 Saturation binding experiment

After determining MuSK IgG concentrations with RIA, the concentration of a sample dilution from the linear binding phase was chosen (IgG4/IgG1-3 purifications: 0.025nM) and samples diluted to this concentration. Healthy control plasma at similar volumes was used as control. A 2-fold serial dilution of

radiolabelled MuSK in PTX was prepared. For each dilution, 50µl sample, 50µl MuSK (radiolabelled, diluted) and 5µl carrier serum (healthy control serum) were mixed and incubated over night at 4°C. 50µl anti-human IgG (RSR) was added and incubated at RT until precipitation occurred (approximately after 60 minutes). Then cps for each sample was measured with a Perkin Elmer Wallac Wizard 1470 automatic gamma counter and multiplied by 60 to obtain total MuSK (cpm). To transform cpm to fmoles per 100µl (the sample volume), the specific activity of radiolabelled MuSK (z) was transformed to cpm/fmol (y):

$$y \frac{\text{cpm}}{\text{fmol}} = z \frac{\text{Ci}}{\text{mmol}} \times 2.22 \times \text{counter efficiency}$$

Next, using the specific activity y in cpm/fmol, total MuSK (cpm/100µl) was divided by y (cpm/fmol) resulting in total MuSK (fmol/100µl).

500µl PTX was added to each sample that was then centrifuged for 5 minutes 13000g at RT, and the pellet was washed twice in 500µl PTX before measuring cpm with a Wallac Wizard gamma counter to obtain bound cpm. Specific bound was calculated:

$$\text{Specific bound cpm} = \text{bound (patient (cpm))} - \text{bound (healthy control) cpm}$$

Then the individual MuSK antibody concentrations in pM were calculated and plotted against total MuSK (fmoles) using GraphPad Prism software, and the data was analysed using nonlinear regression with a saturation binding equation for specific binding to one site to calculates B_{max} (in fmoles) and K_d . (in fmoles/100µl).

The K_d was then transformed from fmoles/100 μ l to pmoles/l (pM) by multiplication with 10.

2.2.10 Cell based assay (CBA)

HEK293 cells were plated at densities of 3×10^5 per well onto poly-L lysine-coated glass coverslips in a 6-well plate, and were transfected the following day with 4 μ g of pIRES2-AcGFP1-MuSK with PEI. 40 hours after transfection, the coverslips were transferred into 24 well plates. Samples containing patient or healthy control plasma diluted 1:20 in incubation medium (DMEM supplemented with 500 units/ml of penicillin, 500 μ g/ml of streptomycin, and 1.25 μ g/ml of Fungizone® (antibiotic-antimycotic), 1% BSA, 20mM HEPES) were applied to the coverslips and incubated 60 minutes at 37°C. The supernatant was then aspirated and the cells were washed 3x with HEK cell growth medium. The cells were fixed for 5 minutes with 3% formaldehyde. Bound human IgG was detected by staining with anti-human IgG Alexa Fluor 568 diluted 1:750 in incubation medium for 45 minutes at RT in the dark. Coverslips were washed with PBS and mounted with confocal-matrix for fluorescence and confocal microscopy onto glass slides. After drying of the slides over night at RT in the dark, red and green fluorescence was analysed (figure 2.3) and the fluorescence intensity of the samples was scored, with scores from 0 = no antibody binding to 4 = strong antibody binding.

2.2.11 Endocytosis studies

HEK293 cells were plated at densities of 3×10^5 cells per well onto poly-L lysine-coated glass coverslips in a 6-well plate, and were transfected the following day with 4 μ g of pIRES2-AcGFP1-MuSK with PEI. 40 hours after transfection, the coverslips

were transferred into 24 well plates. Half of the coverslips were fixed for 10 minutes with 3% formaldehyde prior to incubation with the primary antibody to prevent endocytosis. These will be referred to as “pre-fixed”. The other half was unfixed. Samples containing patient plasma, purified IgG, IgG4 or IgG1-3 were diluted in incubation medium to a final MuSK antibody concentration of 0.17nM; this was limited by the low concentration of MuSK IgG1-3 in the plasma. The samples were blinded, and then applied to the coverslips with living or pre-fixed cells and incubated with gentle rocking for 30 minutes at 4°C to prevent endocytosis. The supernatant was then aspirated and cells were washed 3 times with HEK cell growth medium. In experiments with artificial cross-linking (figure 2.4), the secondary antibody, anti-human IgG Alexa Fluor 568 diluted 1:200 in incubation medium, was added at this point, incubated for 30 minutes at 4°C, with gentle rocking, and washed 3 times with HEK cell growth medium. Next, HEK cell growth medium supplemented with 20mM HEPES was added to the cells. The plates were then moved to either 4°C, or 37°C, a temperature at which endocytosis can occur, and incubated for up to 16 hours. At different time points, the cells were fixed for 5 minutes with 3% formaldehyde. Bound human IgG was identified by staining with anti-human IgG Alexa Fluor 568 diluted 1:750 in incubation medium for 45 minutes at RT in the dark. (This step was omitted in most artificial cross-linking experiments, as secondary antibody had already been added, and only performed as an additional control). Coverslips were washed with PBS, and then the 24-well plates were blinded prior to analysis. Red fluorescence was analysed and the fluorescence intensity of the samples was scored blinded by two independent individuals, with scores from 0 = no antibody binding to 4 = strong antibody binding. Scores at time point 0hr for each sample were set as 100% and scores at subsequent time points were normalised to this.

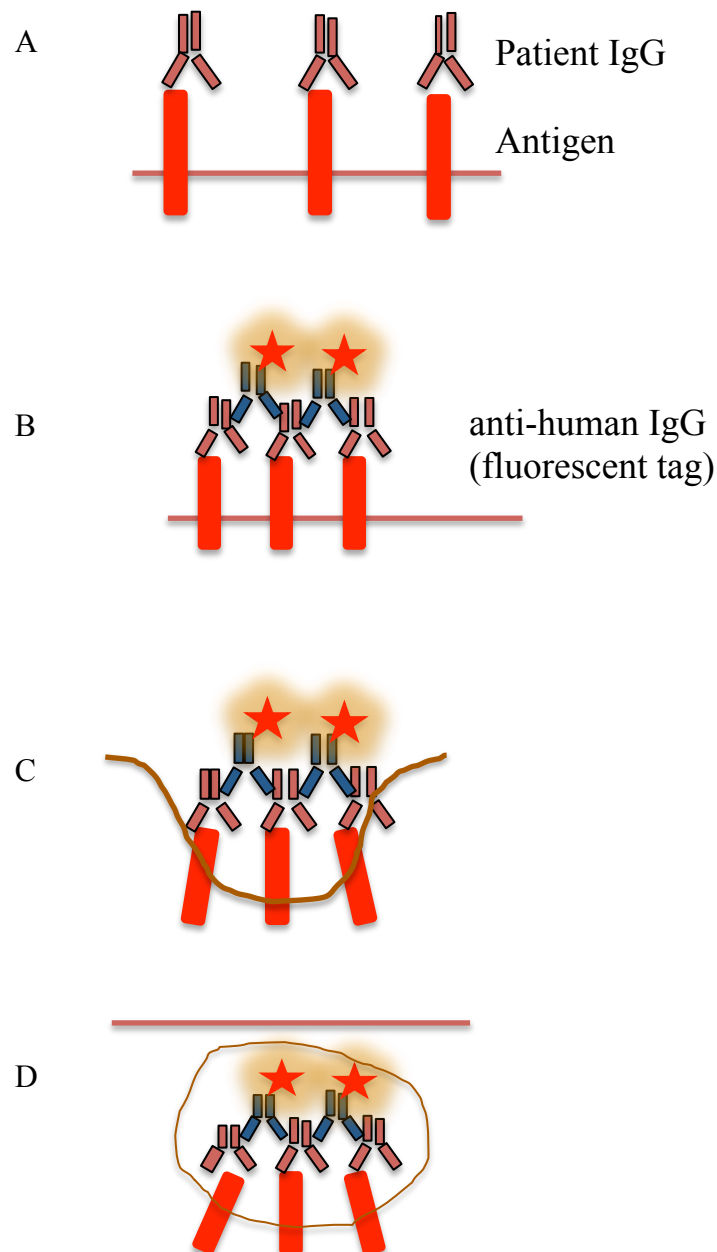


Figure 2.4: The principle of artificial cross-linking and endocytosis. (A) Cells expressing antigen are incubated with human IgG. Patient derived IgG binds to the antigen, in this case in a monovalent manner. (B) A secondary fluorescent antibody against human IgG is added and binds in a divalent manner to human IgG, thus cross-linking the patient IgG and antigen. (C) cross-linked aggregates of antigen, patient IgG and secondary antibody become endocytosed, and (D) are subsequently transported away from the cell surface in intracellular endosomal vesicles. The complex of antigen and antibodies will remain visible throughout the process via the fluorescent tag.

2.2.12 Production of agrin

HEK293 cells were grown to 70% confluency in a T175 tissue culture flask, and were then trypsinised, diluted 1:3 and seeded into a T175 tissue culture flask. After 24 hours incubation at 37°C, the cells were transfected with 42 µg of a construct expressing a c-terminal soluble form of neural agrin with a 4 amino acid insert at the y-splice site and a 8 amino acid insert at the z-splice site (donated by the late Dr. Werner Hoch). The truncated c-terminus of agrin containing an 8, 11 or 19 amino acid insert at the z site was shown to be sufficient to induce AChR clustering in C2C12 myotubes (Ferns et al., 1993). The plasmid was mixed with 42 µl polyethylenimine and 35 µl 20% glucose before adding to the cells. After 24 hours incubation at 37°C, the medium was replaced with HEK cell growth medium. 48 hours later the conditioned medium was centrifuged at 1200g for 5 minutes at room temperature, aliquoted and stored at -20°C.

2.2.13 AChR clustering assay

C2C12 myoblasts were differentiated to myotubes by growth factor depletion. C2C12 myotubes were incubated for 16 hours with or without soluble rat agrin and with purified IgG or Fab fragments from MuSK-MG patients or healthy controls. The concentrations of Fab fragments and IgG for each patient were the same, but differed between patients, and were limited by low yield of Fab fragments (see table 5.1 and table 5.6 for exact concentrations). Healthy control IgG and Fab fragments were used at similar dilutions. Samples were blinded prior to application. AChR clusters were visualized by incubating the myotubes with Alexa Fluor-594 conjugated alpha-bungarotoxin diluted 1:1000 in differentiation medium for 60 minutes at 37°C, 5.5% CO₂. They were then washed with differentiation medium

3x5 minutes and fixed for 20 minutes with 3% paraformaldehyde (at room temperature in the dark). Myotubes were washed with PBS and stored in PBS at 4°C. 30 fields containing myotubes visualised in the bright field were selected and 30 microscopic images of red fluorescence per sample were acquired blinded using Simple PCI and analysed for AChR cluster number and length using ImageJ software.

2.2.14 Generation of DOK7-EGFP overexpressing C2C12 cells

C2C12 myoblasts were infected (see transfection protocol section 2.2.2c) with pBABE-IRES-DOK7-EGFP. The cells were plated in puromycin-containing medium at 1×10^5 cells per well in a 24 well plate, and the medium was exchanged for differentiation medium after 24 hours. The cells were maintained in differentiation medium until they had fused and formed myotubes. The presence of DOK7-induced AChR clusters was verified by staining with 1:1000 alpha-bungarotoxin conjugated to Alexa Fluor 594.

2.2.15 Fab purification

IgG consists of two heavy chains (50kDa) and two light chains (25kDa, figure 2.5A). Light chain and part of the heavy chain build up the Fragment, antigen-binding (Fab) region that has a molecular weight of 50kDa. This is connected by a hinge region to the Fragment, crystallisable (Fc) region that is also 50kDa (see also figure 1.8). Papain cuts specifically in the hinge region, which generates two Fab fragments and one Fc fragment, and so papain was used to generate Fab fragments (figure 2.5B). Aliquots were collected at each step of the purification procedure, which is described below. Purified IgGs (aliquot IgG) were filtered using a 0.2µm syringe filter, desalted using 5ml Zeba Spin desalting columns and diluted to a maximum protein

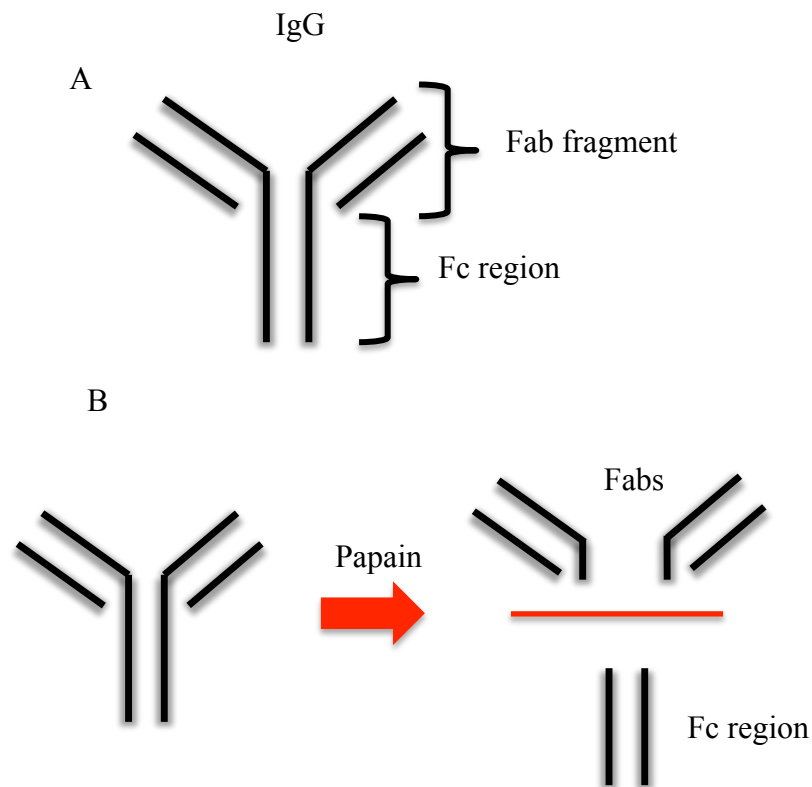


Figure 2.5: Generation of Fab fragments. (A) Human IgG consists of two heavy chains (50kDa) and two light chains (25kDa). The Fc region consists of the constant region of the heavy chains, while the Fab fragments that are connected to the Fc region via the hinge region consist of each a light chain and heavy chain, containing the variable region. (B) Papain cuts within the hinge region, which produces each one Fc region and two Fab fragments per IgG.

concentration of 4mg/ml in Hartmann's solution. Papain beads were pre-washed in digestion buffer from the Pierce Fab preparation kit, and then incubated with two-fold volume of the purified IgGs over night at 37°C with gentle shaking. The digest was centrifuged for 1 minute at 5000g and the supernatant containing Fab fragments was transferred to a fresh tube and incubated for 1h at 4°C with 1ml protein G sepharose to deplete the Fabs of Fc fragments and undigested whole IgG. Flow-through (the aliquot collected at this point was named Fab) and two washes (aliquot wash 1, wash 2) containing Fab fragments were pooled, and the Fc fragments and undigested IgG were eluted from the protein A sepharose with 0.1M glycine pH 2.3 (the aliquots collected were named eluate 1, 2 and 3). Aliquots from all steps were subjected to SDS PAGE with non-reducing conditions and coomassie-staining using PageBlue Protein Staining solution. Functional binding as well as concentrations of Fabs and whole IgG was verified by RIA.

2.2.16 Co-immunoprecipitation

HEK293 cells were seeded at 7.5×10^4 cells per well in 24-well plates. They were transfected the following day with MuSK-mCherry or LRP4-EGFP or both. 48 hours later, each well was incubated with MuSK-MG plasma diluted in HEK growth medium to a final concentration of 1nM MuSK antibodies, or Fab fragments diluted to a final concentration of 0.25nM, or purified IgG4 or IgG1-3 diluted to a final concentration of 0.5nM or corresponding volumes of goat anti rMuSK antibody in 250µl HEK growth medium for 2h at 37°C, 5.5% CO₂ (figure 2.6A). Cells were washed 3 times in growth medium, resuspended in 220µl ice-cold lysis buffer (10mM Tris-HCl, pH 7.4, 100mM NaCl, 1mM EDTA, 1%Triton-X supplemented

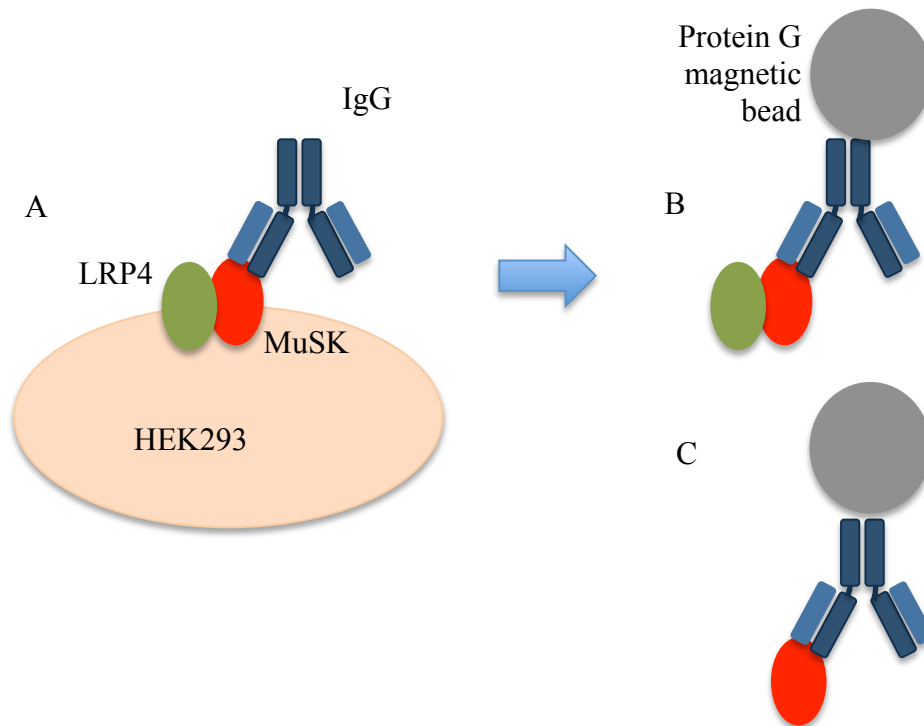


Figure 2.6: The principle of LRP4-MuSK co-immunoprecipitation. (A) HEK293 cells expressing MuSK-mCherry (red) and LRP4-EGFP (green) are incubated with anti-MuSK antibody (commercial or patient-derived). (B,C) The cells are then lysed and antibody bound to MuSK is being precipitated using protein G coupled magnetic beads (grey). (B) If MuSK and LRP4 interact, LRP4 will be co-precipitated by MuSK. (C) If binding between LRP4 and MuSK is disrupted, only MuSK will be precipitated.

with 1:100 protease inhibitor cocktail) and placed on ice for 15 minutes. Cell lysate was centrifuged 15 min 12000g at 4°C. A 10µl aliquot of the supernatant was reserved as whole cell lysate control. 200µl of the supernatant were incubated with 25µl protein G coupled magnetic beads for 20 minutes at room temperature with gentle agitation (figure 2.6B). The beads were washed 6 times with 200µl lysis buffer, resuspended in 15µl of NuPage LDS sample buffer containing NuPage reducing agent, and heated for 10 minutes at 70°C. The precipitate was subjected to SDS PAGE using NuPage Novex Tris-acetate mini gels and transferred to nitrocellulose. HiMark Pre-stained High molecular weight protein standard was also included. MuSK-mCherry was detected using 1:1000 anti-mCherry polyclonal antibody, and LRP4-EGFP was detected with 1:2000 anti-EGFP antibody each diluted in PBS supplemented with 0.1% Tween and 1% milk powder. Primary antibody was detected using HRP-conjugated secondary antibodies and ECL.

For each patient or healthy control sample, the area density of each band corresponding to MuSK on the western blot was measured with VisionWorks®LS Image Acquisition Software. Area density of the patient and healthy control samples was normalised to the area density of the MuSK band immunoprecipitated by the goat anti-rMuSK antibody. Likewise, the area density of each LRP4 band precipitated by patient and control samples was measured and normalised to the area density of the LRP4 band brought down by the goat anti-rMuSK antibody. Since there was some variation in the amount of MuSK precipitated by each patient, a value called “relative LRP4-MuSK binding efficiency” was calculated as a ratio of the normalised area densities of the LRP4 and MuSK bands:

$$\text{Relative MuSK-LRP4 binding efficiency} = \frac{\text{relative area density Lrp4}}{\text{relative area density MuSK}} \times 100$$

2.2.17 Statistics

All data were analysed with GraphPad Prism 6. The relationships between MuSK antibody concentrations and IgG subclasses were analysed by Pearson linear regression. For AChR clustering data and Co-IP, data from at least 3 experiments for each patient preparation were pooled and analysed with one way ANOVA using Bonferroni post-test comparing all columns against a control column. All error bars represent standard error of the mean.

Chapter 3: Characterising MuSK IgG from MuSK-MG patient plasma

3.1 Introduction

MuSK myasthenia gravis (MuSK-MG) is a disease of the neuromuscular junction mediated by autoantibodies against MuSK (Hoch et al., 2001). MuSK Abs have been demonstrated to cause defects in neuromuscular transmission in passive transfer experiments in mice (Cole et al., 2008, Klooster et al., 2012, Viegas et al., 2012), and when mice or rabbits are immunised actively against MuSK (Jha et al., 2006, Mori et al., 2012a, Viegas et al., 2012, Shigemoto et al., 2006, Shigemoto et al., 2008). Furthermore, in MuSK patients disease severity correlates with MuSK Ab concentrations (Bartoccioni et al., 2006).

Interestingly, the majority of MuSK Abs are of the IgG4 subclass (McConville et al., 2004) and one passive transfer study showed that patient derived MuSK IgG4 were pathogenic *in vivo* (Klooster et al., 2012), while MuSK IgG1-3 did not cause any weakness. The mechanisms by which MuSK Abs cause disease, however, are not yet clear and the aim was to investigate potential pathogenic mechanisms of MuSK Abs.

The first step was to identify suitable plasmas, to determine their MuSK Ab concentrations, to analyse the IgG subclass of the MuSK Abs, and to isolate and characterise the IgG4 subclass Abs and the IgG4-depleted IgG1-3 Abs.

3.2 Results

3.2.1 Clinical data

Plasma samples taken at plasma exchange (PEX) had been archived from 14 MuSK patients (table 3.1). Twelve were female and 2 male, with mean age at onset of 22.7 years. Clinical severity was assessed according to the Myasthenia gravis Foundation

of America (MGFA) clinical classification, with scores of I (mild weakness) to V (severe weakness, requiring artificial ventilation). All patients had generalized symptoms with a maximum MGFA score of IIB-V, and MGFA scores between IIB and IVB on the day the plasma samples were taken. All patients had received a combination of treatments, including prednisolone, azathioprine and/or PEX, and most had also received further treatments such as IVIG, rituximab or methotrexate. As controls, sera from 28 healthy controls, aged 23-70 years, 2 healthy control plasmas (male, 31 years and female, 47 years) and 5 AChR-MG sera were used.

To determine accurate MuSK Ab concentrations, the plasmas or sera were assayed by radioimmunoprecipitation assays (RIA, figure 3.1). Increasing volumes of MuSK-MG patient plasmas or control sera were incubated over night with ^{125}I -MuSK and human IgG was precipitated with anti-human IgG. The quantity of IgG-bound ^{125}I -MuSK was plotted against the sample volume. The amount of antibody in the samples was identified from the linear phase of the saturation curve (figure 3.1A shows an example, with the red arrow indicating that the 0.2 μl sample was close to the linear phase), and the MuSK Ab concentration calculated as described in Chapter 2. To determine positivity for a sample, a cut-off of the mean+3SDs of the values from 30 healthy controls was used (0.013nM). All MuSK-MG plasmas were positive for MuSK Abs (figure 3.1B), with concentrations well above the cut-off. All healthy control plasmas and AChR-MG sera were close to zero and below the cut-off.

The samples were also analysed for AChR Ab concentrations with RIA using ^{125}I labelled AChR (figure 3.1C). All MuSK-MG patients and healthy controls were negative for AChR Abs. Four of the AChR MG patients were well above the cut-off and clearly positive for AChR Abs, while one AChR MG patient was only a very low positive.

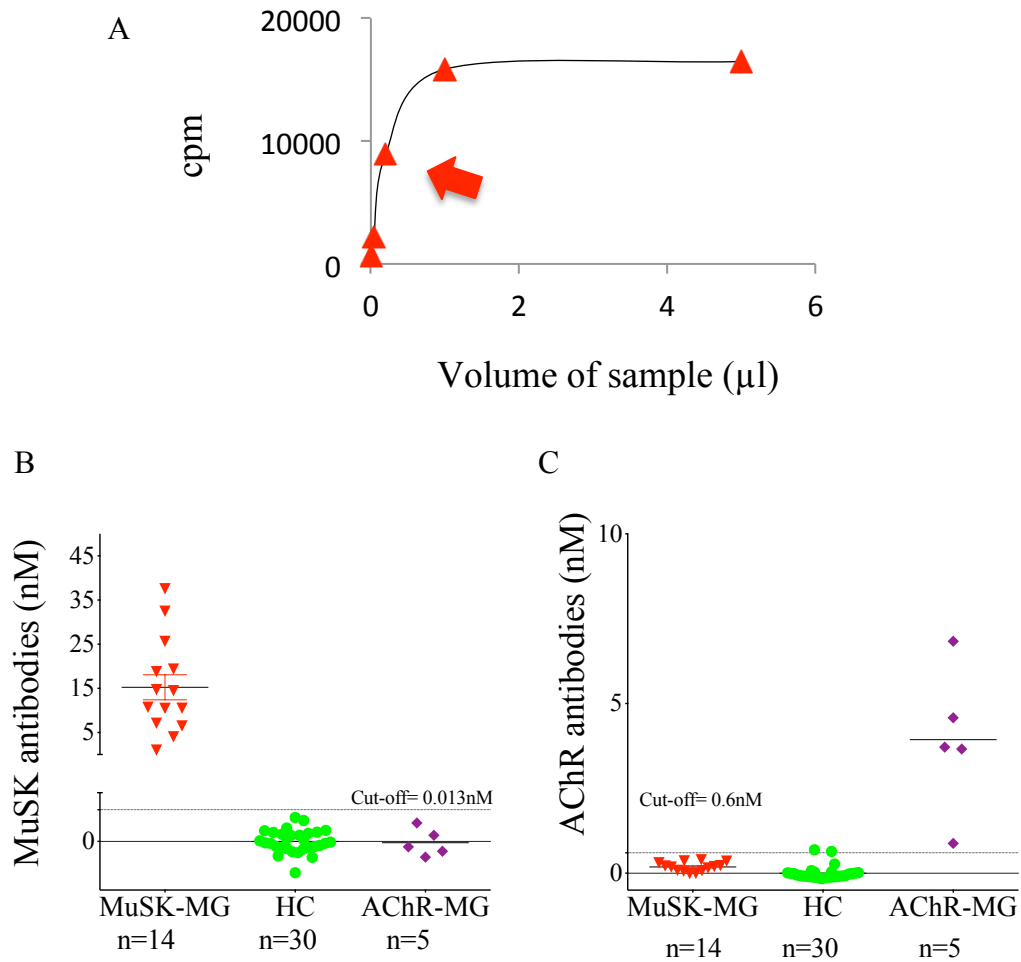


Figure 3.1: Characterisation of antibodies from MuSK-MG patients. (A) Example data of a titration of MuSK-MG patient plasma in radioimmunoassay. A five-fold dilution series of patient plasma was prepared (5, 1, 0.2, 0.04, 0.008 μl plasma), incubated over night with ^{125}I labelled MuSK and precipitated with anti-human IgG. ^{125}I radiolabelled MuSK that was precipitated could be quantified by measuring the cpm. To determine a value within the linear binding phase, the measured cpm of the samples were plotted on the y-axis against the sample volume on the x-axis. The red arrow indicates a sample that is within the linear binding phase and thus could be used to calculate the antibody concentration. (B) MuSK and (C) AChR antibody concentrations of 14 MuSK-MG patients, 30 healthy controls and 5 AChR MG patients as determined by titration in RIA. Cut-off for antibody positivity was the mean antibody concentration of pooled healthy controls + 3x standard deviation.

Table 3.1: Clinical data of fourteen MuSK-MG patients

Patient	MuSK Ab conc. (nM)	Age of onset (years)	^a Sex	Duration at sample day (years)	MGFA at day of sample	Max MGFA	^b Pre- dominant involve- ment	^c Treatment	^d Response to treatment	Outcome ^e (clinical evaluation at last FU)
1	37.57	17	F	1	IVB	IVB	OBR	Py,Pr,Az, PEX,TE	+(except TE)	R
2	32.46	5	F	na	IVB	IVB	OR	Pr,Az,Mt,IVIG,PEX	+++	+I,S
3	25.65	42	M	na	IIIB	IIIB	na	PEX	na	na
4	19.37	23	F	19	IIIB	V	BR	Py,Pr,Az, Cy,IVIG,PEX,TE	+(IS),+(PEX), -(TE)	I,S (T)
5	18.87	23	F	11	IIIB	V	BR	Py,Pr,PEX	++(Pr,PEX)	R
6	14.48	3	F	5	IIIB	IIIB	FB	Py,Pr,Az,Cy,Ri,PEX	+++ (Ri,PEX)	R (T)
7	14.7	27	F	5	IIIB	IIIB	FB	Py,Pr,Az,PEX	+(IS,PEX)	R,(T)
8	10.72	18	F	<1	IVB	IVB	B	Pr,Az,PEX	++	++I
9	10.51	14	F	<1	IIIB	IIIB	B	Py,Pr,Az,PEX	+	++I
10	10.47	25	F	2	IIIB	IVB	L	Pr,Az,Mt,My,PEX	+	Sy (T)
11	7.1	28	F	<1	IIIB	IIIB	OB	PEX,na	na	na
12	6.48	48	F	<1	IIIB	V	BR	PEX,na	++	na
13	4.02	28	M	2	IIIB	IIIB	OF	Py,Pr,Az,PEX	+(IS)	R
14	1.03	17	F	1	IVB	IVB	FB	Pr,Az,PEX	+	++I,Sy

^aF=female, M=male; ^bO=ocular, R=respiratory, F=facial, B=bulbar, L=limbs; ^cPr=Prednisolone, Az=Azathioprine, Mt=Methotrexate, IVIG=intravenous IG, PEX=plasma exchange, Cy=cyclosporine, Ri=Rituximab, TE=thymectomy, My=Mycophenolate, ^d(IS)= immunosuppression; ^eFU=follow up examination, I=improvement, R=remission, (T)=still under treatment, Sy=still showing symptoms, , +=good, ++=very good, +++=remarkably good. na=data not available because some plasmas were sent by colleagues elsewhere, or were from patients who returned to their countries, and follow-up data are not available.

Comparison of MuSK Ab concentration with MGFA clinical classification (figure.3.2) showed a trend towards higher concentrations with higher MGFA scores for both the maximum MGFA scores (figure 3.2A) and also the MGFA scores on the day the samples were taken (figure 3.2B). Not surprisingly, the occurrence of respiratory involvement correlated with MuSK Ab concentrations (figure 3.2C, unpaired t-test, $p=0.0027$).

3.2.2 Purification of whole IgG from human plasma

Human IgG was purified from the plasmas using protein G sepharose. Bound IgG was eluted from the column with glycine buffer pH 2.3, and 12 to 20 1 ml fractions were collected. The fractions containing protein were determined by measuring protein concentrations using a nanodrop (figure 3.3A). The fractions with the highest protein concentrations were then pooled and dialysed against Hartman's buffer to provide a physiological solution for experiments.

To verify that all the IgG has been removed, the flow-through was collected and incubated a second time with protein G sepharose, followed by elution with glycine buffer pH 2.3 (figure 3.3B). The fractions eluted from the beads had very little or no detectable protein, indicating that IgG had efficiently bound to the protein G sepharose during the first step of the purification.

3.2.3 Development of MuSK specific flow cytometry

In order to analyse the IgG subclass profiles of MuSK Abs in patient plasmas, a flow cytometry assay was developed as this allows a quantitative analysis of patient IgG binding to MuSK expressed on the surface of a human non-muscle cell line, and can

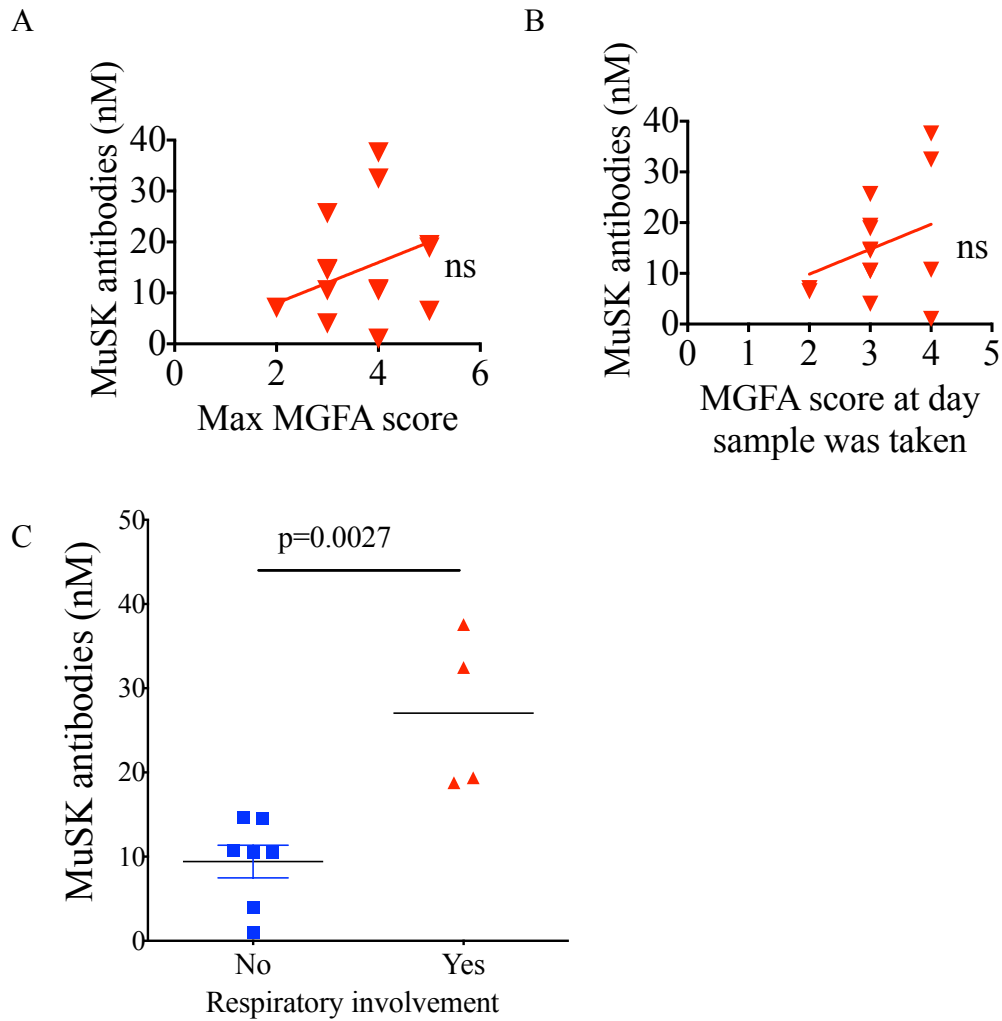


Figure 3.2: MuSK antibody concentration correlated with respiratory involvement. (A,B) MuSK antibody concentrations determined in figure 3.1 were compared to maximum MGFA clinical score (A) or the MGFA score at the day the sample was taken (B). No significant correlation was found (linear regression, Pearson correlation). (C) Patients with respiratory involvement (red triangles) had significantly higher MuSK antibody concentrations than patients without (blue boxes, $p=0.0027$, unpaired t-test).

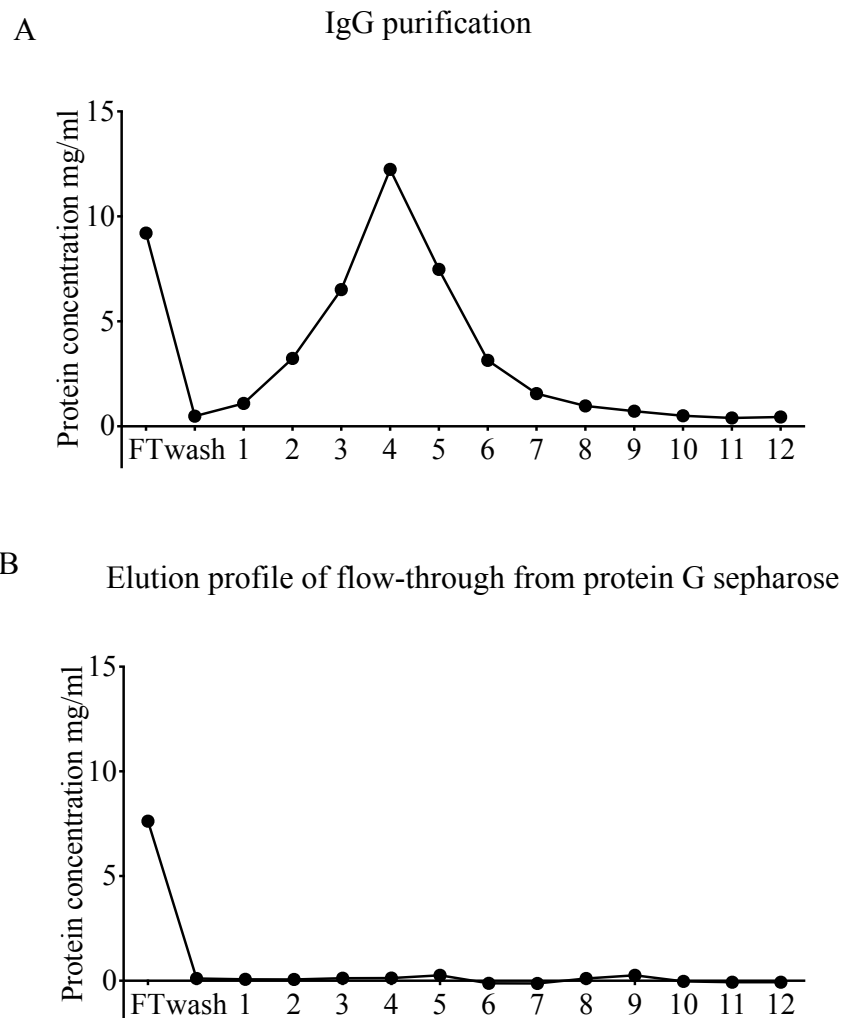


Figure 3.3: IgG purification from patient plasma. Plasma was incubated with protein G sepharose, the flow-through (FT) was collected, and the sepharose was then washed (wash) followed by elution with glycine buffer pH 2.3 in 12 fractions. Protein concentration of all fractions was then determined with a nanodrop. (A) Example data from patient 11 showing a peak in fractions 1-8. (B) To ensure IgG depletion, the FT was then incubated again with protein G sepharose, followed by wash, elution and analysis of protein content in the fractions. Very small amounts of protein were found in the elution steps.

be adapted to measure different IgG subclass antibodies. The assay would also be used to study endocytosis in subsequent experiments.

Optimisation of this assay was carried out using a construct that had already been produced in the laboratory by Dr. Patrick Waters. MuSK had been tagged with mCherry and inserted into the pEGFP-N1 vector, replacing the gene for EGFP (MuSK-mCherry, also see figure 2.2).

To check for MuSK surface expression, the construct was transfected into HEK293 cells. The cells were incubated for 48 hours and then trypsinised before incubation in increasing volumes of goat anti-rMuSK and 0.01mg/ml anti-goat IgG conjugated to allophycocyanin (APC). The results are shown in figure 3.4.

Figure 3.4A shows an example of mCherry fluorescence intensity (log units, measured between 603-623nm) on the x-axis, plotted against the APC fluorescence intensity of the secondary antibody on the y-axis. The median APC fluorescence intensity (mfi) in cells expressing MuSK-mCherry decreased with increasing dilutions of MuSK specific Ab, and pcDNA3.1-transfected cells that did not express MuSK had negligible APC mfi (figure 3.4B), indicating specific binding of the Abs to MuSK rather than non-specific binding to HEK293 cells. A dilution of 1:20 for primary commercial Ab (goat anti-rMuSK) was considered suitable for subsequent experiments, since this produced a value that was well above the control. However, a directly tagged protein had disadvantages for some future experiments, because protein expression and localization of MuSK may be affected by incubation with MuSK-MG patient IgG for several hours. If MuSK was internalised and degraded, this would affect the intensity of the fluorescent tag. Therefore it was decided to produce a construct expressing untagged MuSK.

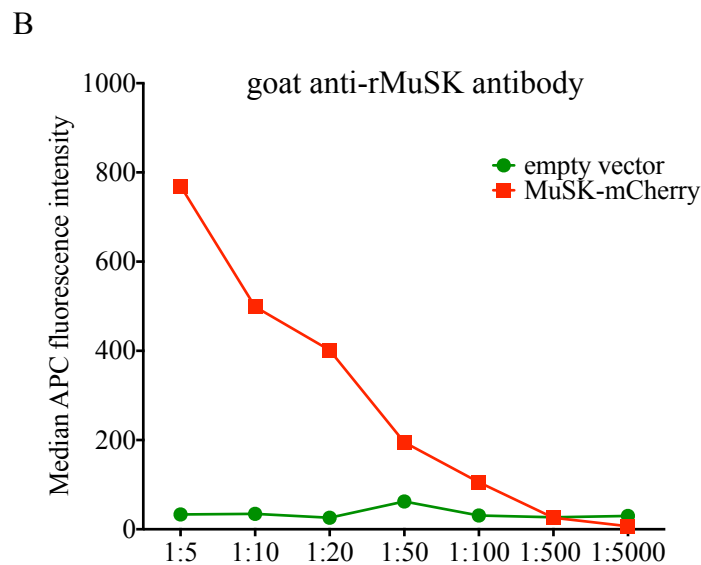
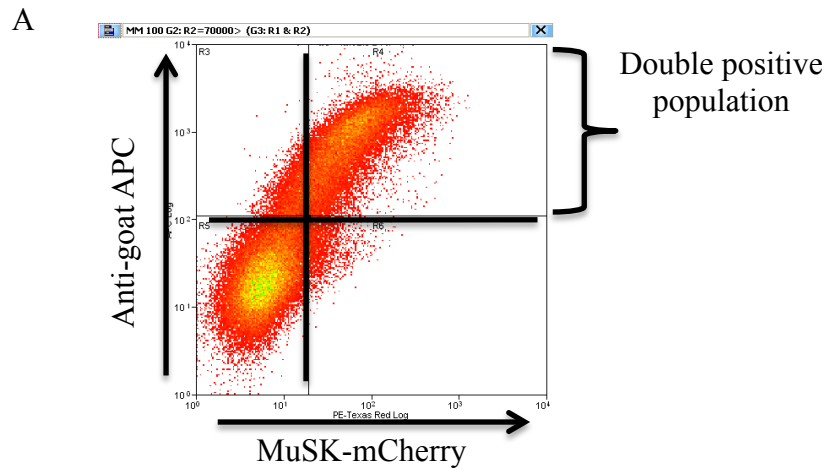


Figure 3.4: Verification of cell surface expression of MuSK using flow cytometry. HEK293 cells transfected with MuSK-mCherry were incubated with increasing concentrations of goat anti-rMuSK and 1:100 dilution of anti-goat APC antibody. (A) Example gating on cells incubated with 1:100 of goat anti-rMuSK with fluorescence intensity of APC in log units depicted on the y-axis and mCherry fluorescence in log units on the x-axis. The upper right quadrant contains the double positive population of transfected cells expressing MuSK-mCherry bound by primary and secondary antibodies to MuSK. The lower left quadrant shows double negative cells that are neither transfected nor bound by antibodies. (B) Mfif of secondary antibody were determined using increasing concentrations of primary anti-rMuSK antibody. Empty vector transfected cells showed very low mfi.

3.2.4 Cloning of pIRES2-AcGFP1-MuSK

Full length human MuSK was cloned into the vector pIRES2-AcGFP1, which expresses AcGFP after an internal ribosomal entry site (IRES, Methods figure 2.2). Full length human MuSK was excised from pcDNA3.1 expression vector (McConville, 2003) by restriction enzymes XhoI and NheI, and inserted into the multiple cloning site (MCS) of the pIRES2-AcGFP1 expression vector (figure 3.5A, insertion site indicated by orange arrows) by ligation of the 2.7kp MuSK insert into the 5.3kb digested and dephosphorylated vector pIRES2-AcGFP1. After transformation in *E.coli* JM109, a test digestion with XhoI and NheI of plasmids isolated from three separate colonies was performed. Figure 3.5B shows agarose gel electrophoresis of the digests; successful insertion of MuSK was judged by the presence of bands of 5.3kb (pIRES2-AcGFP1 vector, purple arrow) and 2.7kb (MuSK insert, red arrow). Both bands were present in the third lane of the gel, and subsequent sequencing of the corresponding plasmid, and BLAST analysis comparing the sequence with MuSK transcript variant 1 mRNA (NCBI reference number NM_005592.3), revealed correct insertion of MuSK.

For expression in HEK293 cells, the DNA was titrated to determine the amount of DNA required for efficient transfection. HEK293 cells were transfected with 0-4µg DNA of pIRES2-AcGFP1 or pIRES2-AcGFP1-MuSK. On the following day, transfection efficiency was assessed by fluorescence microscopy. The vector pIRES2-AcGFP1-MuSK expressed well with 4µg DNA per well in a 6-well plate. The vector pIRES2-AcGFP1 was expressed with higher efficiency than the pIRES2-AcGFP1-MuSK vector, even with 1µg DNA per well. 4µg DNA of pIRES2-AcGFP1-MuSK and 1µg DNA of pIRES2-AcGFP1 were selected as adequate amounts of DNA per well of a 6-well plate for further experiments (data not shown).

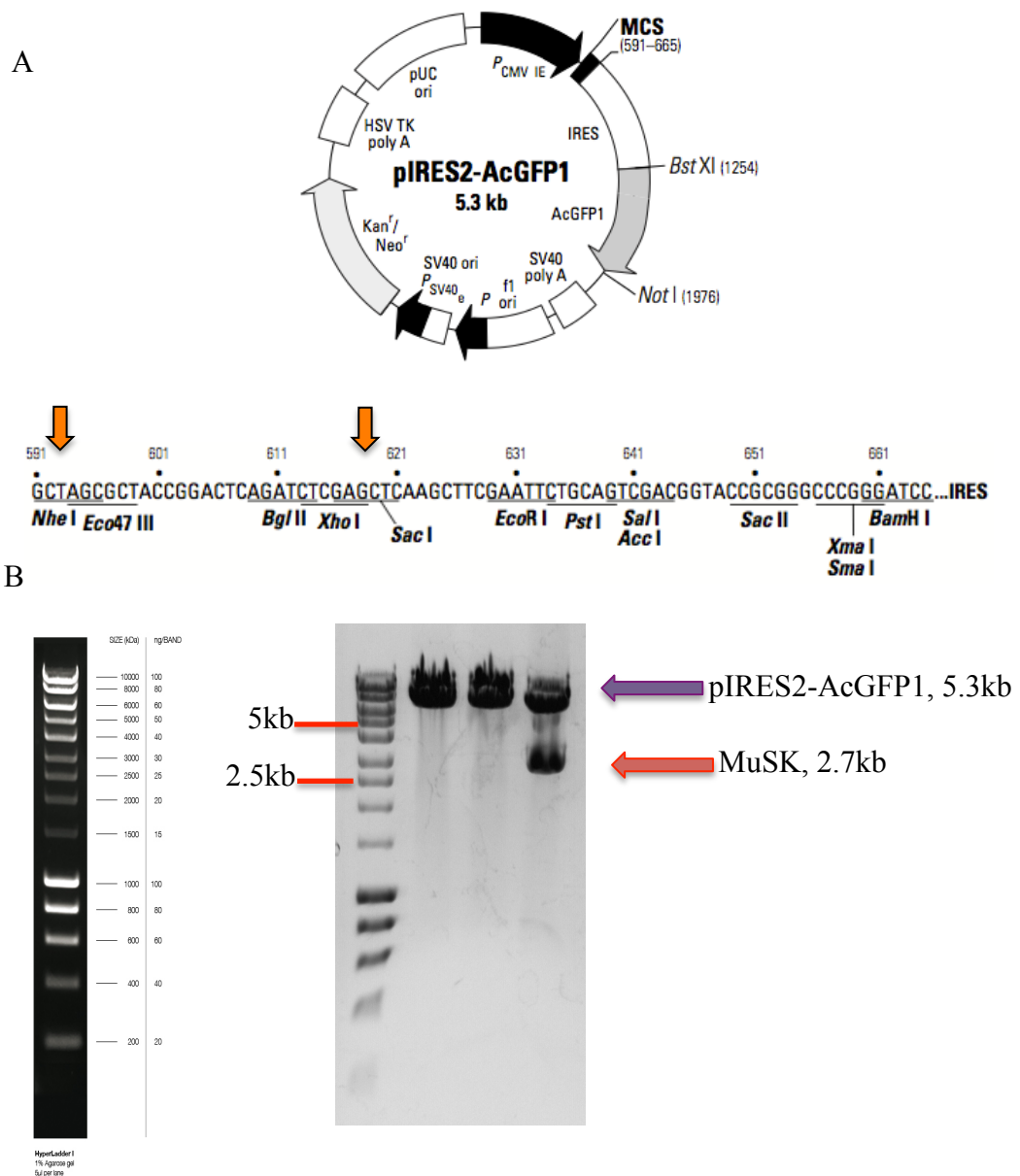


Figure 3.5: Full length human MuSK was cloned into the vector pIRES2-AcGFP1. (A) Map of the vector pIRES2-AcGFP1 indicating the multiple cloning site (MCS) into which MuSK was inserted using the XhoI and NheI restriction sites (orange arrows), the internal ribosomal entry site (IRES) followed by the gene for AcGFP. MuSK (2.7kb) was ligated with the digested pIRES2-AcGFP1 vector, transformed into *E.coli* JM109 and plasmids from three colonies were isolated and digested with XhoI and NheI. (B) Digests were subjected to gel electrophoresis and the presence of bands corresponding to pIRES2-AcGFP1 (5.3kb, purple arrow) and the MuSK insert (2.7kb, red arrow) was assessed. The third lane contains two bands corresponding to the vector and the insert.

3.2.5 Quantification of patient derived MuSK IgG by flow cytometry

Binding of Abs from patient or healthy control plasmas to HEK293 cells expressing pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 was then assessed by flow cytometry. AcGFP (which has an emission peak of 510nm) fluorescence was measured between 510 and 550nm using blue laser excitation (488nm) and human IgG was detected with anti-human IgG Ab conjugated to Alexa Fluor 647 (which has an emission peak of 668nm) by fluorescence measured between 655 and 675nm with 640nm red laser excitation. Alexa Fluor 647 was selected because it has distinct excitation and emission properties to that of AcGFP. The two fluorochromes have very little signal overlap and thus requirements for fluorescence compensation and the errors that can be associated with it are minimised.

Figure 3.6 illustrates binding of patient and healthy control IgG to live pIRES2-AcGFP1 and pIRES2-AcGFP1-MuSK transfected HEK293 cells in flow cytometry. AcGFP expression is depicted on the x-axis, with a shift of cells to the right on the x-axis illustrating cells that were successfully transfected with pIRES2-AcGFP1 or pIRES2-AcGFP1-MuSK. Binding of human IgG to the cells is shown as upward shift on the y-axis. Figure 3.6D shows that cells transfected with pIRES2-AcGFP1-MuSK and incubated with patient plasma shifted to the right on the x-axis and upward on the y-axis and hence produced a double positive population in the upper right quadrant. This was compared to pIRES2-AcGFP1-MuSK transfected cells incubated with healthy control serum (figure 3.6B), and as expected no upward shift of cells along the y-axis could be observed, demonstrating that healthy control IgG did not bind to the cells. Cells transfected with pIRES2-AcGFP1 vector alone did not generally contain double positive populations when incubated in either control or patient samples (figure 3.6A, C). With some samples, a slight upward shift on the y-axis of patient plasma incubated cells could be observed (figure 3.6C) for

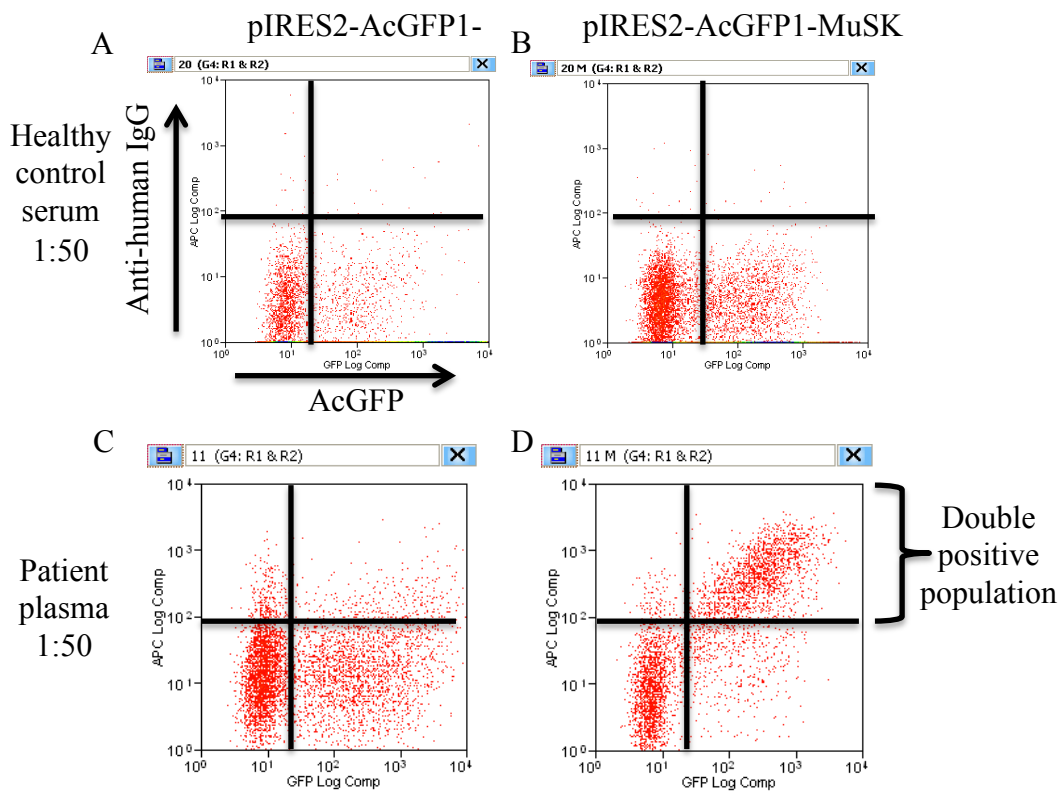


Figure 3.6: Patient derived MuSK IgG bound specifically to pIRES2-AcGFP1-MuSK transfected cells. HEK293 cells were transfected with pIRES2-AcGFP1- (A,C) or pIRES2-AcGFP1-MuSK (B,D) and incubated with 1:50 dilutions of either healthy control serum (A,B) or MuSK-MG patient plasma (C,D) followed by fluorescent-labelled anti-human IgG (Alexa Fluor 647). Fluorescence intensity of AcGFP was depicted on the x-axis, and Alexa Fluor 647 fluorescence of anti-human IgG was depicted on the y-axis. (D) A double-positive population that was AcGFP positive and was bound by human IgG located in the upper right quadrant could only be found in pIRES2-AcGFP1-MuSK transfected cells that were incubated with patient plasma.

transfected cells in the right half and for non-transfected cells in the left half of the figure, illustrating non-specific binding of human IgG. This varied between patients independent of the MuSK Ab concentration but overall, MuSK Abs derived from patients bound to MuSK on the surface of the cells, with a low level of non-specific binding that varied between patients (data not shown).

In order to measure and compare MuSK binding of IgG from different MuSK patients, it was important to determine the range of MuSK Ab concentrations within a linear binding phase. Therefore, patient 2 plasma, with one of the highest MuSK IgG concentrations determined by RIA, was used at increasing volumes (0.04 μ l -8 μ l plasma per 1x10⁵ cells in 40 μ l) and binding of human IgG was quantified as median fluorescence of Alexa Fluor 647 by flow cytometry (figure 3.7). The data was analysed by nonlinear fit with a saturation binding curve with one binding site using GraphPad Prism software. The goodness of the fit was determined as $r^2=0.99$ for cells expressing pIRES2-AcGFP1-MuSK and $r^2=0.97$ for cells expressing pIRES2-AcGFP1-. A volume of 0.8 μ l plasma binding to 1x10⁵ cells, which corresponded to a 1:50 dilution of patient plasma, produced binding within the linear phase. Thenceforth, plasmas from all 14 patients and 7 healthy control sera were used at a 1:50 dilution (0.8 μ l sample per 1x10⁵ cells per 40 μ l).

Binding of patient IgG to pIRES2-AcGFP1 transfected cells was negligible in this example, but was variable (although low) in other patients. Therefore specific binding of patient derived IgG to cell surface MuSK was determined: the populations of pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 transfected cells were selected by gating on AcGFP⁺ cells, and human IgG binding was quantified by the median fluorescence intensity (mfi) of Alexa Fluor 647 fluorescence. Then the mfi of pIRES2-AcGFP1 transfected cells was subtracted from the mfi of pIRES2-AcGFP1-MuSK transfected cells, resulting in a specific MuSK binding value called the Δ mfi.

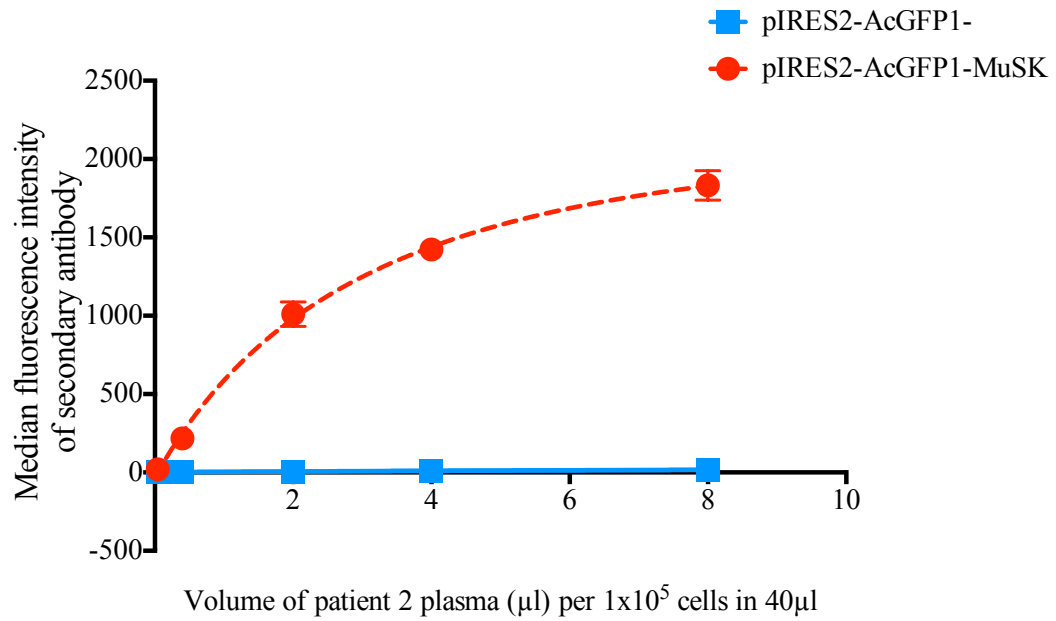


Figure 3.7: Titration of patient 2 plasma using flow cytometry. HEK293 cells expressing pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 were incubated with increasing volumes of patient 2 plasma and fluorescent-labelled secondary antibody. Transfected cells were selected by gating on AcGFP positive cells. Median fluorescence intensity of the secondary antibody was determined. Analysis: Nonlinear fit saturation binding curve with one binding site. Goodness of fit: pIRES2-AcGFP1-MuSK: $r^2 = 0.99$, pIRES2-AcGFP1: $r^2 = 0.96$

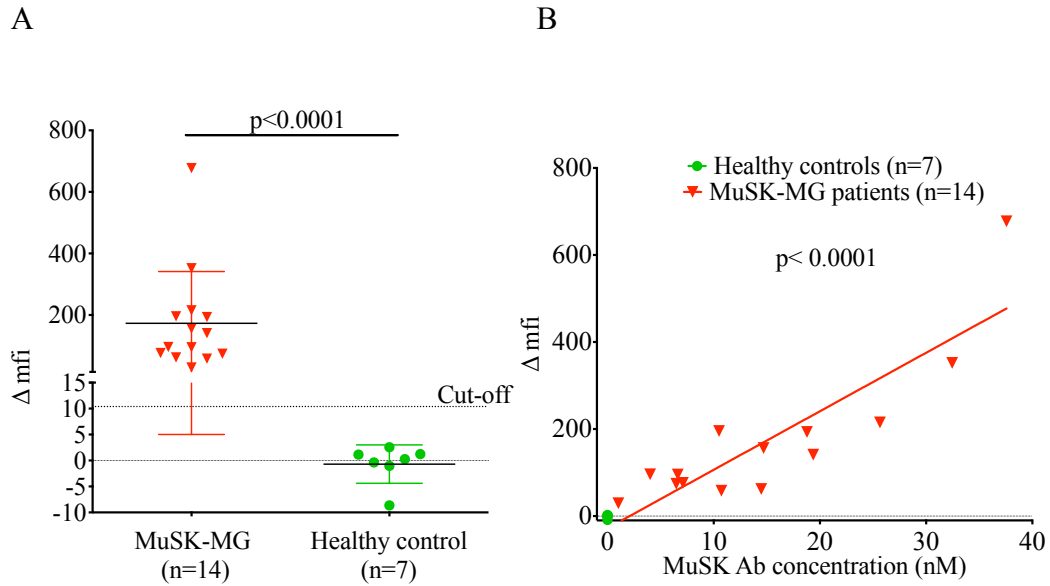


Figure 3.8: MuSK antibody binding detected by flow cytometry correlated with MuSK antibody concentrations determined by RIA. Fourteen MuSK-MG patient plasmas and seven healthy control sera were analysed for MuSK antibody binding to MuSK by flow cytometry using 1:50 dilution of patient sample on 1×10^5 cells in 40 μ l volume. Median fluorescence intensity (mfi) of pIRES2-AcGFP1 and pIRES2-AcGFP1-MuSK transfected cells was determined. The mfi of pIRES2-AcGFP1 transfected cells was subtracted from the mfi of pIRES2-AcGFP1-MuSK transfected cells to obtain Δmfi . (A) Pooled Δmfi s of MuSK-MG patients have significantly higher mean Δmfi than healthy controls (green squares, $p < 0.0001$, unpaired t-test). A cut-off was calculated as mean of healthy controls + $3 \times$ standard deviation of the healthy controls. (B) The Δmfi of fourteen patient plasmas and seven healthy control sera were compared to MuSK antibody concentrations determined by RIA. MuSK patient Δmfi correlated with MuSK antibody concentrations ($p < 0.0001$, linear regression, Pearson correlation).

In this way the Δmfi for all 14 patient plasma and 7 healthy control sera was obtained (figure 3.8A). A cut-off of 10 Δmfi was calculated as mean Δmfi of seven healthy controls plus 3SDs. All healthy controls (green) were below the cut-off, and all MuSK plasmas (red) were above the cut-off. Unpaired t-test showed a significant difference in Δmfi between healthy controls and MuSK patients ($p < 0.0001$).

A good correlation between Δmfi and MuSK concentration measured by RIA was found ($p < 0.0001$, figure 3.8B), demonstrating that flow cytometry could be used to quantify binding of MuSK-specific IgG to MuSK. However, as autofluorescence of the cells varied in each flow cytometry experiment, and different flow cytometry instruments with slightly different performance had to be used sometimes, the gates were set up anew for each experiment. However, the relative difference in binding between different patients' antibodies, or between IgG subclasses (analysed below), was predicted to stay the same, which was demonstrated to be the case when the Δmfi was normalised to percent (see section 3.2.6).

3.2.6 IgG subclass profiling using flow cytometry

IgG subclass profiles of MuSK patient plasmas were then measured by flow cytometry. Instead of using anti-human IgG as the secondary Ab, subclass specific Abs (mouse anti-human IgG1, 2, 3 and 4) were used and detected using an anti-mouse Ab conjugated to APC. Anti-mouse-APC was used at a 1:200 dilution according to the manufacturer's instructions, and each secondary Ab was titrated, maintaining a 1:50 dilution of patient 2 plasma as primary Ab (figure 3.9A).

The IgG4-specific Ab resulted in notably higher Δmfis than the IgG1, 2 and 3-specific Abs at all dilutions, confirming that IgG4 was the predominant subclass in this patient. For subsequent experiments, a 1:50 dilution was selected for all of the

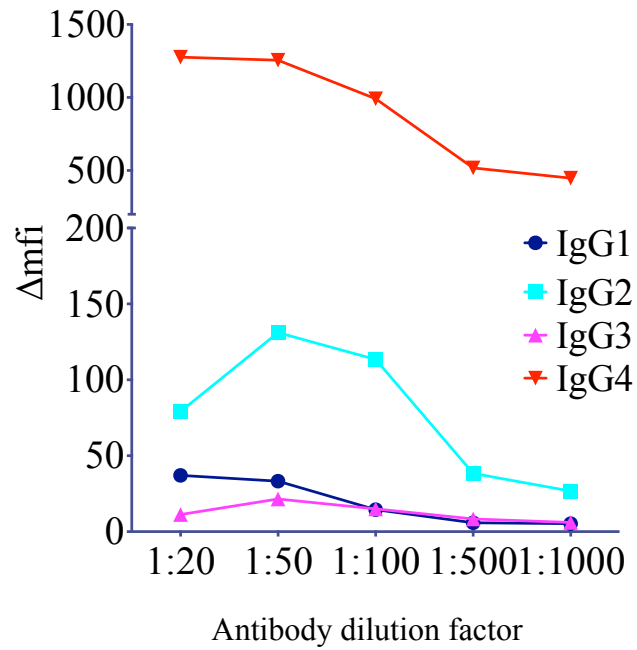


Figure 3.9: IgG subclass profiling with flow cytometry. Titration of IgG subclass specific antibodies using flow cytometry. HEK293 cells transfected with pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 were incubated with a 1:50 dilution of patient 2 plasma, increasing concentrations of subclass specific antibodies raised in mouse and a 1:200 dilution of anti-mouse APC. The Δmfi s were determined for each dilution.

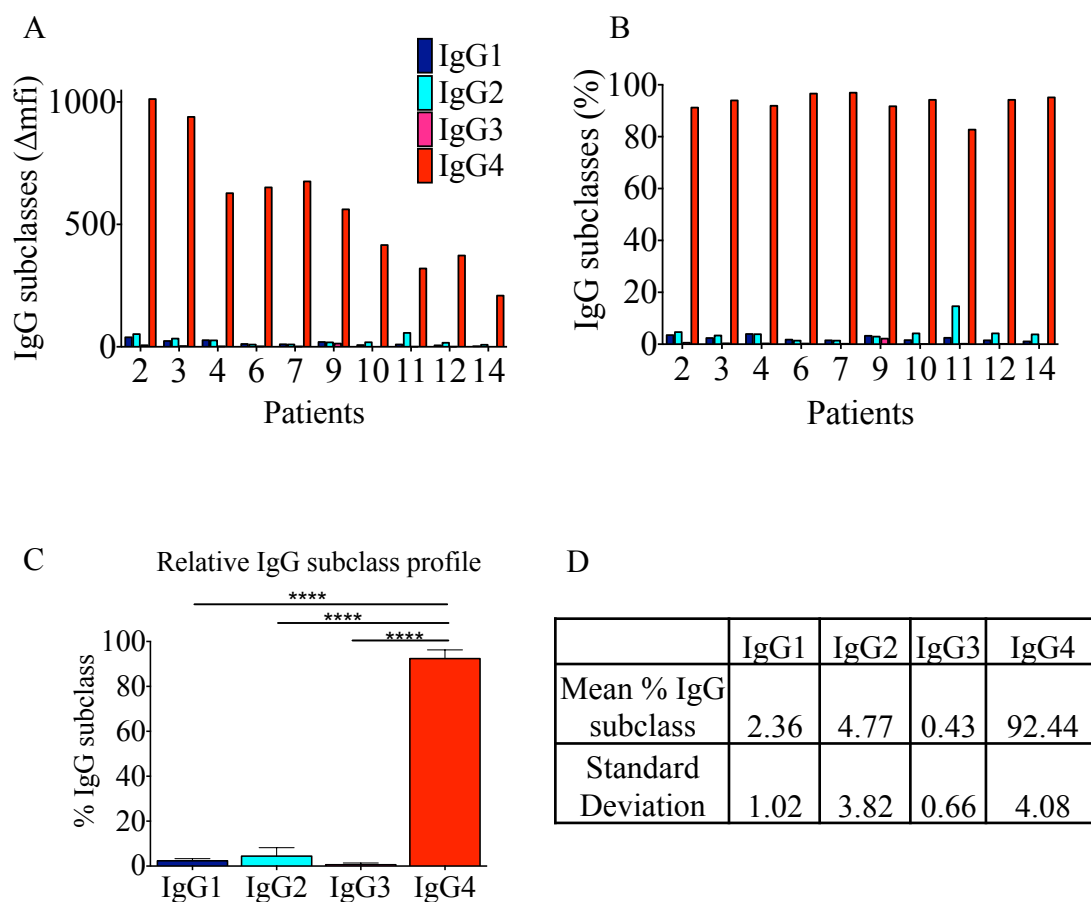


Figure 3.10: IgG4 was the predominant subclass in MuSK-MG plasma. HEK293 cells expressing pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 were incubated with 1:50 dilutions of patient plasma followed by 1:50 dilution of mouse anti-human IgG1, 2, 3 or 4 and 1:200 dilution of anti-mouse APC. (A) IgG1, 2, 3 and 4 subclasses of ten patients were measured by flow cytometry. (B) The Δmfi s of IgG1-4 were added up to obtain total Δmfi s and Δmfi s of individual subclasses were calculated as percent of total Δmfi . (C) IgG subclass data from all ten patients in percent was pooled and analysed with one-way ANOVA and Bonferroni post-test. **** $p \leq 0.0001$. Error bars depict standard deviation. (D) Mean and standard deviation of (C).

anti-human IgG1, 2, 3 and 4 Abs. IgG1, 2, 3 and 4 subclass Δmfi values of ten patients were measured (figure 3.10A). All of the samples were set up at the same time and measured on the same instrument using the same settings, so that the levels of subclasses could be compared. The absolute levels of MuSK-specific subclasses varied from patient to patient, and to be able to compare patients' subclass profiles more easily the Δmfi values were expressed as percent of total Δmfi as shown in figure 3.10B (see also table 3.2). The predominant subclass in all ten patients was IgG4 with values above 80% in all patients. IgG2 levels varied between patients and was unusually high (14.64%) in patient 11. The % values of each patient were then measured and pooled to obtain a relative IgG subclass profile (figure 3.10C) and analysed by one-way ANOVA and Bonferroni post-test. IgG4 was significantly higher than IgG1, 2 and 3 (****, $p \leq 0.0001$). Figure 3.10D depicts the mean IgG subclass percentages. IgG4 is the predominant subclass in the analysed MuSK-MG patients.

The data shown in figure 3.10A hinted that the levels of each MuSK-specific subclass antibody might correlate with the overall MuSK Ab concentration, because the patient samples were arranged in order of decreasing MuSK Ab concentration. Therefore, to investigate this, Δmfi of IgG1, 2, 3, 4 subclasses of all fourteen patients and three healthy controls were assessed individually, one subclass at a time. It is important to understand that the four subclasses were assessed on four different days and cannot be directly compared to each other. For this experiment fresh aliquots of antibody were used, and the samples were also assayed by RIA. Figure 3.11 depicts the pooled Δmfi s for IgG1-4 of the fourteen patients in red and of the three healthy controls in green. Note that IgG4 samples of patient 8 and 11 are not shown due to a loss of cells and thus low sample quality. Despite very low levels of IgG1-3, the means of the pooled Δmfi s of the patients were significantly higher than the means

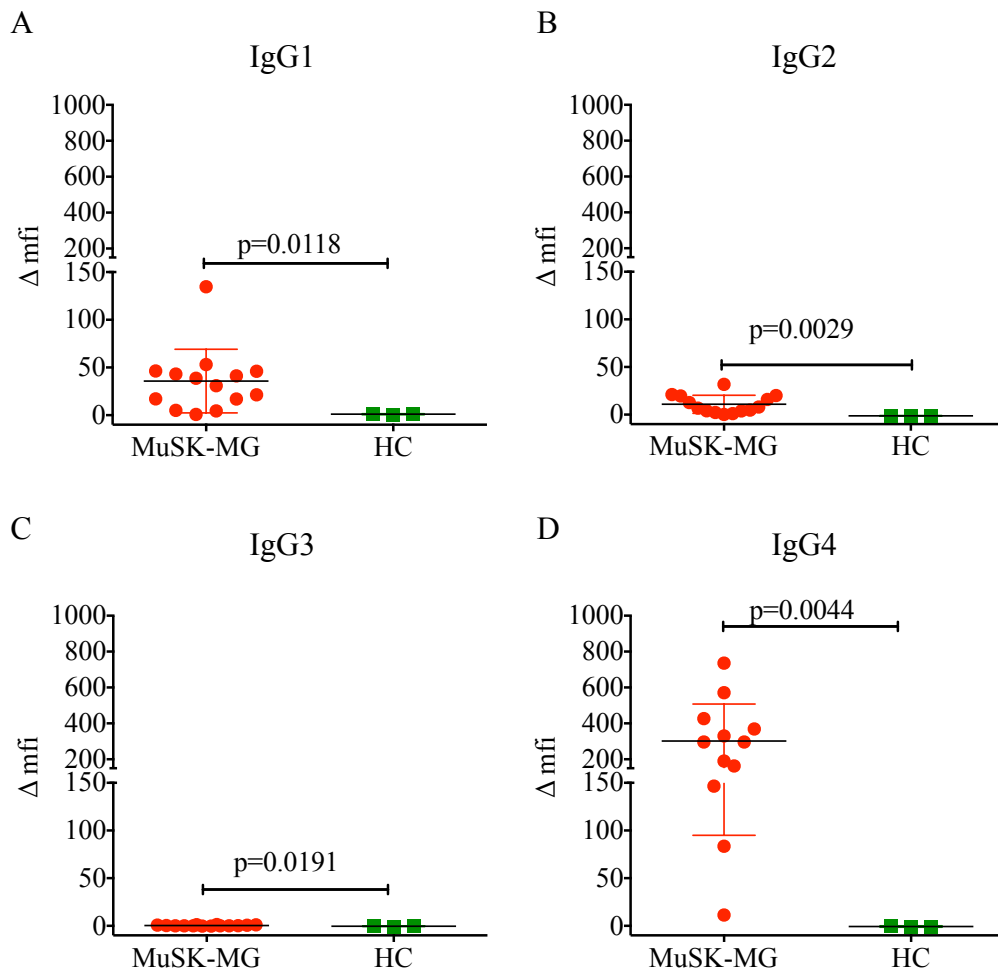


Figure 3.11: Quantification of IgG subclass profiles of fourteen MuSK-MG patients. The Δmfi s of fourteen MuSK-MG patients (red circles) and three healthy controls (green squares) were obtained as described in figure 3.10 and pooled. Mean patient Δmfi s were significantly higher than mean healthy control Δmfi s in all IgG subclasses using Mann Whitney t-test: (A) IgG1: $p=0.0118$, (B) IgG2: $p=0.0029$, (C) IgG3: $p=0.0191$ and (D) IgG4: $p=0.0044$. IgG4 Δmfi s were considerably higher than Δmfi s of IgG1, 2 or 3.

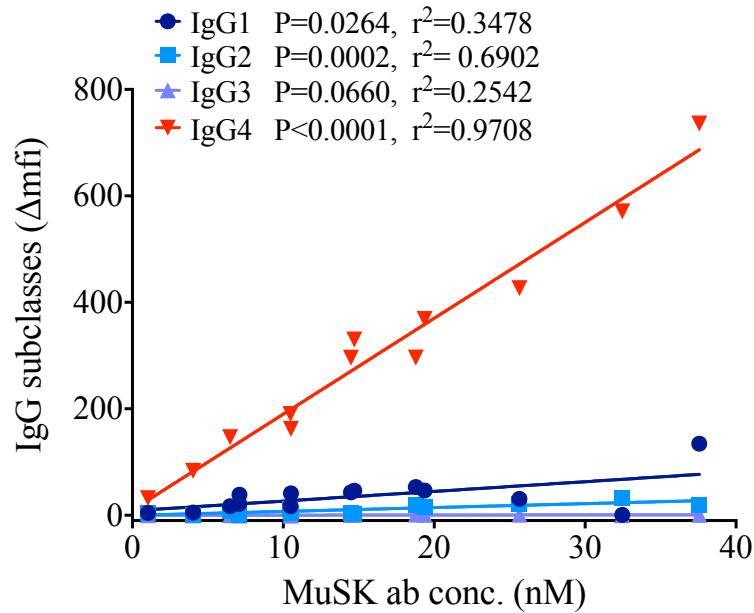


Figure 3.12: IgG1, 2 and 4 subclass correlated with MuSK antibody concentration. IgG subclasses of 14 MuSK patient plasmas were measured by flow cytometry (figure 3.11). The MuSK antibody concentrations of the plasmas were measured by RIA and compared to the Δmfi s. IgG1, 2 and 4 Δmfi correlated significantly with MuSK antibody concentration. Statistic analysis: linear regression followed by Pearson correlation (Gaussian distribution was determined by D'Agostino and Pearson test).

of the pooled $\Delta m f_i$ s of the healthy controls for all four IgG subclasses (Mann Whitney t-test: IgG1: $p=0.0118$, IgG2: $p=0.0029$, IgG3: $p=0.0191$ and IgG4: $p=0.0044$).

The results of the four individual IgG subclass measurements ($\Delta m f_i$ of IgG1, 2, 3, 4) were then compared to MuSK concentrations measured by RIA (figure 3.12), and a significant correlation between the MuSK Ab concentration and $\Delta m f_i$ of IgG1, 2 and 4 could be found (linear regression and correlation analysis, Pearson correlation). Thus the IgG subclass profiles of all patients were similar, and IgG4, which correlated most significantly with MuSK Ab concentrations, was the predominant IgG subclass in all patients.

Unfortunately, two IgG4 values, of patient 8 and 11 (figure 3.11-13), had to be discarded in the IgG4 subclass assay, due to a loss of cells and thus low sample quality, but IgG4 values from the IgG subclass profiling experiments (figure 3.10, table 3.2) was available for patient 11.

None of the $\Delta m f_i$ s of the IgG subclasses correlated with the MGFA scores at the times the samples were obtained (figure 3.13A) or the maximum MGFA scores (figure 3.13B, linear regression followed by Pearson correlation). However, many samples had very low $\Delta m f_i$ s, close to zero or negative, due to low MuSK IgG1-3 concentrations and low overall MuSK IgG concentrations in some of the patients; this makes the analysis unreliable.

3.2.7 IgG4 and IgG1-3 subclass purification

To isolate IgG4 subclass and IgG4 depleted IgG1-3 subclass Abs from patient plasmas, an IgG4 affinity matrix (CaptureSelect) was used. The matrix contained a ligand directed against a region in the IgG4 Fc part, unique to this Ab subclass, conjugated to sepharose beads. The IgG4 affinity matrix was used in a similar

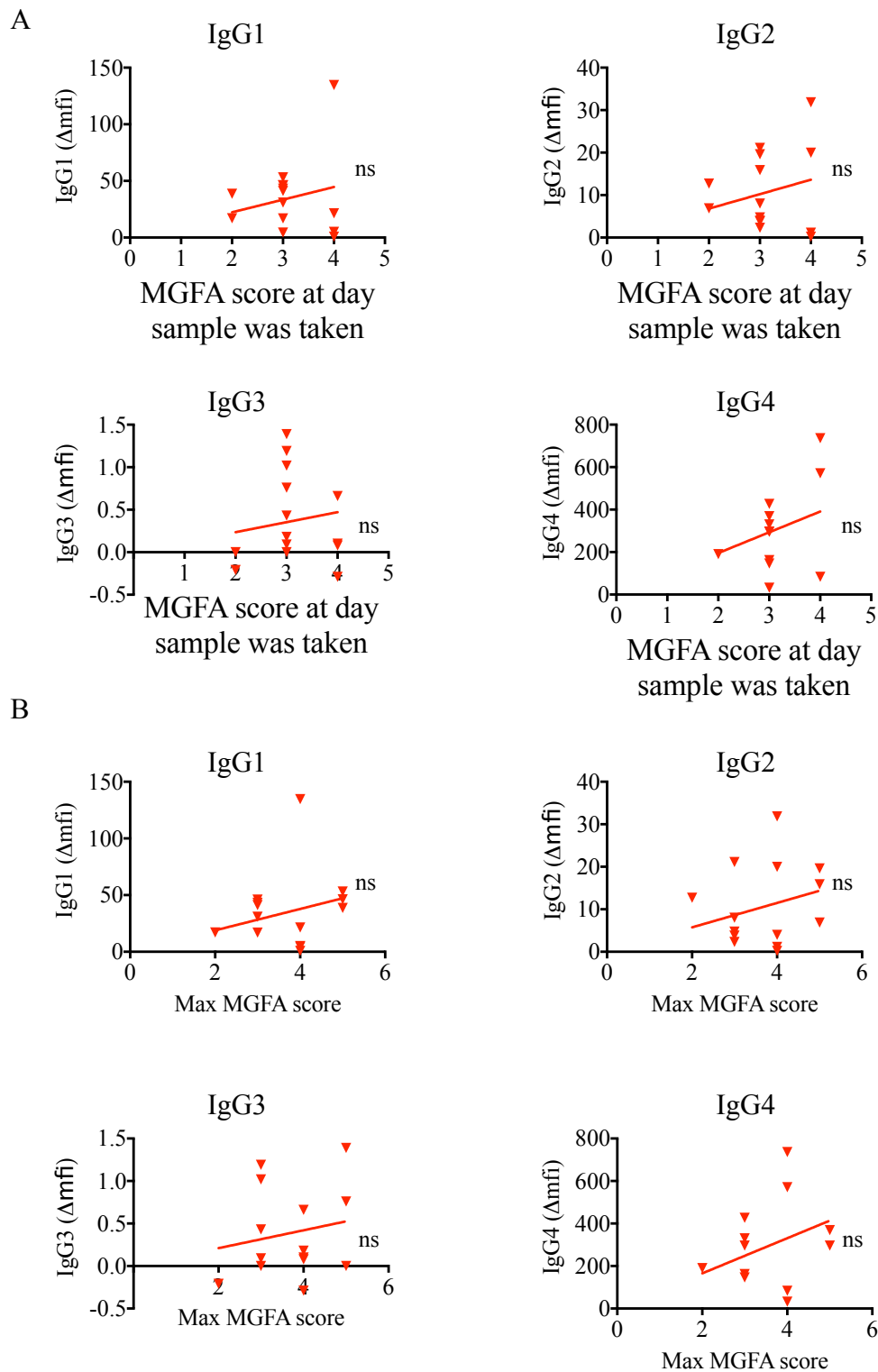


Figure 3.13: IgG subclasses did not correlate with disease severity. IgG1-4 subclass proportions measured in flow cytometry were plotted against (A) the MGFA at the day the sample was taken and (B) the maximum MGFA scores. Statistic analysis: linear regression followed by Pearson correlation.

Table 3.2: IgG subclass profiles of ten patients

Patient	MuSK Ab concentration (nM)	IgG1		IgG2		IgG3		IgG4	
		Δmfi	%	Δmfi	%	Δmfi	%	Δmfi	%
2	32.46	38.98	3.51	52.02	4.69	6.22	0.56	1011.89	91.23
3	25.65	23.73	2.37	33.43	3.34	3.17	0.32	939.13	93.96
4	19.37	26.99	3.95	26.33	3.86	1.97	0.29	627.45	91.90
6	14.48	11.81	1.75	9.23	1.37	1.76	0.26	651.07	96.62
7	14.7	10.42	1.50	9.51	1.36	1.25	0.18	675.57	96.96
9	10.51	19.71	3.22	18.01	2.94	13.12	2.14	561.47	91.70
10	10.47	7.01	1.59	18.34	4.16	0.31	0.07	415.67	94.19
11	7.1	9.60	2.48	56.62	14.64	0.68	0.18	319.77	82.70
12	6.48	5.96	1.51	16.47	4.16	0.56	0.14	372.99	94.19
14	1.03	2.36	1.07	8.31	3.78	0.00	0.00	209.25	95.15

manner to protein G sepharose for purification of whole IgG, but with a shorter incubation time (one hour for IgG4 as opposed to 16 hours for whole IgG). Figure 3.14A shows the OD₂₈₀ of the eluted fractions containing IgG4, with a peak in fractions 2 and 3. IgG1-3 subclass Abs were then purified from the IgG4 depleted flow-through by binding and elution from protein G sepharose (figure 3.14B). As expected from the low concentrations of total IgG4 in serum, the peak for IgG4 elution (OD₂₈₀ value of approximately 2) was lower than the peak for IgG1-3 elution (OD₂₈₀ value of 10).

Protein concentrations and MuSK Ab concentrations in the purified IgG4 and IgG1-3 were assessed with a nanodrop and RIA respectively (figure 3.14C). Notably, though protein concentration of IgG1-3 was 6-10 fold higher than IgG4, the MuSK Ab concentration in IgG1-3 was 6-10 fold lower than in IgG4, demonstrating the high percentage of MuSK Abs in the IgG4 subclass compared with the low fraction of MuSK Abs in the IgG1-3 subclasses. Purified healthy control IgGs, that were used as a control in subsequent experiments, had protein concentrations of 4.48mg/ml (HC 1) and 3.23mg/ml (HC 2).

3.2.8 IgG subclass profiles of purified IgG4 and IgG1-3

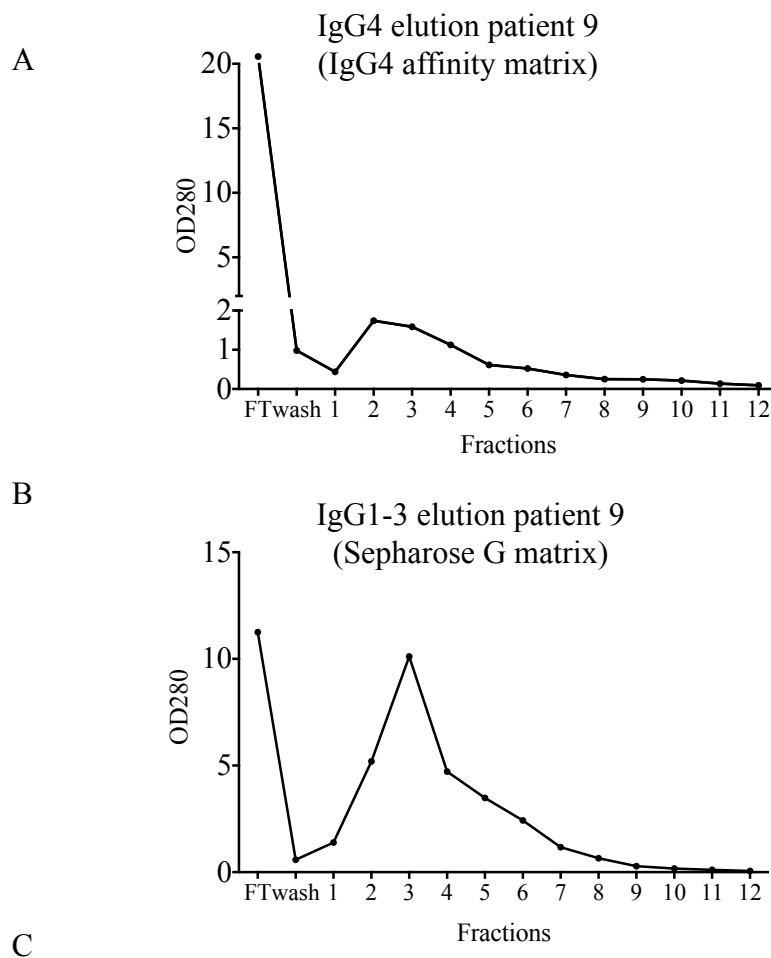
To ensure adequate isolation of IgG4 from total IgG, IgG subclass profiles of the isolated IgG subclass fractions were assessed with flow cytometry. Purified IgG4 and IgG1-3 were analysed for the presence of IgG1, 2, 3 and 4 subclass MuSK Abs (figure 3.15A, B). Purified IgG4 contained mainly IgG4 Abs and low levels of IgG2. The IgG4 depleted IgG1-3 subclasses had low levels of MuSK Ab IgG1, 2 and 3 and IgG4 was undetectable. This indicates efficient IgG4 purification from MuSK patient plasma with the remaining IgG1-3 subclass successfully depleted of IgG4 but still containing detectable MuSK Abs.

3.2.9 Determination of binding affinities of IgG4 and IgG1-3 to MuSK

To compare the binding affinities of IgG4 and IgG1-3 to MuSK, a saturation binding experiment was conducted. Measuring the K_d of polyclonal IgG to a complex antigen is difficult, and precise values can only be obtained of monoclonal antibodies binding to hapten antigens. Therefore the calculated K_d below has to be considered as an estimate.

The dissociation constant K_d was determined as the concentration of MuSK at half maximum binding of IgG4 or IgG1-3. Increasing concentrations of radiolabelled MuSK were exposed to a constant concentration of IgG4 or IgG1-3. Total MuSK (fmoles per 100 μ l) was defined as the concentration of available radiolabelled MuSK for each sample before immunoprecipitation. Bound MuSK (fmoles) was defined as the amount of radiolabelled MuSK that was immunoprecipitated. To exclude non-specific binding, healthy control serum was used at an equal volume as patient IgG4 or IgG1-3 and resulting values were subtracted from bound MuSK to obtain specific bound MuSK (fmoles).

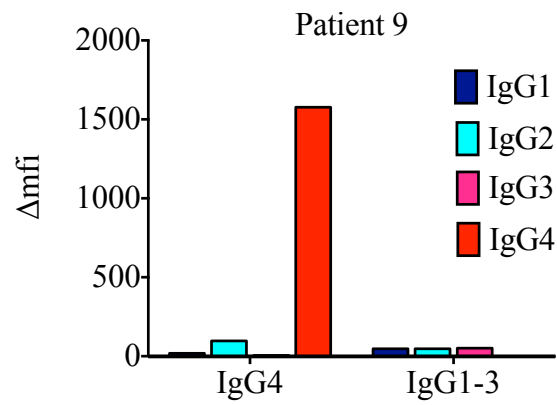
Figure 3.16 depicts results of a saturation binding experiment with IgG4 and IgG1-3 of two patients. Specific bound MuSK was plotted against total MuSK (figure 3.16A), and K_d and B_{max} were calculated with nonlinear regression using a saturation binding curve with one site binding analysis and the equation $Y = B_{max} * X / (K_d + X)$ in GraphPad Prism (figure 3.16B). The K_d values were found to be in a picomolar range, thus both IgG4 and IgG1-3 subclass Abs were of high affinity.



Patient	MuSK Ab conc. purified IgG4 (nM)	MuSK Ab conc. purified IgG1-3 (nM)	Protein conc. purified IgG4 (mg/ ml)	Protein conc. purified IgG1-3 (mg/ml)	MuSK Ab/ protein IgG4 (nmol/g)	MuSK Ab/ protein IgG1-3 (nmol/g)
9	6.47	1.5	0.41	2.59	15.78	0.57
12	5.77	0.51	0.26	2.08	22.19	0.24

Figure 3.14: IgG4 and IgG1-3 purification from patient plasma. MuSK-MG patient plasma was incubated with an IgG4 affinity matrix. (A) The flow-through (FT) containing IgG1-3 was collected, and after a washing step (wash), bound IgG4 was eluted in 12 fractions (1-12) with pH2.3 glycine buffer. (B) To obtain IgG4 depleted IgG1-3 subclass antibodies, the FT was incubated with protein G sepharose. FT and wash were collected, and 12 fractions were eluted with glycine buffer pH2.3. (C) MuSK antibody concentrations (nM) and total protein concentrations (mg/ml) of patient derived IgG4 and IgG1-3 were determined by RIA and a nanodrop, and MuSK antibody concentrations in nmol/g were calculated.

A



B

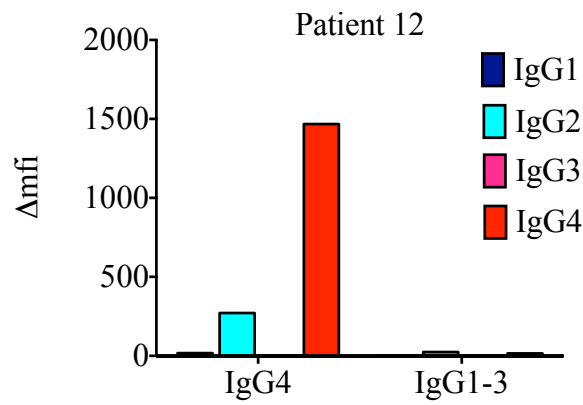
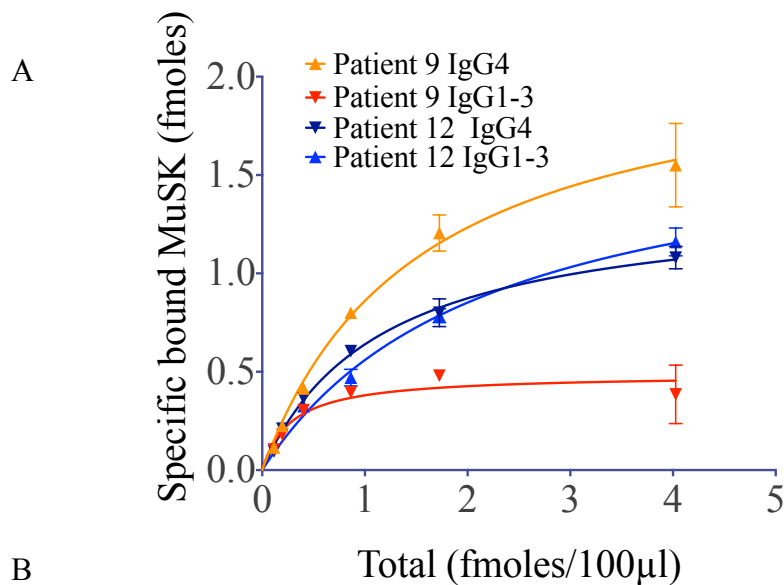


Figure 3.15: IgG subclass profiles of purified IgG4 and IgG1-3 from two patients. IgG1-3 and IgG4 subclasses were purified (figure 3.14) from MuSK-MG patients 9 (A) and 12 (B). The purity of the products was analysed by flow cytometry and profiles depict Δmfi s of IgG4 and IgG1, 2 and 3. Purified IgG4 contained mainly IgG4 and low levels of IgG2. The IgG1-3 fraction contained low levels of IgG1, 2 and 3, while IgG4 levels were negligible.



	B_{max} (fmoles) IgG4	K_d (pM) IgG4	B_{max} (fmoles) IgG1-3	K_d (pM) IgG1-3
Patient 9	2.17	15.31	0.48	2.79
Patient 12	1.37	11.56	1.72	20.27

Figure 3.16: Determination of binding affinities of IgG4 and IgG1-3. To calculate the K_d of IgG4 and IgG1-3, a saturation binding experiment was performed. (A) IgG4 or IgG1-3 were exposed to increasing concentrations of radiolabelled MuSK. Total MuSK was determined for each sample. After precipitation, unbound MuSK was washed away and specific bound MuSK was determined. (B) K_d and B_{max} derived from nonlinear regression analysis with one site binding using GraphPad Prism software. The goodness of the fit was determined: $r^2 = 0.9796$ (patient 9 IgG4), 0.7965 (patient 9 IgG1-3), 0.9900 (patient 12 IgG4) and 0.9819 (patient 12 IgG1-3).

3.3 Discussion

Fourteen MuSK-MG patients with generalized symptoms were studied for MuSK Ab concentrations and IgG subclass composition, and IgG4 was found to be the predominant subclass that correlated with overall MuSK Ab concentration. Ab products, namely whole IgG, IgG4 and IgG4-depleted IgG1-3, were purified from MuSK-MG patient plasma and analysed for protein and MuSK Ab concentration. Purified IgG4 and IgG1-3 were analysed for IgG subclass profiles and MuSK binding affinity (K_d), with both IgG4 and IgG1-3 preparations harbouring high binding affinities for MuSK with K_d values in 10^{-12} to 10^{-11} range. Disease severity was compared with MuSK Ab concentrations in plasmas, and a trend for higher MGFA clinical classification score with increasing MuSK Ab levels could be found, and a correlation between MuSK Ab concentration and respiratory involvement was noted.

MuSK Abs were reported to be mainly of the IgG4 subclass (McConville et al., 2004), which was confirmed in this study by flow cytometry which showed a strong correlation between IgG4 subclass and overall MuSK Ab concentration (see also table 3.2). Although a previous study found that IgG4 correlated with disease severity in individual patients (Niks et al., 2008a), no significant correlations between maximum MGFA scores and total IgG or IgG4 MuSK Abs were found in the cohort of patients examined. However, most patients had MGFA scores of IIIB or IVB, and this limited the potential for a reliable correlation. Within patient correlations as performed in the Dutch study (Niks et al., 2008a) were not performed.

A limiting factor in the flow cytometry experiments was that samples with relatively low Ab populations, such as MuSK IgG1, 2 or 3 subclass, were close to the detection limit of the instruments (figure 3.11). Thus they could not be detected with the same accuracy as samples with high Ab concentrations, such as IgG4 subclass

MuSK Abs. Although the IgG1, 2 and 4 subclasses were found to correlate with the MuSK antibody concentration (figure 3.12), in a different experiment (figure 3.10, table 3.2) the Δmfi values of IgG2 varied independent of the MuSK antibody concentration. This again suggests that the values for these Ab populations, especially for IgG2 and 3, are too low to provide consistent trends. In retrospect higher concentrations (e.g. 1:20) of the samples could have been used for the IgG1-3 analysis in order to avoid this problem. However, a 1:20 dilution could also confound the data as the high concentration of IgG4 MuSK Abs might saturate the MuSK binding sites and prevent binding of the IgG1-3 Abs. Another difficulty of the flow cytometry experiments was the inter-experimental variation, which was caused by a variation in autofluorescence of cells and also due to the use of different individual instruments, so that the flow cytometry acquisition and gating had to be set up anew for every experiment. Therefore, the resulting Δmfi could vary between experiments. To be able to compare different patients with one another, all patient samples had to be analysed at the same time. By setting up large experiments of this kind, it was possible to show that the MuSK IgG4 subclass antibodies were higher than any other IgG subclass for each patients' plasma. This was also evident from the very high MuSK Ab IgG4 concentration in the IgG4 fractions compared with the corresponding values for IgG1-3.

To study MuSK IgG4 in more detail, total IgG4 subclass and the IgG4 depleted IgG1-3 subclasses were purified with an IgG4 affinity matrix; this was the same as used to demonstrate pathogenicity of MuSK IgG4 by passive transfer into mice (Klooster et al., 2012). The IgG4 elution yielded lower levels of protein than the IgG1-3 elution, which was expected as plasma levels of IgG4 (0.5-1mg/ml) are approximately 10-fold lower than that of other IgG subclasses, such as IgG1 (5-12mg/ml) (French, 1986). However, the IgG4 and IgG1-3 purification data from

patient 9 showed only a six-fold difference in protein yield, not a ten-fold difference as expected (and indeed seen in the IgG4 and IgG1-3 purification data of patient 12). It is unlikely that this is due to a reduced amount of IgG1-3 subclass in patient 9, compared to patient 12, because the IgG subclass profiles of patients 9 and 12 and thus the ratios between relative amount of IgG4 and IgG1-3 were determined before and were similar (figure 3.10A, table 3.2). This suggests that the ratio between the yield of total IgG4 and total IgG1-3 should also be similar in patient 9 and 12. A possible explanation could be a loss of overall protein during the preparation of IgG1-3 from patient 9.

As expected, MuSK Ab concentrations of purified IgG4 and IgG1-3, measured with RIA, had up to 100 fold higher concentrations of MuSK Abs per mg of purified IgG4 compared to IgG1-3 (see also table 3.3). This difference should be taken into account when the subclasses are being assessed for pathogenicity. Experiments in the study by Klooster et al (Klooster et al., 2012) used a total amount of 40mg IgG1-3 subclass and 40mg IgG4 subclass Abs. Under these conditions the IgG1-3 MuSK-Ab concentration would be approximately 1% compared to the IgG4 concentration. Furthermore, MuSK Abs were high in mice injected with patient IgG4, but not detectable in mice that were injected with patient IgG1-3 at the end of the experiment. Thus, it is questionable whether MuSK IgG1-3 subclass Abs in their experiments were tested under conditions which could determine whether they were pathogenic.

Table 3.3: Properties of purified IgG4 and IgG1-3

	IgG4		IgG1-3	
Patient	9	12	9	12
MuSK Ab conc. (nM)	6.47	5.77	1.50	0.51
Protein conc. (mg/ml)	0.41	0.26	2.59	2.08
MuSK Ab conc. (nmol/g)	15.78	22.19	0.57	0.24
K _d (pM)	15.31	11.56	2.79	20.27
IgG4 Δmfi	1576.77	1468.13	-4.65	15.87

The binding affinities of both IgG4 and IgG1-3 Ab subclasses were very high, with K_d values of approximately 10^{-11/-12}M, which is in the range of the findings of a previous study on total MuSK Abs that described K_d values of 5.5-10.8 x10⁻¹¹M (McConville et al., 2004). The high affinity for IgG4 MuSK Abs suggests an affinity-matured IgG since IgG4 antibodies are thought to be monovalent. It is generally thought that IgGs switch subclasses during their affinity maturation, which would suggest that the IgG4 MuSK Abs would be of higher affinity than the IgG1-3 MuSK Abs. However, this was not the case in the two patients examined.

The IgG1-3 Abs from patient 9 had lower K_d values than IgG1-3 from patient 12 or IgG4 from either patient, but these were relatively small differences given the high K_d values overall. Nevertheless, other differences were found between these two MuSK Ab preparations that were used for subsequent functional experiments.

Chapter 4: Characterising MuSK IgG in cell-based assays

4.1 Introduction

It is unknown how MuSK autoantibodies cause neuromuscular transmission failure and muscle weakness. In AChR MG, cell surface AChRs are lost by divalent binding of antibodies and cross-linking, followed by internalisation. In addition, complement-mediated pathology is very evident (Engel et al., 1977). MuSK IgG are mainly of the IgG4 subclass (McConville et al., 2004), which is an unlikely subclass to cause pathogenicity by these mechanisms, as its Fc region does not bind C1q (reviewed by (Nirula et al., 2011)). Complement involvement in MuSK patients has only been studied in one report in which patient biopsies of biceps brachii showed no evidence for complement deposition, although muscle weakness had been confirmed by electrophysiology (Shiraishi et al., 2005b).

IgG4 has been described as functionally monovalent by Fab arm exchange (van der Zee et al., 1986, Margni and Binaghi, 1988, van der Neut Kolfshoten et al., 2007), which makes it unlikely to cross-link and internalise its target. One publication attempted to show that patient derived MuSK IgG induced rapid internalisation of MuSK-EGFP *in vitro* (Cole et al., 2010). However, the presented data were not entirely convincing, because it was difficult to distinguish between endocytosis of MuSK-EGFP, the change of localisation of MuSK-EGFP with the movement of the membrane upon cell movement, such as the retraction of cell protrusions, and the homeostatic intracellular vesicular transport of MuSK-EGFP.

It is thus unclear if endocytosis of MuSK is a pathogenic mechanism in MuSK-MG, and this was investigated using a slightly different methodology. The basic principle of the method is the quantification of cell surface binding of patient IgG over time using flow cytometry and cell based assays. In previous studies,

similar methods have been used to demonstrate that autoantibodies cause internalisation of its target, for example with aquaporin-4 (Hinson et al., 2007), with N-methyl-D-aspartate (NMDA)-receptor antibodies (Dalmau et al., 2008, Hughes et al., 2010) and most importantly with AChR antibodies (Drachman et al., 1978).

The methods used in this study are based on the principles of antibody feeding techniques (Arancibia-Carcamo et al., 2006). Antibody feeding utilizes the binding of antibodies to cell surface expressed receptors to label these, and it is assumed that the antibody stays attached to the receptor if it is internalised and throughout the endosomal pathway until both receptor and antibody are detached and degraded in the lysosomes. The antibody binding is carried out at 4°C to reduce endocytosis, then unbound antibody is removed and the cells are moved to physiological temperature (37°C) to allow endocytosis, or retained at 4°C as a control. A reduction in cell surface bound IgG to MuSK at 37°C over time would be associated with either detachment of the antibody, or endocytosis of IgG bound to MuSK.

The study by Drachman et al (1978) is the most striking as they demonstrated that patient antibodies bind to their target, followed by cross-linking and endocytosis, and that patient derived Fabs that bind monovalently are unable to do so, unless artificially cross-linked by a Fab specific antibody. The cross-linking of patient antibody by anti-human IgG was thus used as a positive control for MuSK endocytosis.

4.2 Results

4.2.1 *MuSK endocytosis with flow cytometry*

HEK293 cells expressing MuSK were incubated with patient IgG at 4°C, then unbound antibody was washed off with medium, and the cells were incubated at 4°C or 37°C for up to six hours (figure 4.1A). As an additional control, some cells were fixed in 3% paraformaldehyde prior to incubation with patient IgG (figure 4.1B). The Δmfi for each sample was determined by flow cytometry (figure 4.1).

The living cells at 37°C showed some decrease of cell surface bound human IgG over time at 37°C (approximately 30%, from 300 Δmfi at 0h to 200 Δmfi at 6h) than at 4°C (300 Δmfi at 0h and 6h). However, the pre-fixed cells showed a similar loss of IgG binding to that of the live cells (figure 4.1B,C). It was possible that the experimental setup with flow cytometry was not adequate to study endocytosis, because the cells were kept in solution for up to, or sometimes over, 6 hours, which may have influenced the cell metabolism and protein expression. Therefore, a cell based assay (CBA) in which the cells remain adhered to a plastic surface was used (Leite et al. 2008) to study MuSK endocytosis.

4.2.2 *MuSK-MG patient IgG binding to MuSK in a CBA*

The CBA is not different from flow cytometry except that in flow cytometry the cells are in solution and the results are quantitatively assessed with a flow cytometer, while in CBA the cells stay attached to glass coverslips and the results are subjectively assessed with fluorescence microscopy and visual observation and scoring.

Ideally, the expression of a fluorescent marker as a transfection control should be independent of the expression of the protein of interest. Therefore, the

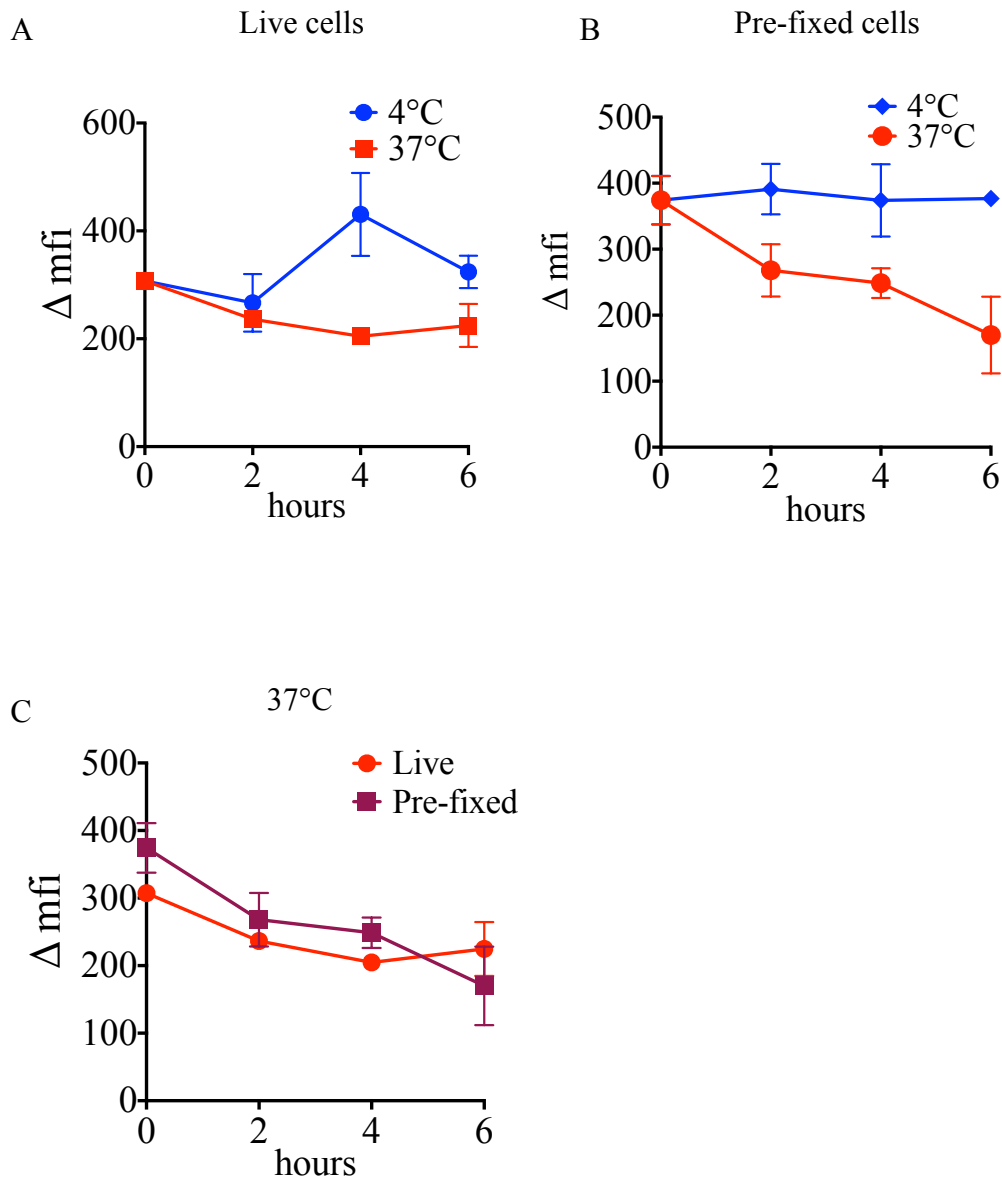


Figure 4.1: No evidence for MuSK endocytosis by flow cytometry. (A) Binding of patient 2 antibodies to MuSK expressed at the cell surface of pIRES2-AcGFP1-MuSK transfected HEK293 cells over time at 37°C or 4°C was measured with flow cytometry. A loss of cell surface bound patient IgG at 37°C, but not at 4°C, could indicate antibody detachment or MuSK endocytosis. (B) As a control for antibody detachment at 37°C, cells were fixed with 3% PFA prior to incubation with patient antibodies, rendering the cells incapable of endocytosis. (C) Comparing loss of cell surface bound patient IgG in fixed and live cells at 37°C reveals no difference and suggests antibody detachment rather than endocytosis.

vector pIRES2-AcGFP1-MuSK was used, which expresses AcGFP independently of MuSK after an internal ribosomal entry site (IRES), a nucleotide sequence that allows for translation initiation in the middle of a messenger RNA. MuSK and AcGFP are thus encoded within the same plasmid, but expressed separately. It was important not to use a directly tagged MuSK, because protein expression and localization of MuSK may be affected when the cells are incubated for up to 16 hours with MuSK-MG patient IgG. If MuSK was internalised and degraded, this would affect the intensity of the fluorescent tag.

HEK293 cells were transfected with the expression vectors pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 and incubated with MuSK-MG patient plasma at 1:20 dilution followed by anti-human IgG (heavy and light chain specific) conjugated to Alexa Fluor 568 (figure 4.2A). Red fluorescent staining, depicting anti-human IgG binding, was observed in cells expressing pIRES2-AcGFP1-MuSK. The red fluorescence was only found in cells that were positive for AcGFP fluorescence. Cells transfected with pIRES2-AcGFP1 showed no specific staining.

As a further control, healthy control serum at the same dilution was incubated with the cells expressing pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 (figure 4.1B), and no specific staining was found. This suggests that MuSK IgG derived from patient plasma binds specifically to MuSK expressed at the cell surface of pIRES2-AcGFP1-MuSK transfected HEK293 cells. Several patient plasmas and healthy control sera were then analysed at the same dilution (1:20) in the CBA (figure 4.3). Cells incubated with plasmas derived from patients with high MuSK antibody concentrations (e. g. patient 3, 25.65nM) showed brighter staining than cells incubated with patients with low MuSK antibody concentrations (e. g. patient 14, 1.03nM). This is in accordance with the flow cytometry data that showed a

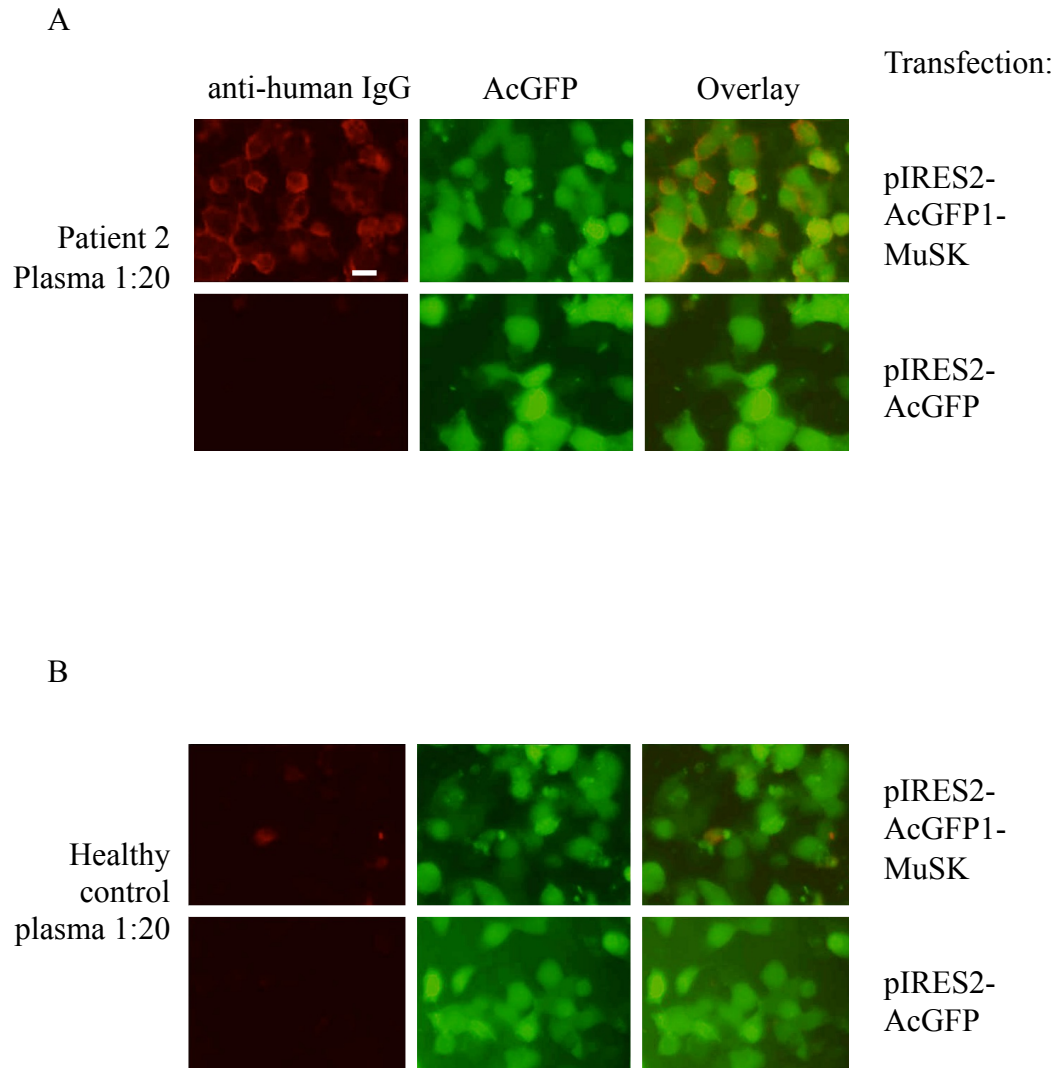


Figure 4.2 : MuSK-MG patient IgG bound specifically to pIRES2-AcGFP1-MuSK transfected HEK293 cells. HEK293 cells were transfected with pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1, and incubated with 1:20 dilutions of (A) patient plasma or (B) healthy control plasma. Images of red fluorescence (anti-human IgG), green fluorescence (AcGFP) and the overlay were taken at 20x magnifications. Patient derived antibodies specifically bound to pIRES2-AcGFP1-MuSK transfected cells. Scale bar= 50µm.

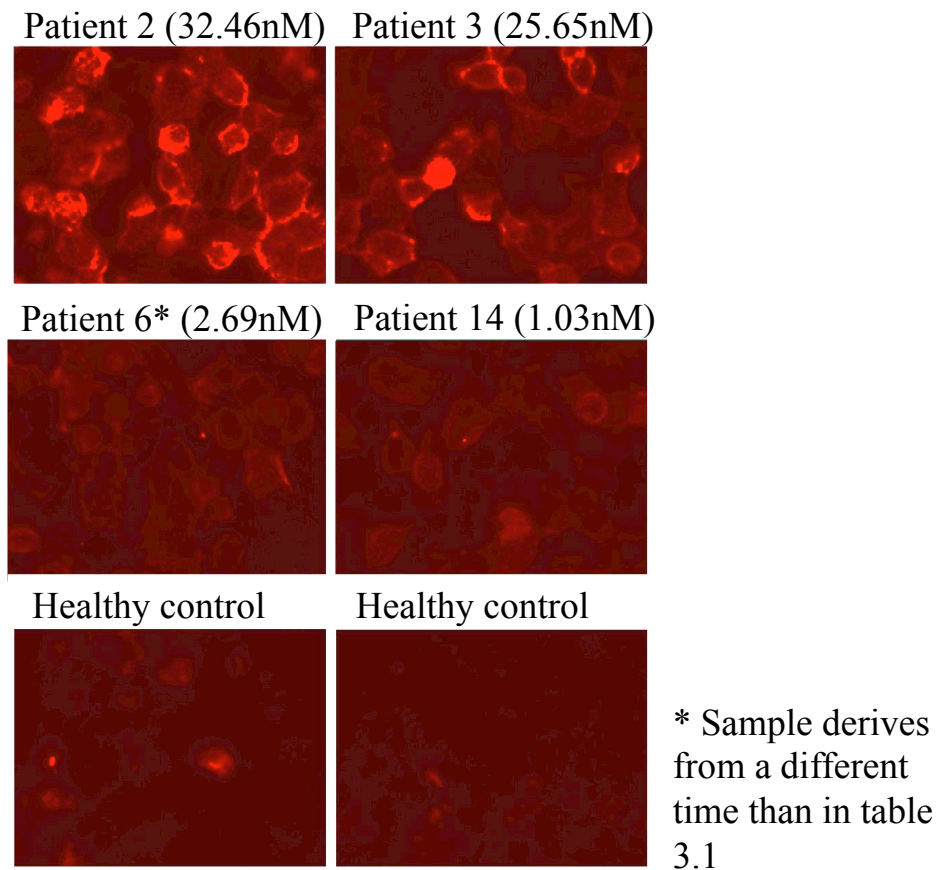


Figure 4.3: Binding of patient IgG in CBA correlated with MuSK antibody concentration. HEK293 cells transfected with pIRES2-AcGFP1-MuSK were incubated with sera from healthy controls or different patient plasmas at 1:20 dilution. MuSK antibody concentrations of the MuSK-MG patient plasmas are depicted in brackets after the patient designation. Images of red fluorescence (anti-human IgG) were acquired at 20x magnification. Patients with higher MuSK antibody concentration (Patient 2, 3) had brighter fluorescence than patients with lower MuSK antibody concentrations (Patient 6,14).

correlation between MuSK antibody concentration and binding to MuSK at the cell surface of transfected HEK293 cells (Chapter 3, figure 3.8B).

4.2.3 Investigating MuSK endocytosis with the CBA

HEK293 cells were transfected with pIRES2-AcGFP1-MuSK, and live cells or cells fixed with 1% paraformaldehyde were incubated with patient plasma at 1:50 dilutions at 4°C. Unbound IgG was removed by washing, and the cells were incubated at 4°C or 37°C for 0-6 hours. Cell surface bound IgG was stained with fluorescent-labelled anti-human IgG and analysed by fluorescence microscopy. Example images are shown in figure 4.4A.

The experiment was repeated using MuSK-MG plasma at 1:20 dilution, to test a higher concentration of MuSK IgG, in case a 1:50 dilution did not provide sufficient amount of MuSK antibody. The result was very similar. The conditions were blinded, and fluorescence intensity was quantified by scoring 0 (no binding) to 4 (strong binding, very bright fluorescence). The scores for two experiments were normalised to the score at time point 0h and pooled (figure 4.4B,C).

The visual scores of red fluorescence of the live cells at 4°C and 37°C showed a minimal decrease of approximately 10%. The pre-fixed cells showed an average loss of approximately 75% cell surface bound antibody at 37°C (with large standard deviation of 31.25), but only a loss of approximately 25% (and standard deviation of 12.50) at 4°C. This suggests that the antibodies detach from the pre-fixed cells.

4.2.4 Investigating the effect of IgG4 and IgG1-3 subclasses on MuSK endocytosis

It is possible that only a fraction of the patient antibodies is able to cross-link MuSK and induce endocytosis. Likely antibody subpopulations would be the divalent

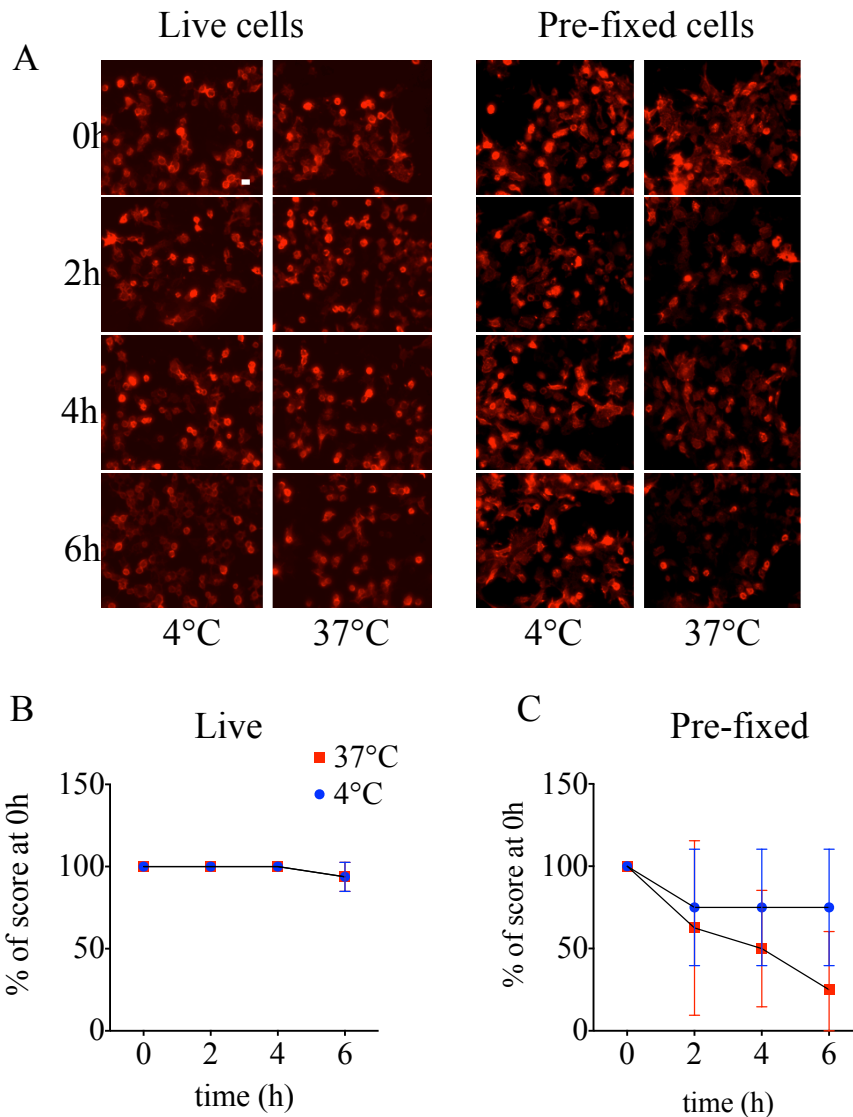


Figure 4.4: No evidence for MuSK endocytosis with a 6 hour time course CBA with MuSK-MG patient plasma. (A) HEK293 cells were transfected with pIRES2-AcGFP1-MuSK and incubated with 1:50 dilution of patient 2 plasma. Bound human IgG was visualized at the end of the time course by fluorescent-labelled anti-human IgG. Binding of antibodies to MuSK expressed at the cell surface of pIRES2-AcGFP1-MuSK transfected HEK293 cells over time at physiological temperature (37°C) or at 4°C was assessed by fluorescence microscopy. As a control for antibody detachment, cells were fixed with 3%PFA prior to incubation with patient antibodies (pre-fixed cells). MuSK antibody binding to live (B) or pre-fixed (C) cells was assessed by scoring the brightness of fluorescence (0 = no binding – 4 = strong binding). The scores were normalised to the score at 0h and expressed as %. Loss of cell surface bound IgG at 37°C was more prominent in fixed cells than in living cells, which suggests antibody detachment rather than endocytosis. Scale bar=50µm.

IgG1-3 subclasses. It was possible that this effect could be only observed with isolated subclasses *in vitro*. To explore this possibility, purified IgG1-3 and IgG4 (Chapter 3, figure 3.14) were studied in the CBA.

First, to ensure that MuSK IgG subclasses bound to MuSK, and not non-specifically to HEK293 cells, purified IgG4 and IgG1-3 from patient 9 were tested. Both IgG4 (figure 4.5A) and IgG1-3 (figure 4.5B), each used at 0.17nM, bound specifically to pIRES2-AcGFP1-MuSK transfected cells but not to pIRES2-AcGFP1 transfected cells. Endocytosis was then investigated in cells incubated with purified IgG4, IgG1-3 or plasma from two patients at final concentrations of 0.17nM, which was the highest concentration possible for all samples including the IgG1-3 with the lowest MuSK Ab concentration (Chapter 3, figure 3.14). Example data using patient 9 IgG subclasses are shown in figure 4.6A, data from patient 12 were very similar (data not shown). Both pre-fixed and live cells showed loss of cell surface bound IgG over time.

The cells were scored, blinded by two investigators (Inga Koneczny, Dr. Judith Cossins) and normalised to scores at 0h (figure 4.6C). Loss of patient IgG binding to the cell surface over time at 37°C (approximately 25% in the live cells), but not at 4°C, could be observed in all samples, but again this effect was most prominent in pre-fixed cells (up to 75% reduction with IgG4). In case endocytosis would require more than 6 hours of incubation time, the incubation time was extended to 16 hours (figure 4.7). Antibody binding was reduced after 16 hours at 37°C in all samples, but the loss of cell surface bound antibody was similar or even more pronounced in pre-fixed cells.

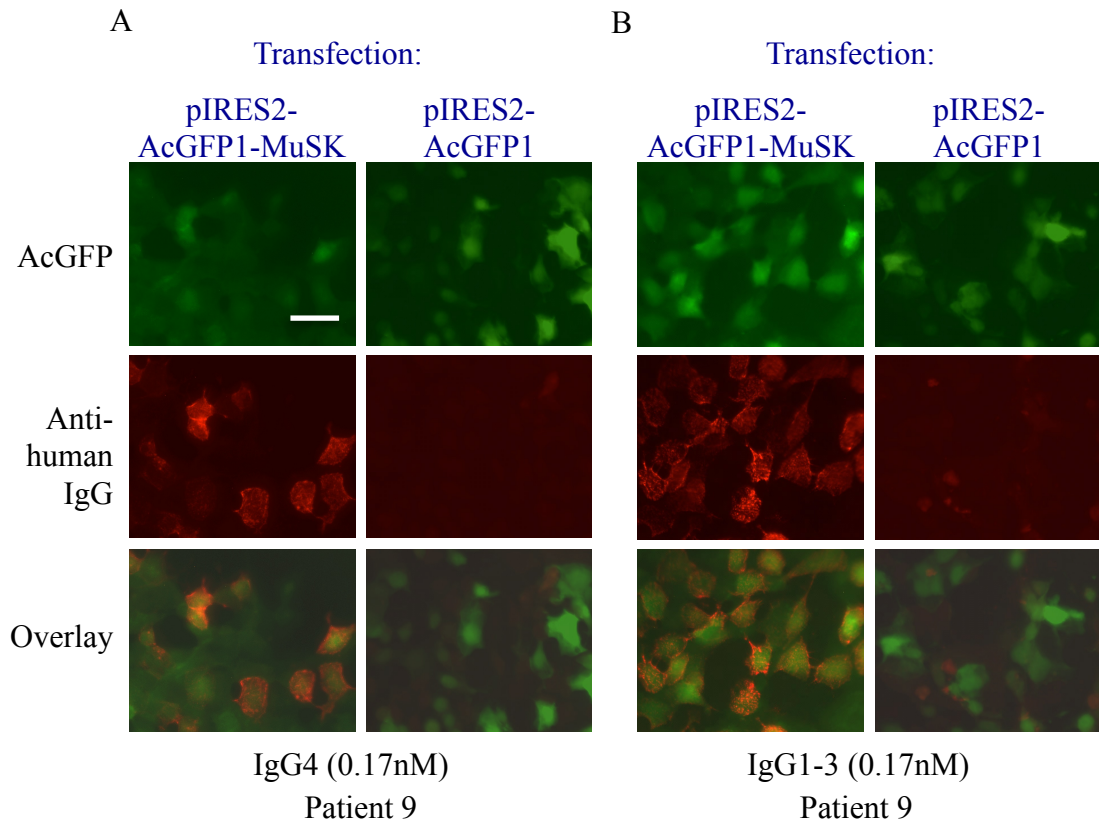


Figure 4.5: Purified IgG4 and IgG1-3 from MuSK-MG patient plasma bound specifically to MuSK in CBA. HEK293 cells transfected with pIRES2-AcGFP1-MuSK or pIRES2-AcGFP1 (green, EGFP fluorescence) were incubated with 0.17nM (A) purified IgG4 or (B) IgG1-3 from patient 9 followed by incubation with fluorescent-labelled anti-human IgG (red fluorescence, anti-human IgG). The overlay shows specific binding of IgG4 and IgG1-3 only in pIRES2-AcGFP1-MuSK transfected cells. Scale bar = 50µm.

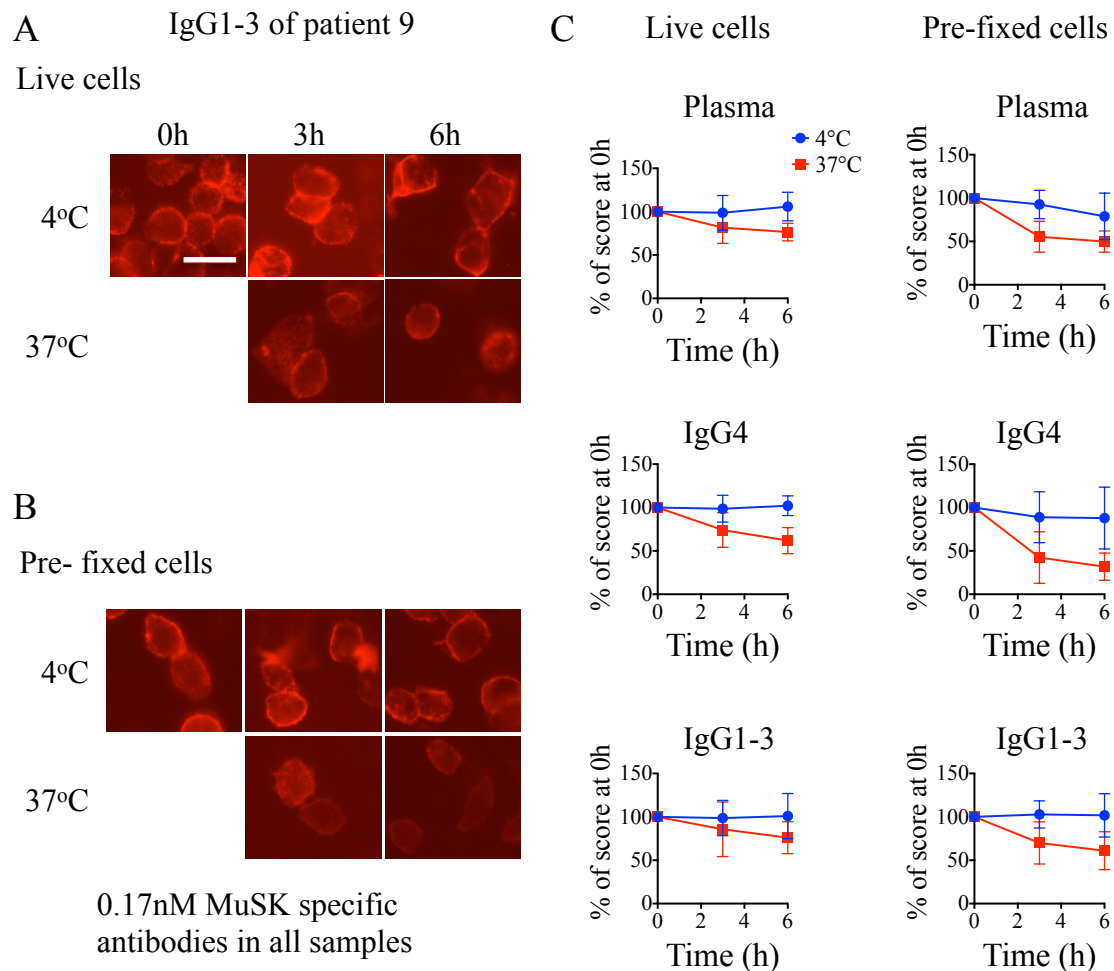


Figure 4.6: Endocytosis assay with IgG4 and IgG1-3 subclasses. Binding of patient derived MuSK antibodies to human MuSK expressed on the cell surface of HEK293 cells over time. Living or pre-fixed cells were incubated with patient antibodies for 0, 3 or 6 hours, at 4°C or 37°C. (A,B) Example images of cells incubated with IgG1-3 at 20x magnifications. (A) living cells, (B) pre-fixed cells. (C) quantification of antibody binding. Images were scored blinded by two investigators (0 = no binding – 4 = strong binding), normalised to score at 0h and expressed as %. Cell surface bound IgG decreased at 37°C both in living and fixed cells over time, suggesting antibody detachment. n = 3. Scale bar = 50µm.

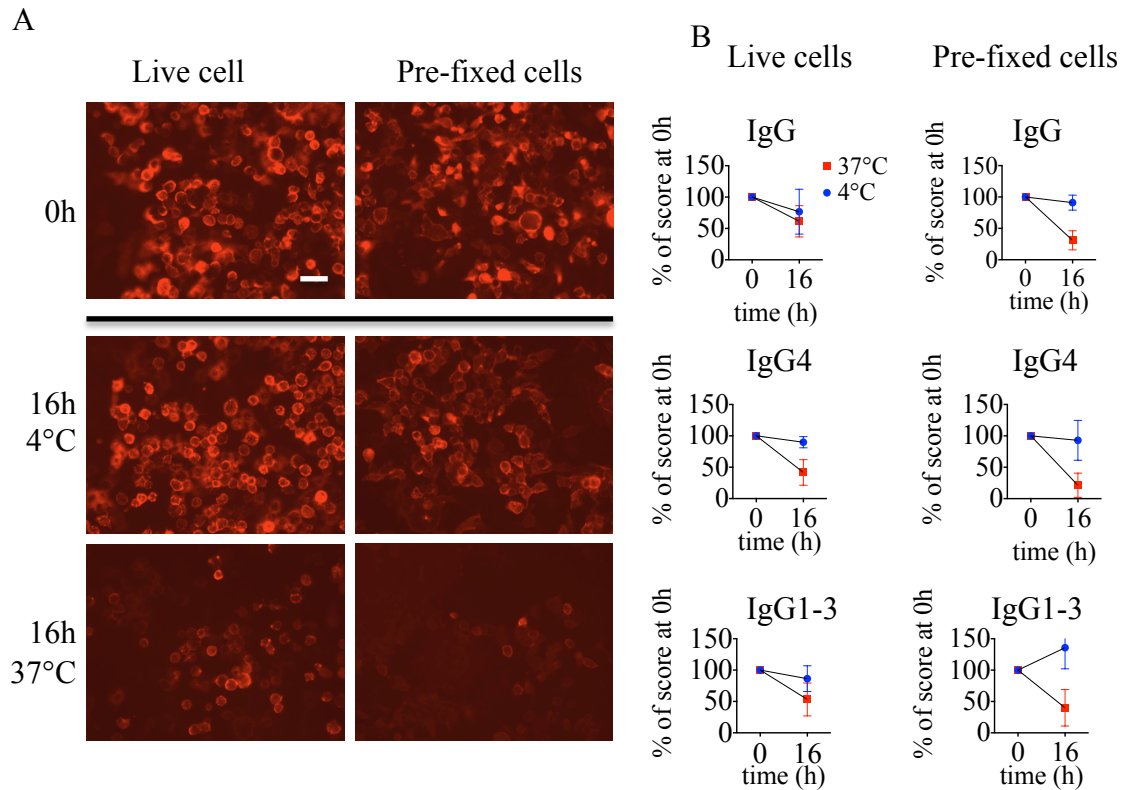


Figure 4.7: MuSK endocytosis assay with 16h incubation time at 4°C or 37°C with MuSK-MG patient derived IgG, IgG1-3 and IgG4. (A) Living or pre-fixed HEK293 cells were incubated with patient derived IgG, IgG4 or IgG1-3 for 0 or 16 hours, at 4°C or 37°C. (A) Example images of cells incubated with IgG at 20x magnification. (B) Quantification of antibody binding. Images were scored blinded by two investigators, normalised to score at 0h and expressed as %. Cell surface bound antibodies decreased over time in most samples, but the loss at 37°C for both living and fixed cells was pronounced, suggesting antibody detachment. Scale bar = 50µm. n = varies as follows: IgG live cells = 3, IgG pre-fixed cells = 2, IgG4/IgG1-3 live and pre-fixed = 1

4.2.5 MuSK endocytosis study with muscle cell-like conditions

It was surprising that MuSK endocytosis was not found using MuSK transfected HEK293 cells even with divalent IgG1-3. One possibility could be that MuSK endocytosis requires specific conditions found in muscle cells. MuSK closely interacts with LRP4, which is the agrin co-receptor (Kim et al., 2008, Zhang et al., 2008). Binding of LRP4 to MuSK, following agrin binding to LRP4, leads to MuSK dimerisation, autophosphorylation and activation of its kinase function. It was possible that the presence of LRP4 was required in order to enable MuSK endocytosis.

HEK293 cells were transfected with MuSK and also with LRP4, and the time course experiment over 6 hours was repeated. Three dilutions of patient 9 and patient 2 plasmas were used: pure patient plasma dialysed against medium and 1:5 and 1:10 dilutions of plasma in medium. Example data of patient 9 can be found in figure 4.8. All dilutions from both patients showed similar results, with a loss of cell surface bound IgG observed at 37°C, both in live and fixed cells.

Because no evidence for endocytosis was found in these experiments, it was attempted to reproduce the experimental settings deployed by Cole et al. (Cole et al., 2010). MuSK-mCherry (figure 2.2) was introduced by electroporation into C2C12 myoblasts, which do not express endogenous MuSK at this stage (Glass et al., 1996). Patient 9 plasma was incubated at a 1:10 dilution with the cells. The cells were incubated for 0, 10 and 90 minutes, as in the study by Cole et al. However, more control conditions, namely incubating the cells at 4°C or 37°C, and using live or fixed cells, were included. Fluorescence intensity of anti-human IgG Alexa Fluor 488 was assessed, blinded and images at 20x magnification were taken (data not shown).

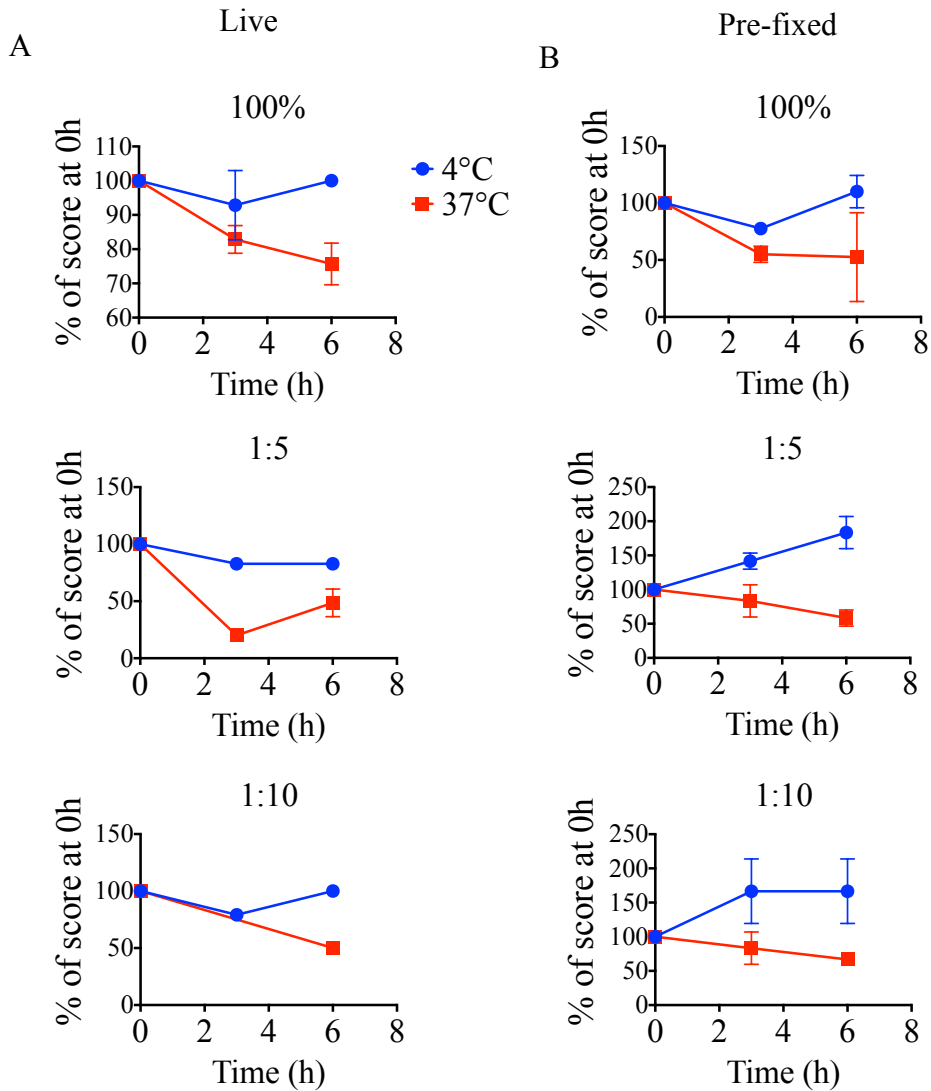


Figure 4.8: No evidence for MuSK endocytosis in HEK293 cells co-expressing MuSK and LRP4. HEK293 cells were transfected with pIRES2-AcGFP1-MuSK and LRP4 and (A) living cells or (B) cells fixed with 3% PFA were incubated with patient 9 plasma dialysed against medium (100%) or at dilutions of 1:5 and 1:10 in medium. Unbound antibody was washed away and cells were incubated at 4°C or 37°C for 0-6 hours. Images were scored (0 = no binding – 4 = strong binding), blinded by two investigators and normalised to the score at 0h. Cell surface bound IgG decreased over time at 37°C in both living and fixed cells, suggesting antibody detachment. n = 1.

The experiment was only conducted once, and cell surface binding of IgG was found to be minimally reduced after 90 minutes at 37°C, both in live and fixed cells. In conclusion, no evidence for MuSK endocytosis was found.

4.2.6 Induction of MuSK endocytosis by cross-linking of IgG

It was important to ask whether MuSK and MuSK IgG endocytosis was possible in HEK293 cells. Ground breaking work by Drachman et al. used the method of cross-linking monovalent patient Fabs to AChR with Fab-specific secondary antibody, thus inducing cross-linking and endocytosis (Drachman et al., 1978), which demonstrated the requirement of divalent binding and cross-linking for endocytosis of AChR. Here, patient MuSK IgG was cross-linked by incubation with anti-human IgG secondary antibody to induce cross-linking and endocytosis.

In the previous experiments the secondary antibody was added at the end of the time course. In this experiment the secondary antibody was added at the beginning of the time course for the dual purpose of detection and cross-linking of the human IgG. Briefly, HEK293 cells transfected with pIRES2-AcGFP1-MuSK were incubated with Patient 9 and 12 derived IgG4 or IgG1-3 at concentrations of 0.17nM at 4°C. Unbound antibody was removed by washing, then fluorescence labelled secondary antibody (anti-human IgG, Alexa Fluor 568) was incubated on the living cells at 4°C, and unbound antibody was removed by washing. Then the cells were moved to 37°C or kept at 4°C and incubated for 0h, 3h and 6h, before fixing with paraformaldehyde for observations. As a control, cells were fixed prior to primary antibody incubation. Images of red (anti-human IgG) and green fluorescence (cytosolic AcGFP) were acquired at 20x magnification. In IgG4 and IgG1-3

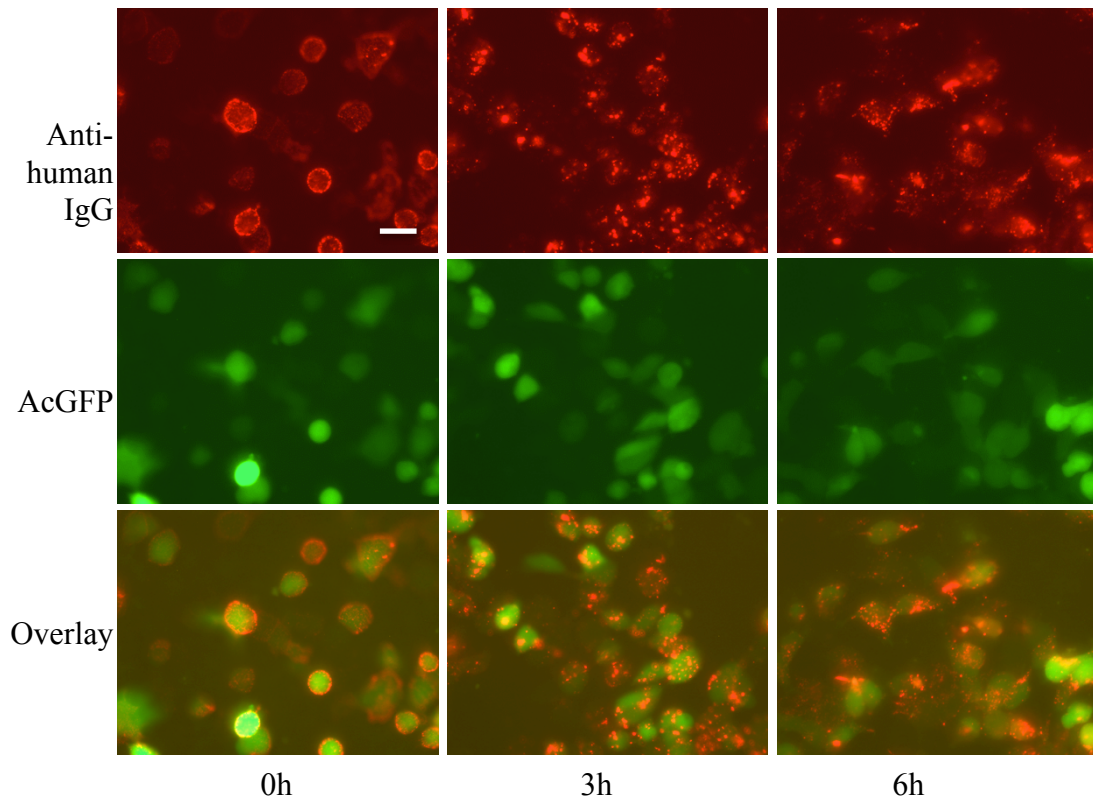


Figure 4.9: Artificial cross-linking by anti-human IgG caused endocytosis of human IgG4. HEK293 cells were transfected with pIRES2-AcGFP1-MuSK and incubated with 0.17nM IgG4 from patient 9 followed by incubation with fluorescent-labelled anti-human IgG at 4°C. Unbound antibody was removed by washing and cells were moved to 37°C. After 0-6h, cells were fixed with 3% PFA and analysed by fluorescence microscopy for binding and localisation of human IgG (red, anti-human IgG) in transfected cells (green, AcGFP). Anti-human IgG was evenly distributed on the cell membrane at point 0h, but was found in aggregates after 3 and 6h. Scale bar = 50µm.

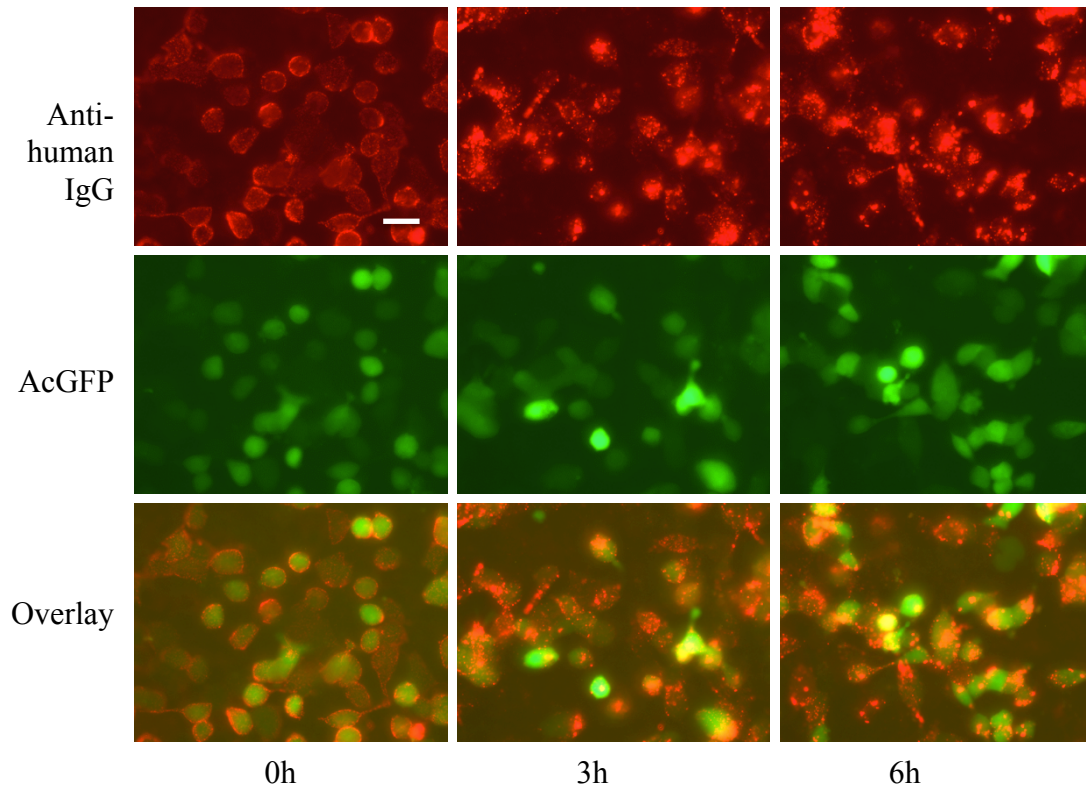


Figure 4.10: Artificial cross-linking by anti-human IgG caused endocytosis of human IgG1-3. HEK293 cells were transfected with pIRES2-AcGFP1-MuSK and incubated with 0.17nM IgG1-3 from patient 9 as described in figure 4.9. After 0-6h, cells were fixed with 3% PFA and analysed by fluorescence microscopy for binding and localisation of human IgG (red, anti-human IgG) in transfected cells (green, AcGFP). Anti-human IgG was evenly distributed on the cell membrane at point 0h, but was found in aggregates after 3 and 6h. Scale bar = 50 μ m.

treated samples of both patients, a striking difference in IgG localization between time points 0h and 3h/6h was observed (figures 4.9, 4.10). At time point 0h, patient IgG (red) was evenly distributed at the cell surface, around the cytoplasm (green, AcGFP). After 3h incubation, the anti-human IgG staining was very fragmented. The difference in IgG localization could be observed as early as 10 minutes in repeats of the experiment (data not shown). Images at 40x magnification of IgG1-3 treated cells at 0 and 3h demonstrated the difference in IgG binding over time in more detail (figure 4.11A).

To compare the difference in IgG location in cells that were artificially cross-linked and those that were not, images were acquired at 60x magnification after 6 hours incubation with IgG4 or IgG1-3 and with or without anti-human IgG present during the time course (figure 4.11B). In samples without anti-human IgG present during the time course, in which fluorescent-labelled anti-human IgG was used only at the end of the experiment to visualize human IgG, the patient IgG was clearly localized at the cell surface. In samples with fluorescent-labelled anti-human IgG present throughout the time course, the patient IgG appeared to be localized both at the cell surface and intracellular in small, round vesicles. These findings were similar for IgG4 and IgG1-3 treated cells.

In order to verify that the aggregates found were intracellular, and that MuSK was internalised as well, the experiment was repeated with EGFP tagged MuSK (MuSK-EGFP) and analysed for co-localisation of MuSK-EGFP and human IgG within the cell using confocal microscopy. After incubation at 37°C for 30 minutes, the artificially cross-linked cells were fixed and incubated with anti-human IgG again. Z-stack images were acquired using Zen software by Zeiss and a three dimensional image was created using Imaris. MuSK-EGFP is shown in green and anti-human IgG in red (figure 4.12).

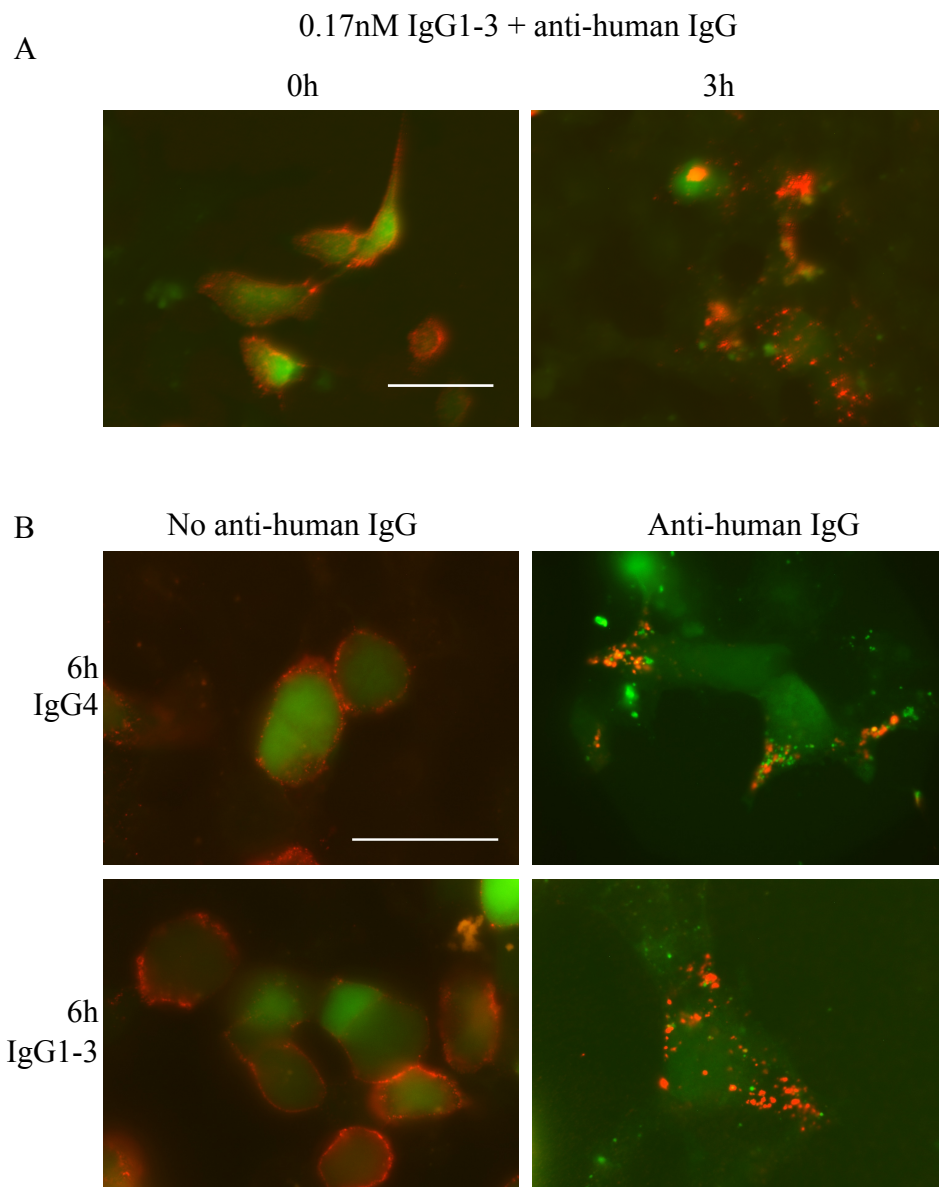


Figure 4.11: Anti-human IgG induced cross-linking of IgG4 and IgG1-3. (A) HEK293 cells transfected with pIRES2-AcGFP1-MuSK were incubated with 0.17nM IgG1-3 derived from patient 9 and fluorescent-labelled anti-human IgG. After removing unbound antibody by washing, cells were incubated for 0 or 3h at 37°C, fixed and analysed by fluorescence microscopy. Images were acquired at 40x magnification. Red fluorescence: anti-human IgG, green fluorescence: cytosolic AcGFP. While human IgG is localised at the cell surface at 0h, it is found in aggregates after 3h. Scale bar= 50µm. (B) HEK293 cells transfected with pIRES2-AcGFP1-MuSK were incubated with 0.17nM IgG4 or IgG1-3 from patient 9 in the presence or absence of fluorescent-labelled anti-human IgG. Example images of cells incubated with IgG1-3 and IgG4 at 60x magnification, red = anti-human IgG, green = cytosolic AcGFP. Without anti-human IgG, patient IgG4 and IgG1-3 remain located at the cell surface. Scale bar = 50µm.

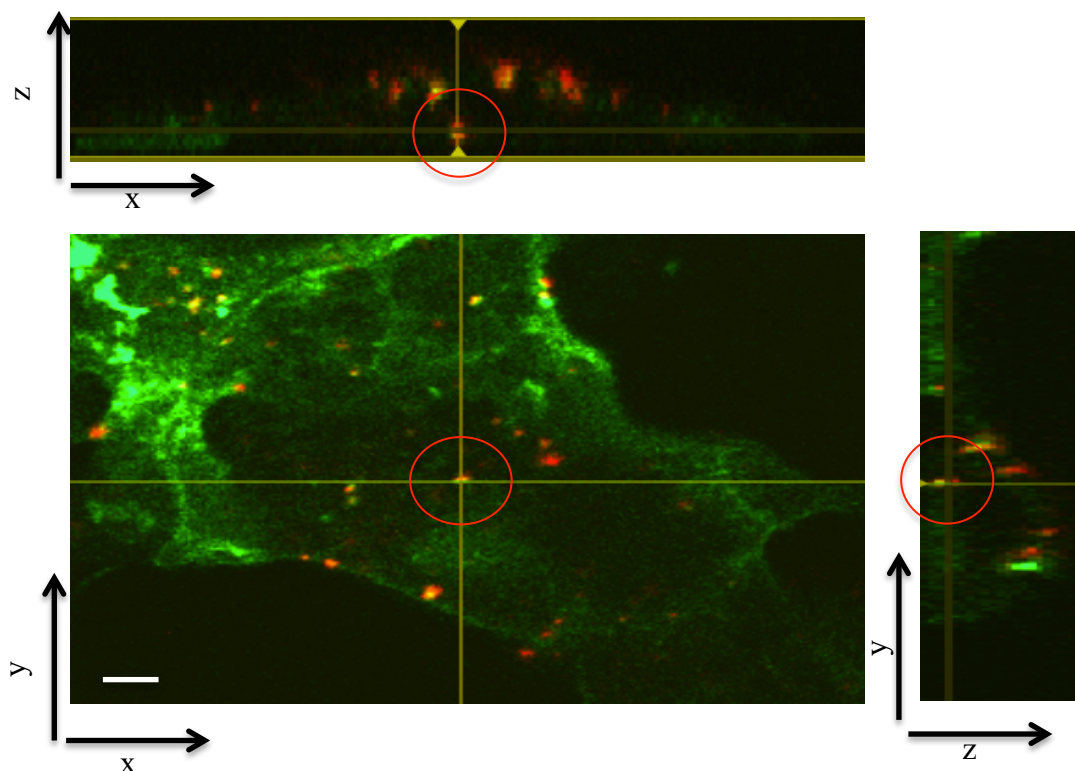


Figure 4.12: Confocal microscopy verified intracellular localisation of artificially cross-linked human MuSK IgG and MuSK-EGFP. HEK293 cells were transfected with MuSK-EGFP (green) and incubated with 0.17nM IgG1-3 from patient 9 and fluorescent-labelled anti-human IgG (red), and after removing unbound antibody by washing, the cells were incubated for 30 minutes at 37°C, then fixed with 3% PFA and incubated a second time with anti-human IgG. The large image shows a single slice from a z-stack of images. The x and y axes are shown on this image. One aggregate at the intersection of the yellow lines was selected. A cross section through the x and z axis of the cell is positioned in the uppermost image, and likewise a cross-section through the y and z axis of the cell is shown on the right hand side. The selected aggregate is shown in both cross-sections where the two yellow lines intersect (also marked by a red circle). Thus it can be seen that this aggregate is in the centre of the cell in the xy plane (main image) and is neither at the top nor the bottom of the cell in the cross-sections. Hence this aggregate is inside the cell. Scale bar = 5µm.

Vesicles or aggregates with red staining containing anti-human IgG were found in the cell and on the cell surface. Most red vesicles overlapped with green, which suggests that vesicles containing human IgG also contained MuSK. Vesicles that were only green, and not red, were also present, which may be vesicles transporting newly synthesised MuSK-EGFP. Imaris software (Bitplane) was used to make orthogonal slices through the three dimensional image of the cell, indicated by a yellow hair cross (figure 4.12). A red and green vesicle located centrally in the xy-plane (red circle, figure 4.12) was selected to determine its three-dimensional orientation in the cell. The yellow hair cross was focused on the vesicle. The image on top shows that the vesicle is below the cell surface in the xz-plane (red circle), because the cell membrane with other vesicles was located above the focused vesicle. The image on the right shows the orientation of the vesicle (red circle) in the yz-plane, with further vesicles and cell membrane visible on the right end side next to the vesicle, which suggests that the vesicle is below the cell surface in the yz-plane. Thus vertical and horizontal slices through the cell showed that the vesicle was located below the cell membrane and hence inside the cell. Several vesicles were analysed and at least three found to be located intracellular.

As a further control, the experiment with MuSK-GFP transfected cells was repeated, and at the end of the time course, half of the cells were permeabilised with 0.1% Triton X. All cells were (re-) incubated with fluorescent-labelled anti-human IgG, and the localisation of human IgG in the cells was determined with confocal microscopy. Permeabilised cells incubated with patient IgG that were not artificially cross-linked with anti-human IgG did not show intracellular human IgG (data not shown). This suggests that MuSK IgG was not internalized during the time course.

4.3 Discussion

MuSK endocytosis was explored as a potential pathogenic mechanism using flow cytometry and cell based assay (CBA). The CBA was adapted from a previously published method (Leite et al., 2008) to quantify binding of MuSK patient IgG over time in live and pre-fixed cells at 37°C and 4°C. Plasma, purified IgG, IgG4 and IgG1-3 subclass antibodies from MuSK-MG patients were investigated. At 37°C, but not at 4°C, cell surface binding of all patient IgG decreased over time, both in live and pre-fixed cells. This suggests that the decrease of cell surface binding may derive from antibody detachment rather than endocytosis, which may have been caused by epitope damage or reduced accessibility of the epitope due to the presence of the paraformaldehyde.

Flow cytometry and CBA were then used to investigate MuSK endocytosis as a potential pathogenic mechanism of divalent IgG1-3 subclass IgG. Intriguingly, IgG4 can be functionally monovalent (van der Zee et al., 1986, Margni and Binaghi, 1988) by Fab arm exchange (van der Neut Kolfshoten et al., 2007). A monovalent binding antibody would be unlikely to cross-link their target, although it should be noted that a population of IgG4 might contain both mono- and bi-specific antibodies, which has not yet been determined. On average, 92 % of the MuSK antibodies belong to the IgG4 subclass (Chapter 3, figure 3.10), and it has not been investigated yet whether IgG4 is able to cross-link MuSK. Even if MuSK IgG4 was functionally monovalent and unable to cross-link MuSK, there would still be 8% of MuSK IgG1-3 subclass antibodies that are divalent. One publication attempted to show endocytosis of MuSK by IgG from one patient (Cole et al., 2010), but the results were not entirely convincing. Thus endocytosis was studied by quantification of patient IgG bound to cell surface expressed antigen over time.

Table 4.1: Endocytosis study. Loss of cell surface bound IgG over time in live cells compared to pre-fixed cells found (yes/no).

Samples	Flow cytometry time course 6h	CBA time course 6h	CBA time course 16h	CBA C2C12 6h	CBA time course MuSK+LRP4 in HEK293 cells 90 min	CBA time course 6h +anti-human IgG
Plasma patient 2	No	No	-	-	-	-
Plasma patient 9	-	No	-	No	No	-
Plasma patient 12	-	No	-	-	No	-
IgG patient 9	-	-	No	-	-	-
IgG patient 12	-	-	No	-	-	-
IgG4 patient 9	-	No	No	-	-	Yes
IgG1-3 patient 9	-	No	No	-	-	Yes
IgG4 patient 12	-	No	No	-	-	Yes
IgG1-3 patient 12	-	No	No	-	-	Yes

All cell based assays were carried out in cells transfected with constructs expressing full length human MuSK unless otherwise stated.

Several studies used similar experimental procedures, by monitoring the localisation of fluorescent tagged cell surface antigen (Hinson et al., 2007), or labelling the cell surface expressed antigen with patient or commercial antibodies (Hughes et al., 2010, Dalmau et al., 2008), or in case of the AChR with radioactive labelled alpha-bungarotoxin (Drachman et al., 1978), to detect endocytosis induced by patient autoantibody binding to their target.

Here, fluorescent-tagged anti-human IgG was used to investigate binding of patient IgG to MuSK at the cell surface over time. A reduction in fluorescence would be an indication of loss of cell surface bound antibody, by antibody detachment or endocytosis, and indeed a reduction of fluorescence was observed. Loss of cell surface bound patient IgG, IgG4 and IgG1-3 was found at 37°C over incubation periods of up to 16 hours (table 4.1). However, cells that were fixed with paraformaldehyde prior to the experiment showed a marked loss of cell surface bound antibodies as well. This observation was surprising, as MuSK Abs were found to be of very high binding affinity (figure 3.16, (McConville et al., 2004)).

A possible cause could have been that the initial fixing procedure at the beginning of the experiment was not stringent enough to prevent endocytosis and induce cell death, with 5 minutes incubation of 1% paraformaldehyde on the cells, though the same fixing procedure was applied in different experiments and showed good preservation of cell structures after one week storage at 4°C. Another concern was that the fixing may have altered MuSK epitope structure or accessibility, thus reducing the binding strength between the patient Abs and MuSK. This would explain the loss of cell surface bound human IgG at 37°C in the fixed cells as antibody detachment rather than endocytosis. In this case it may be possible that patient IgG bound with high affinity to MuSK on live cells and induced endocytosis, while patient IgG bound with reduced affinity to MuSK on pre-fixed cells and

detached. Furthermore, it cannot be excluded that endocytosis occurred during the initial steps of the experiments, where the cells were incubated with patient IgG at 4°C prior to the time course. Thus it may be possible that the methodology used in this study is not adequate to study endocytosis. In retrospect, it would have been important to use a positive control for endocytosis to explore the adequacy of the method to study endocytosis. Such a control could have been aquaporin-4 autoantibodies that were shown to induce endocytosis of aquaporin-4 and aquaporin-4 transfected HEK293 cells (Hinson et al., 2007), although it is not clear whether all antigens are endocytosed at the same rate, or by the same mechanism.

However, when cross-linking and endocytosis were induced by anti-human IgG, the Abs showed a different localisation and binding pattern than in non cross-linked cells, visible by prominent vesicles that were also found intracellular and that colocalised with MuSK-EGFP. In non cross-linked cells, patient IgG did not form such vesicles. Drachman et al. showed that whole IgG of AChR antibodies induce endocytosis of AChR. No endocytosis could be observed when monovalent Fabs were used, but could be regained when the Fabs were cross-linked by an anti-Fab antibody (Drachman et al., 1978). Thus, in retrospect it would have been interesting to test Fabs for cross-linking with anti-human IgG.

Another limiting factor of the experiments was that human IgG and not MuSK expression at the cell surface was monitored. In retrospect, it would have been reasonable to determine cell surface expression of MuSK at the different time points, perhaps with a commercial antibody. However, in one experiment, MuSK-GFP expressing HEK293 cells incubated with patient IgG were permeabilised with Triton-X at the end of the experiment and re-incubated with fluorescence-labelled anti-human IgG and analysed by confocal microscopy. The cells showed no increase in cytosolic staining (data not shown) unless cross-linked with anti-human IgG. The

absence of intracellular human IgG suggests that no endocytosis of human IgG bound to MuSK had occurred, but it cannot be excluded that MuSK alone was endocytosed. It is possible that the human IgG detached before MuSK endocytosis, or that human IgG was degraded in endolysosomes. This was not very likely though, as MuSK IgG was of high binding affinity (figure 3.16) and anti-human IgG was detected in intracellular vesicles after six hours of incubation in the artificial cross-linking experiments.

It also may be that endocytosis of MuSK would require a muscle cell environment, although other antigens have been shown to be endocytosed by patient IgG when expressed in HEK293 cells, such as Aquaporin 4 (Hinson et al., 2007). To address this, experiments were performed using cells that featured more muscle like conditions, such as HEK293 cells co-transfected with full length human MuSK and LRP4, but they also did not show any evidence for endocytosis.

Because none of the experiments in this study show evidence for MuSK endocytosis, it was then attempted to reproduce the experiment by Cole et al. In retrospect, it would have been reasonable to start the project with the reproduction of the data of this study. Cole et al. used undifferentiated C2 myoblasts, which were stably transfected with MuSK-EGFP. In that study, C2C12 myoblasts were transiently transfected with fluorescent-tagged full length human MuSK, and cell surface binding of human IgG was analysed over a period of 90 minutes. However, it was not possible to reproduce the data from Cole et al. Although a loss of cell surface bound IgG over time was observed in live cells at 37°C, a similar loss of cell surface binding was observed in the pre-fixed cells.

Cole et al. note that MuSK-EGFP was found both at the cell membrane and in the cytosol, and that intracellular trafficking of MuSK could be observed. It is important to mention that in the study by Cole et al. tagged MuSK was used and the

fluorescence of the tag was observed. Using tagged MuSK has the disadvantage that all MuSK, whether at the cell membrane or in intracellular compartments, e. g. in transport vesicles, would be monitored. This would make it more difficult to interpret whether MuSK from the cell membrane internalises or if intracellular MuSK is being transported within the cell. Furthermore, fluorescent tags may interfere with the structure or function of the protein, and could induce dimerisation.

In their study, a supplementary video shows the effect of control or MuSK-MG patient IgG on the localisation of MuSK-EGFP. The cells exposed to patient IgG show movement of MuSK-EGFP in the cells, but a main concern is that this movement was synchronous and close to the retraction of cell protrusions, and may not be caused by MuSK-EGFP internalisation but rather by the movement of the plasma membrane. IgG from only a single MuSK-MG patient was used. It is possible that MuSK endocytosis was a pathogenic mechanism of this patient, but may not be for all patients, therefore more patients need to be investigated. In addition, Cole et al. did not use control conditions such as incubation at 4°C, which is a temperature that prevents endocytosis.

With regard to the limitations of the experiments described here, it was concluded that no evidence for MuSK endocytosis was found in the present study. An explanation may be that the epitopes of MuSK bound by the human antibodies do not allow inter-MuSK cross-linking. Only after addition of anti-human IgG could cross-linking of patient IgG and MuSK endocytosis be observed. This was a rapid and very obvious process in which intracellular vesicles containing MuSK and human IgG could be found after only 10 minutes. After 30 minutes, cell surface bound human IgG outside of aggregates and vesicles was lost, which was established by additional staining with anti-human IgG.

Chapter 5: Analysis of MuSK function *in vitro*

5.1 Introduction

When MuSK antibodies were first described (Hoch et al., 2001), it was found that the antibodies reduced agrin-induced AChR clustering in C2C12 myotubes. This was not due to a reduction in the levels of cell surface expressed AChR (Farrugia et al., 2007). In addition it was found that MuSK antibodies from patients (Cole et al., 2010) and animals (Shigemoto et al., 2006) also alter MuSK and AChR β phosphorylation in C2C12 myotubes. Paradoxically, MuSK antibodies were reported to induce AChR clustering in the absence of agrin (Hoch et al., 2001). Taken together, this evidence suggests that MuSK autoantibodies affect MuSK function, but which IgG subclass is responsible for this effect, and whether divalent binding is required to affect AChR clustering, have not been investigated. These two issues were therefore studied *in vitro* using C2C12 myotubes.

The agrin-induced AChR cluster assay in C2C12 myotubes is very well established and widely used to study AChR clustering in health and disease. Overexpression of DOK7 in C2C12 cells has also been shown to induce stable AChR clustering and does not require the addition of agrin (Okada et al., 2006, Cossins et al., 2012b). In contrast to the agrin-induced AChR assay, the DOK7-induced AChR clusters are already pre-formed in differentiated myotubes and are more complex than agrin-induced AChR clusters, thus may be more indicative of mature endplates than agrin-induced AChR clusters. The effects of MuSK-MG patient plasmas, IgG and IgG subclasses, and Fab fragments on agrin-induced or DOK7-induced AChR clusters were studied.

5.2 Results

5.2.1 MuSK-MG plasma effects on agrin-induced and agrin-independent AChR clustering in C2C12 myotubes

C2C12 myoblasts were differentiated to myotubes by depletion of growth factors (figure 1.5, figure 5.1). Agrin was produced in HEK293 cells using a construct expressing soluble c-terminal splice form of neural agrin (Ferns et al., 1993). Incubation with agrin for 16 hours induced dense aggregations of AChR to form clusters that could be visualized using fluorescent-labelled alpha-bungarotoxin. Agrin was titrated to find a suitable dilution that induced AChR clusters in a concentration dependent manner, which in most cases was a 1:50 dilution.

MuSK-MG patient plasmas and healthy control sera were dialysed against Hartmann's solution using a molecular weight cut-off of 10kDa to remove small molecules from plasmas that may interfere with AChR clustering on C2C12 myotubes. Patient plasmas or healthy control sera were diluted 1:10 in differentiation medium (DM) and applied to differentiated C2C12 myotubes. After 30 minutes incubation, agrin was added to the cells. In some experiments the effect of plasmas/sera on induction of AChR clusters was investigated by omission of agrin. After incubation over night, agrin-independent AChR clusters were visualized with fluorescent-labelled alpha-bungarotoxin, fixed and analysed by fluorescence microscopy.

Figure 5.1 shows example images at 20x magnification of myotubes after incubation with or without agrin, and with patient 11 plasma or healthy control serum. In contrast to healthy control serum, MuSK-MG patient plasma reduced the number of AChR clusters in the presence of agrin, and was in some instances able to induce spontaneous AChR clusters.

To quantify AChR clustering of a single experiment, 30 microscopic images per sample (randomly selected in bright field) of red fluorescence were acquired at 20x magnification, and AChR cluster number (figure 5.2) was analysed manually using ImageJ. Unless otherwise stated, only AChR aggregates that were 5µm or longer in diameter were considered as AChR clusters, as aggregates below this size may occur independent of agrin stimulation.

The mean AChR cluster numbers of the patient or healthy control samples in the presence (figure 5.2A) or absence (figure 5.2B) of agrin were pooled. Agrin induced 20-30 AChR clusters per microscopic field in the healthy control treated myotubes, but AChR cluster levels in patient treated myotubes were reduced to 1-10 AChR clusters per field (unpaired t-test, $p=0.0005$, figure 5.2A). Plasmas from approximately half the patients (e.g. patient 11 see figure 5.1) were able to induce AChR clustering in the absence of agrin, but overall there was no significant difference between patient and healthy control treated myotubes (figure 5.2B, unpaired t-test), because MuSK antibody-induced clustering of AChR was variable between experiments.

Next the AChR cluster numbers were related to the MuSK antibody concentration of the patient plasmas (figure 5.3A,B). The mean number of AChR clusters for each patient in the presence or absence of agrin was normalised to the mean number of AChR clusters of pooled healthy controls in the presence or absence of agrin and expressed as a percentage. There was no correlation between AChR cluster number and antibody concentration in the presence or absence of agrin (figure 5.3A, B, linear regression, correlation: Pearson). However, the reduction of agrin-induced AChR clustering did correlate inversely with spontaneous AChR clusters ($p=0.0023$, linear regression, Pearson correlation, figure 5.3C).

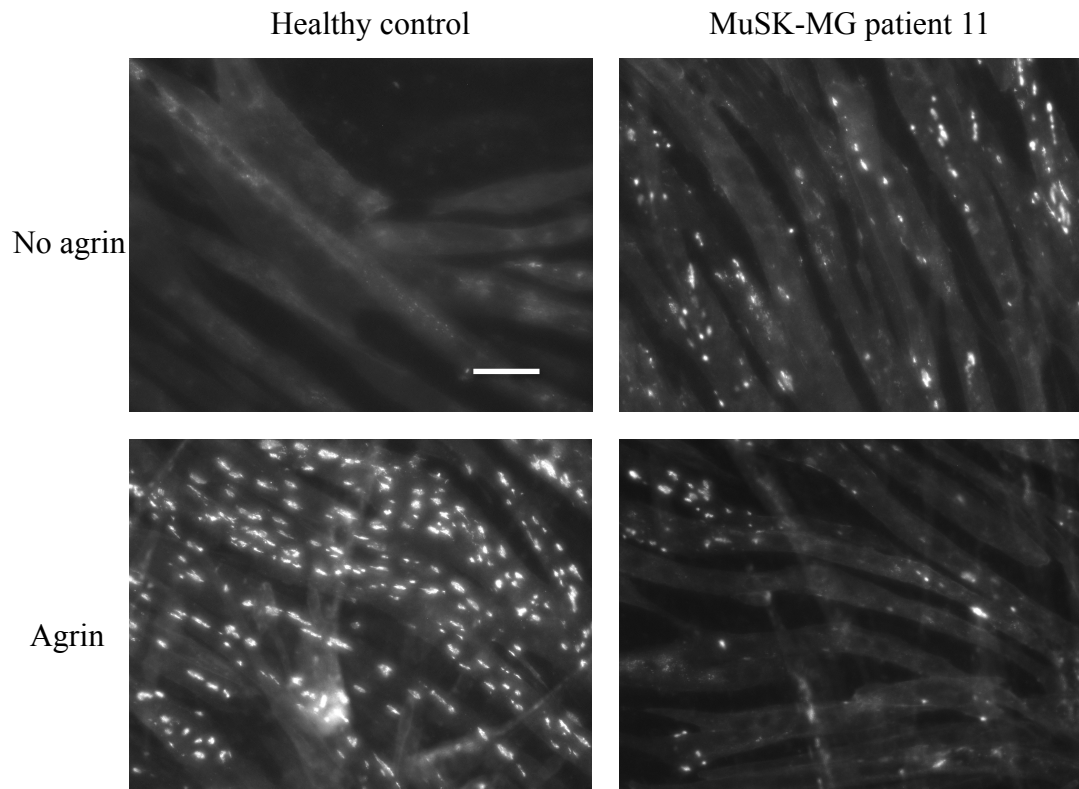


Figure 5.1: MuSK-MG patient plasma affected AChR clustering *in vitro*. C2C12 myotubes were incubated over night with a 1:10 dilution of healthy control serum or MuSK-MG patient 11 plasma in the presence or absence of neural agrin. AChR clusters were visualised with Alexa Fluor 594-conjugated alpha-bungarotoxin, the cells were then fixed and analysed by fluorescence microscopy. MuSK-MG patient plasma reduced AChR clusters in the presence of agrin, and in some cases induced AChR clusters in the absence of agrin. Scale bar = 50 μ m.

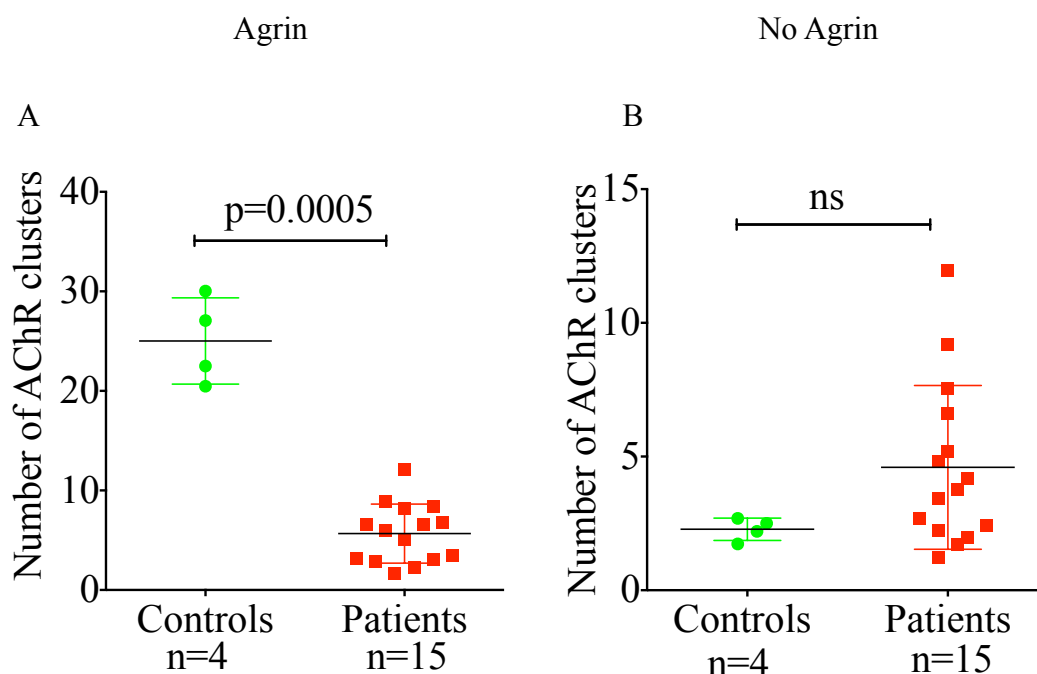


Figure 5.2: MuSK-MG patient plasma reduced the number of agrin-induced AChR clusters. Fifteen plasma samples of fourteen MuSK-MG patients and four healthy control sera were analysed in the AChR clustering assay. 30 microscopic images were acquired at 20x magnification and analysed blinded for AChR numbers using ImageJ software. Mean AChR numbers of healthy control and patient samples in the presence (A) or absence (B) of agrin were pooled. (A) The mean of the pooled mean AChR cluster numbers of agrin treated samples was reduced in patient-treated samples ($p = 0.0005$, Mann-Whitney). (B) No significant difference in mean AChR cluster number between pooled patient and healthy control treated samples was found in the absence of agrin (Mann-Whitney).

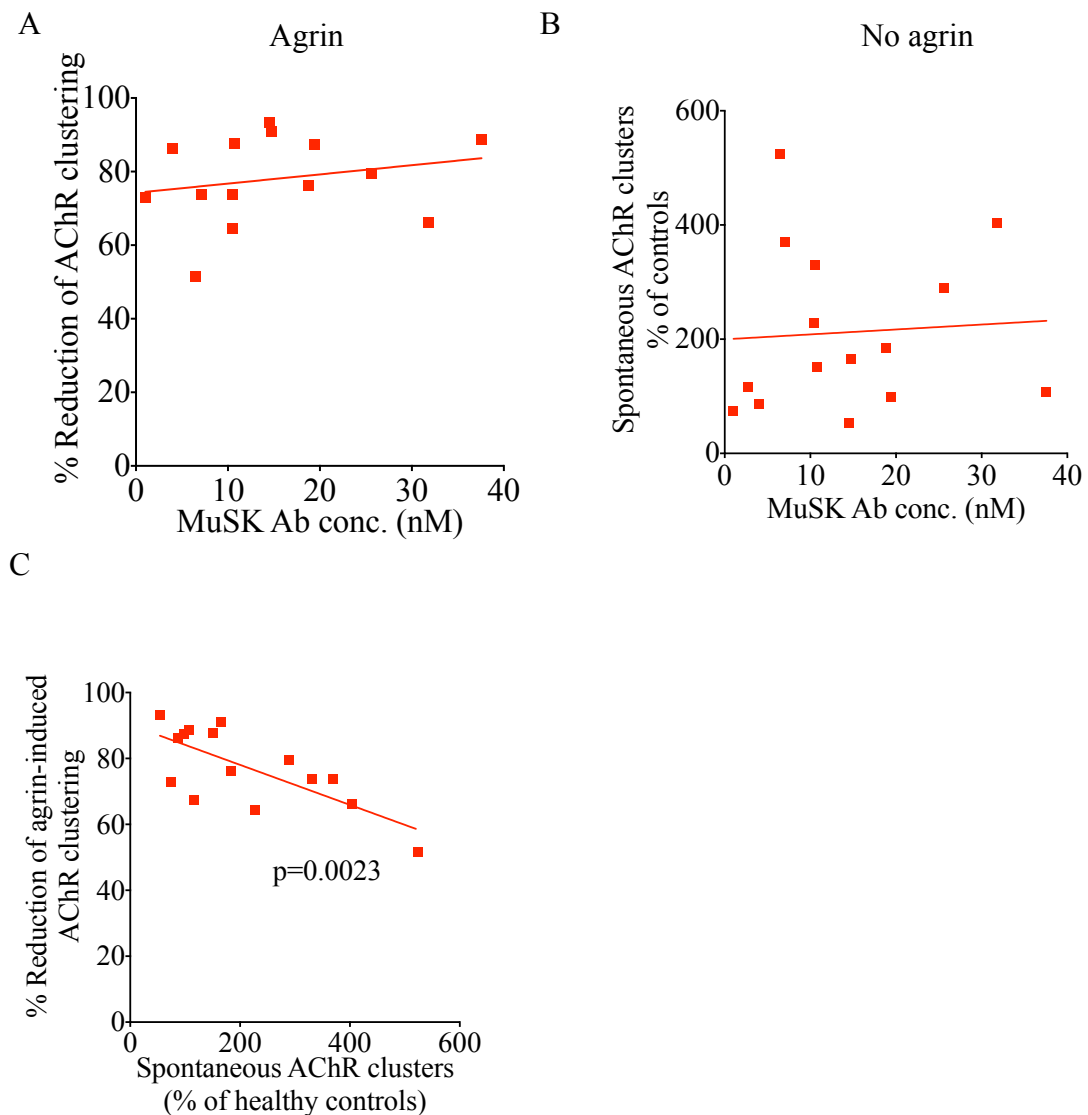


Figure 5.3: The ability of MuSK-MG plasma to reduce agrin-induced AChR clustering and to induce spontaneous AChR clustering negatively correlated. (A) Number of AChR clusters (see figure 5.2) from patient-treated C2C12 myotubes in the presence of agrin was normalised to the number of AChR clusters of healthy control treated samples, expressed as percentage of reduction of AChR clustering and compared to MuSK antibody concentrations of patient plasmas measured by RIA. (B) AChR cluster numbers of patient-treated C2C12 myotubes in the absence of agrin was expressed as percentage of healthy control treated C2C12 myotubes and compared to the MuSK antibody concentrations of patient plasmas measured by RIA. No significant correlation was found (linear regression, Pearson correlation). When the reduction of agrin-induced AChR clustering (A) was related to the spontaneous AChR clustering (B), a significant negative correlation was found (C, $p=0.0023$, linear regression, Pearson correlation).

Next the length of AChR clusters was analysed (figure 5.4). AChR clusters from samples treated with agrin and patient plasma were shorter than AChR clusters from healthy control and agrin-treated samples (figure 5.4A, unpaired t-test, Mann-Whitney, $p=0.0055$). Spontaneous AChR clusters in the absence of agrin (figure 5.4B) showed no difference but there was greater variability in AChR cluster length. When AChR cluster length of agrin-induced and spontaneous AChR clusters was compared, a positive correlation was found (figure 5.4C, $p=0.0200$, linear regression, Pearson correlation).

5.2.2 Whole IgG from MuSK patients reduces agrin-induced AChR clustering

To confirm that the effects of MuSK-MG patient plasmas on agrin-induced AChR clustering in C2C12 myotubes were caused by IgG rather than unrelated components of the plasma, purified IgG from seven patients and two healthy controls were analysed for their effect on AChR clustering in C2C12 myotubes (figure 5.5). The conditions of the experiment were similar to the preceding experiment, but this time instead of 1:10 dilution of plasma, specific concentrations of MuSK IgG for each patient were used. The concentrations had been chosen to match the concentrations of purified Fab fragments in later experiments (table 5.1). Healthy control IgG was used at the same volume as the volume of the lowest dilution of patient IgG, to take effects of the IgG buffer into account. The final protein concentrations of the healthy control IgG (0.65mg/ml, 0.90mg/ml) were within the range of the final protein concentrations of the patients (0.17-1.74mg/ml).

The patient IgG samples were able to reduce agrin-induced AChR clustering compared to the pooled healthy controls, as expected (figure 5.5B, one-way ANOVA, $p<0.0001$, Bonferroni-post test). Induction of AChR clustering without agrin was

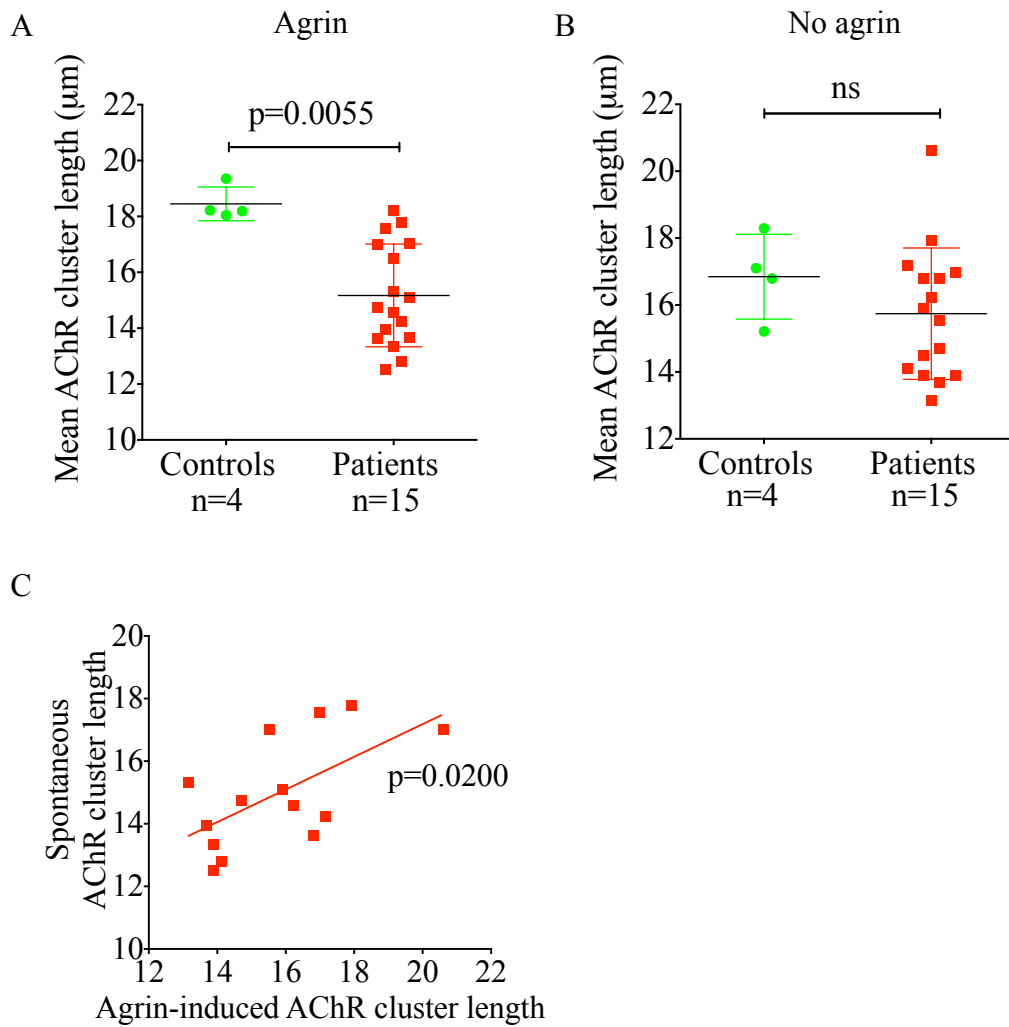


Figure 5.4: Agrin-induced AChR clusters were shorter in MuSK-MG plasma treated samples than in healthy control treated samples. The length of AChR clusters in the C2C12 AChR cluster assay (figure 5.2, 5.3) in the presence (A) or absence (B) of agrin was assessed from 30 microscopic fields at 20x magnification using ImageJ software. (A) MuSK-MG patient plasma treated AChR cluster length was significantly reduced compared to healthy control treated AChR cluster length ($p = 0.0055$, Mann-Whitney). (B) No significant difference between patient and healthy control treated AChR cluster length in the absence of agrin (ns, Mann-Whitney). (C) AChR cluster length of MuSK-MG patient plasma-treated samples in the presence of agrin was compared to AChR cluster length of MuSK-MG patient plasma-treated samples in the absence of agrin, and a positive correlation was found ($p = 0.0200$, linear regression, Pearson correlation). Data from one experiment ($n = 1$), one repeat of the experiment showed similar results.

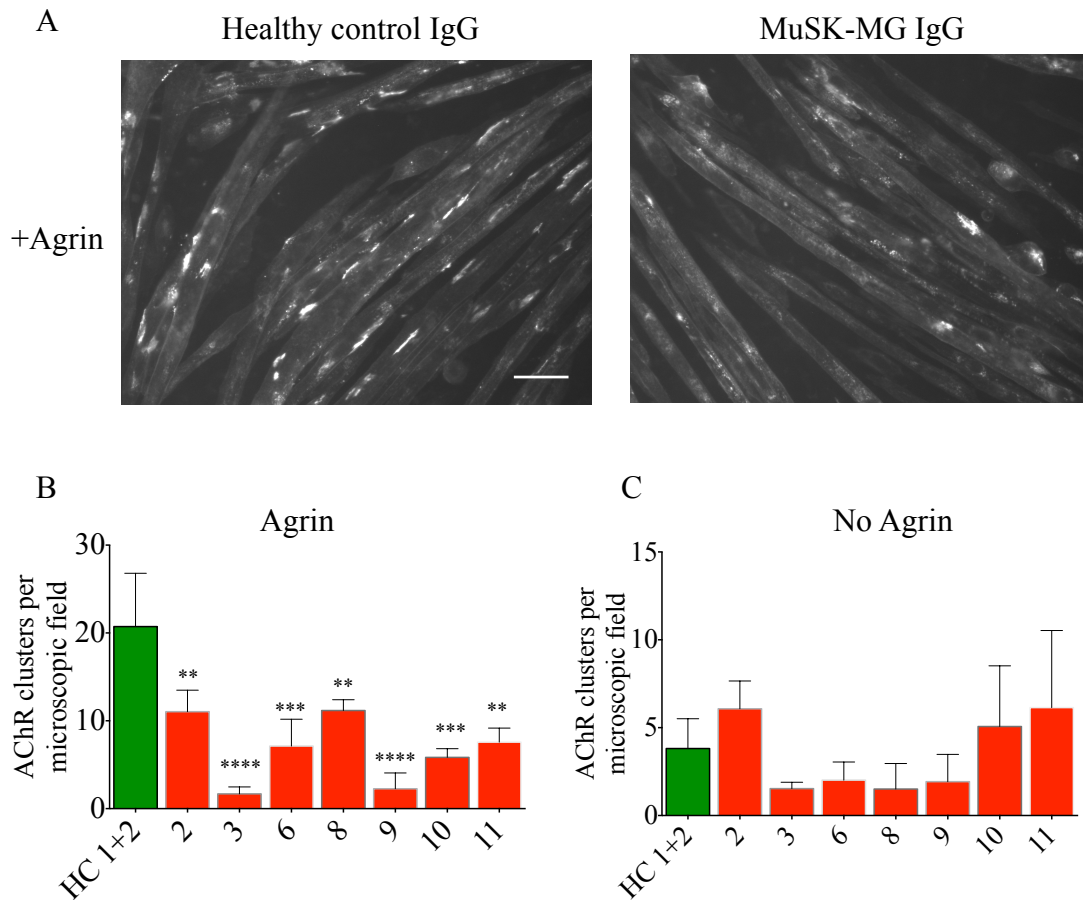


Figure 5.5: Purified IgG from MuSK-MG patients reduced agrin-induced AChR clustering in C2C12 myotubes. C2C12 myotubes were incubated with healthy control IgG or MuSK-MG patient IgG in the presence or absence of agrin over night and analysed as in previous experiments (figure 5.2). (A) Example images of healthy control or patient 3 IgG treated C2C12 myotubes in the presence of agrin. Scale bar = 50 μ m. (B) AChR cluster numbers in MuSK-MG patient-treated samples in the presence of agrin were significantly reduced compared to pooled healthy control samples ($p < 0.0001$, one way ANOVA, Bonferroni post-test, $n = 3$). (C) No significant difference between MuSK-MG patient-treated samples and healthy controls in the absence of agrin was found ($p = 0.0070$, one way ANOVA, Bonferroni post-test, $n = 3$). * $p \leq 0.05$, ** $p \leq 0.01$. *** $p \leq 0.001$, **** $p \leq 0.0001$.

Table 5.1: Final concentrations of MuSK IgG and total protein in AChR clustering assay

Patient	MuSK IgG (nM)	Protein concentration (mg/ml)
2	0.81	0.64
3	4.57	0.87
6	1.37	0.44
8	0.75	0.17
9	1.13	1.74
10	0.21	0.75
11	0.11	0.23
HC 1	0	0.90
HC 2	0	0.65

only found in three patients (patients 2, 10, 11) in one of three experiments, and as with the plasmas, the low levels of spontaneous AChR clustering induced by patient IgGs was difficult to reproduce. Overall, purified patient IgG did not cause spontaneous AChR clustering compared to pooled healthy controls (one-way ANOVA, $p=0.007$, Bonferroni post test, figure 5.5C).

5.2.3 IgG4 and IgG1-3 subclass of MuSK patients reduce agrin-induced AChR clustering

To investigate the effect of IgG4 and IgG1-3 subclasses on agrin-induced AChR clustering in C2C12 myotubes, purified IgG4 and IgG1-3 from two patients (9 and

Table 5.2: Protein concentrations of IgG subclass preparations used in AChR assay

Sample	Protein concentration (mg/ml)	MuSK Ab conc. (nM)
Patient 9 IgG4	0.27	2.40
Patient 9 IgG1-3	3.65	1.5
Patient 12 IgG4	0.13	4.15
Patient 12 IgG1-3	4.00	0.70
HC 1 IgG	4.48	0
HC 2 IgG	3.23	0

12) were used at the highest concentrations possible for each patient (1.5nM for patient 9, 0.7nM for patient 12), and purified IgG of two healthy controls were used at the same dilution as the lowest dilution of patient sample used. IgG4 and IgG1-3 were not purified from healthy controls, because the IgG4 affinity matrix was very expensive and it was considered unnecessary. The protein concentration of healthy control IgG was slightly higher than of patient IgG1-3 (table 5.2, this is a different batch of IgG subclass preparations than described in chapter 3 and used in chapter 6). The patient IgG4 protein concentration was considerably lower than patient IgG1-3 protein concentration, as expected.

Both IgG4 and IgG1-3 reduced agrin-induced AChR clustering compared to pooled healthy control IgGs (figure 5.6A,B, one-way ANOVA, $p=0.0001$, Bonferroni post-test). Neither IgG4 nor IgG1-3 were able to induce spontaneous AChR clusters (figure 5.6C, one-way ANOVA, $p=0.0771$, Bonferroni post test). IgG1-3 of patient 9 appeared to reduce spontaneous clustering, although the level of spontaneous AChR clusters was very low.

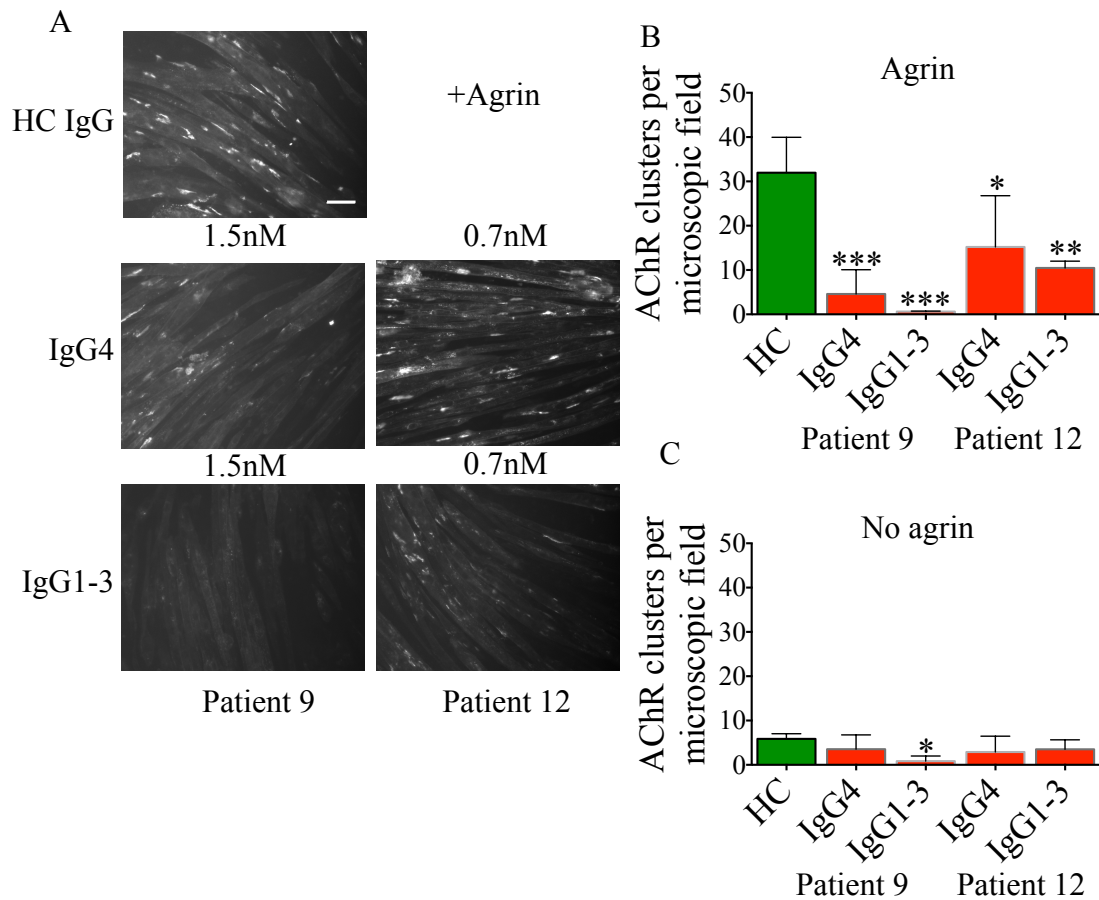


Figure 5.6: When used at the same MuSK Ab concentration, both IgG4 and IgG1-3 subclass antibodies impaired agrin-induced AChR clustering in C2C12 myotubes. C2C12 myotubes were incubated overnight with 1.5nM purified MuSK IgG4 or IgG1-3 from patient 9, or 0.7nM purified IgG4 or IgG1-3 from patient 12, or with purified IgG from a healthy control at the highest used volume, in the presence or absence of agrin. AChR clusters were visualised and analysed as described (figure 5.2). (A) Example images showing AChR clusters in the presence of agrin, scale bar = 50 μ m. (B,C) The AChR clusters per microscopic image were analysed blinded in the presence (B) or absence (C) of agrin using ImageJ software (n=3). Statistical analysis: one way ANOVA (p=0.0001) followed by Bonferroni post test, n=3. * p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001.

To see how IgG4 and IgG1-3 subclass antibodies affect clustering when they are used at a ratio that reflect their different concentrations in the patients' plasmas, the concentrations of MuSK specific IgG4 and IgG1-3 subclasses in plasma were calculated (table 5.3) using the MuSK antibody concentration of the patient plasma determined by RIA (figure 3.1) and the relative proportions of IgG subclasses as determined by flow cytometry in a preliminary IgG subclass profiling experiment (data not shown, patient 9 IgG4: 88.5%, patient 12: 83,8%).

Table 5.3: MuSK antibody concentrations calculated to be in whole plasma

Sample	Whole plasma (nM)	Relative IgG4 in plasma (nM)	Relative IgG1-3 in plasma (nM)
Patient 9	10.5	9.3	1.2
Patient 12	6.4	5.7	0.7

Table 5.4: MuSK antibody concentration at 1:10 dilution of plasma

Patient	IgG4 (nM)	IgG1-3 (nM)
Patient 9	0.9	0.1
Patient 12	0.6	0.07

A dilution of 1:10 of MuSK-MG plasma had been found to affect AChR clustering in C2C12 myotubes (figure 5.1, 5.2). Therefore, IgG4 and IgG1-3 subclasses were used in a C2C12 clustering assay at concentrations that would be present in plasma at a 1:10 dilution (table 5.4), and healthy control plasma was used at a 1:10 dilution. The samples were incubated with the C2C12 myotubes over night in the presence or absence of agrin. Example images in the presence of agrin are depicted in figure 5.7A. The experiment was conducted three times and the data were

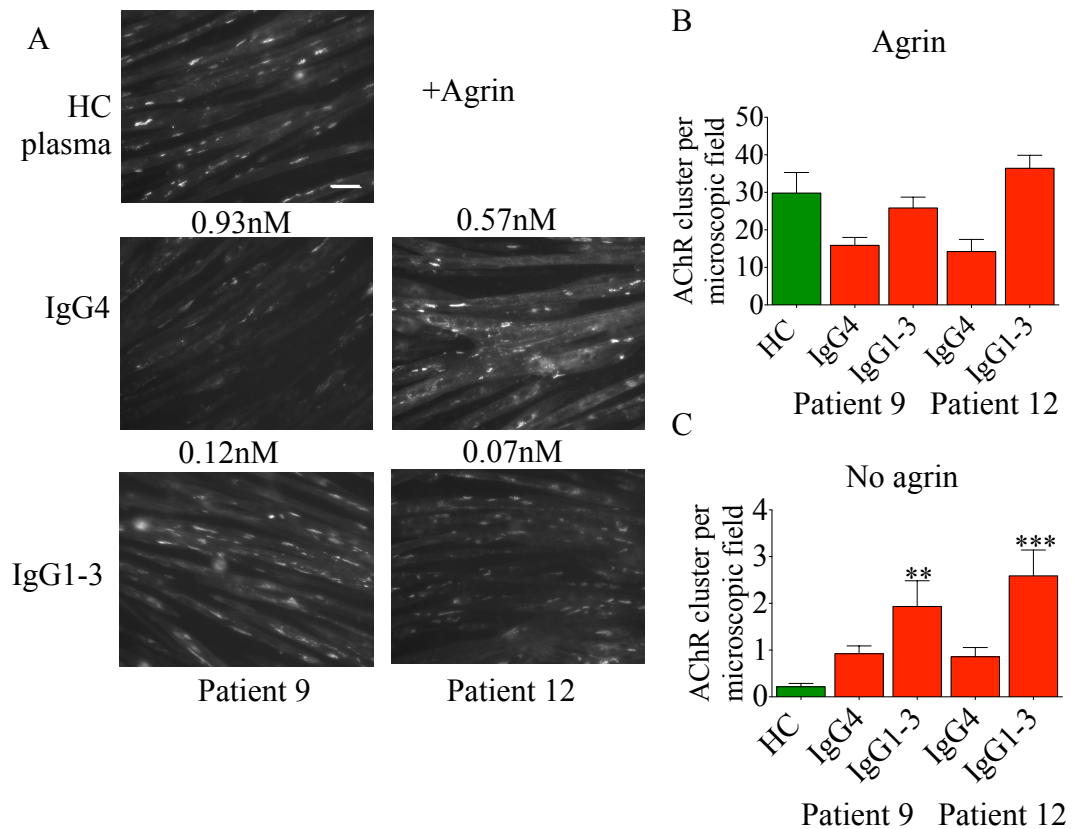


Figure 5.7: In contrast to figure 5.6, IgG4 and IgG1-3 subclass antibodies used at MuSK Ab concentrations corresponding to a 1:10 dilution of plasma did not impair agrin-induced AChR clustering in C2C12 myotubes. C2C12 myotubes were incubated overnight with purified IgG4 or IgG1-3 at final concentrations correlating to a 1:10 dilution of patient plasma (0.93nM IgG4, 0.12nM IgG1-3 of patient 9, 0.57nM IgG4, 0.07nM IgG1-3 of patient 12) or a 1:10 dilution of healthy control plasma in the presence or absence of agrin. AChR clusters were analysed as described (figure 5.2). (A) Example images of AChR clusters in the presence of agrin, scale bar = 50 μ m. (B,C) The number of AChR clusters per field in the presence (B) or absence (C) of agrin was analysed blinded using ImageJ software (n=3). Statistical analysis: one way ANOVA (p=0.0001) followed by Bonferroni post test, n=3. ** p $\leq$ 0.01, *** p $\leq$ 0.001.

pooled. With these conditions, only IgG4 reduced agrin-induced AChR clustering, but the reduction was not significant (figure 5.7B, one-way ANOVA, Bonferroni post test). IgG1-3 diluted to 1:10 of their concentration in plasma did not affect agrin-induced AChR clustering (figure 5.7B), but were capable of inducing minimal AChR clustering with approximately 2.2 AChR clusters per field (figure 5.7C, one-way ANOVA, $p < 0.0001$, Bonferroni post-test).

5.2.4 IgG4 and IgG1-3 subclass reduce DOK7-induced AChR clustering

MuSK-MG presents after birth when neuromuscular junctions are already formed, thus the effect of IgG4 and IgG1-3 subclass antibodies on DOK7-induced AChR clustering was investigated. DOK7 was introduced into C2C12 cells with a retrovirus (derived from Phoenix Eco cells) that also expresses EGFP and a puromycin resistance gene (see also figure 2.2). The myoblasts were maintained in selection medium containing puromycin. Successful infection, determined by green fluorescence of EGFP, was verified by fluorescence microscopy and the myoblasts were differentiated to myotubes by growth factor depletion. Differentiated myotubes harboured a variable number of DOK7-induced AChR clusters, with a subset of AChR clusters showing complex structures such as perforated (figure 5.8A), c-shaped (figure 5.8B) or branched (figure 5.8C).

The myotubes were incubated with purified IgG4 or IgG1-3 from patient 9 at the highest possible common concentration, which was 1.5nM, or healthy control IgG at the same volume as before (figure 5.8D). At these concentrations, both IgG4 and IgG1-3 subclass antibodies from the patient reduced numbers of AChR clusters, and fewer complex AChR clusters were visible. The observed AChR clusters on the

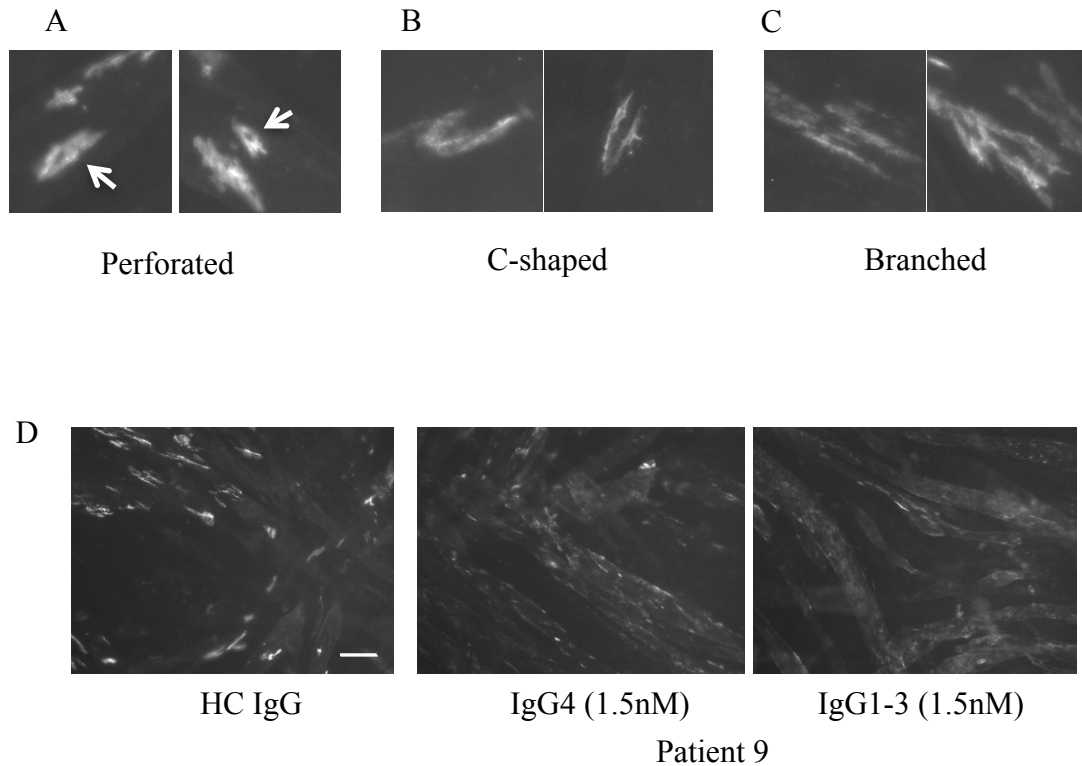


Figure 5.8: MuSK-MG patient IgG1-3 and IgG4 disrupted DOK7-induced AChR clustering on C2C12 myotubes and reduced complex AChR clustering. (A-C) Examples of complex AChR clusters. DOK7 was overexpressed in C2C12 myotubes and AChR clusters were visualised using Alexa Fluor 594-conjugated alpha-bungarotoxin. (A) Arrows indicate perforated clusters. (B) C-shaped clusters. (C) Branched clusters. (D) C2C12 myotubes were incubated overnight with 1.5nM MuSK specific IgG1-3 or IgG4 from patient 9. Representative images of AChR clusters, visualised with Alexa Fluor 594-conjugated alpha-bungarotoxin. Scale bar = 50 μ m.

myotubes in the presence of patient antibodies were also dimmer than those on the healthy control treated myotubes.

To quantify the observed effects, 30 microscopic images at 20x magnification were acquired, and AChR cluster number and the number of complex AChR clusters per microscopic field were analysed using ImageJ software. The samples were blinded throughout the experiments. Aggregates of dim fluorescence were only included in the quantification if they had well-defined edges and could be measured. Data from three consecutive experiments were pooled (figure 5.9A-C). In addition, IgG4 and IgG1-3 from patient 12 at the highest possible common concentration, which was 0.11nM, were also analysed, although the experiment was only conducted twice due to limited amounts of sample (figure 5.9D-F).

The mean AChR number per field in healthy control IgG treated samples varied between 25 and 65, depending on the batch of infected cells and time after infection. AChR cluster numbers were reduced by both IgG4 and IgG1-3 from both patients (one-way ANOVA with Bonferroni post-test, figure 5.9A,D). The number of complex AChR clusters and total AChR cluster number was used to determine the percentage of complex AChR clusters (figure 5.9B,E). Approximately 15-20% of DOK7-induced AChR clusters were found to be perforated, branched or c-shaped, and thus defined as complex, in the presence of healthy control IgG or Fabs. Incubation of DOK7-infected C2C12 myotubes with either 1.5nM (patient 9) or 0.11nM (patient 12) MuSK IgG4 and IgG1-3 significantly reduced the percentage of complex AChR clusters (one-way ANOVA with Bonferroni post-test).

There also appeared to be a larger proportion of small clusters on myotubes treated with patient antibodies. Thus the number of AChR clusters shorter than 5µm was quantified (figure 5.9C,F). Treatment with IgG4 of patient 9 generated a considerable increase in the presence of small AChR clusters. IgG1-3 of patient 9, as

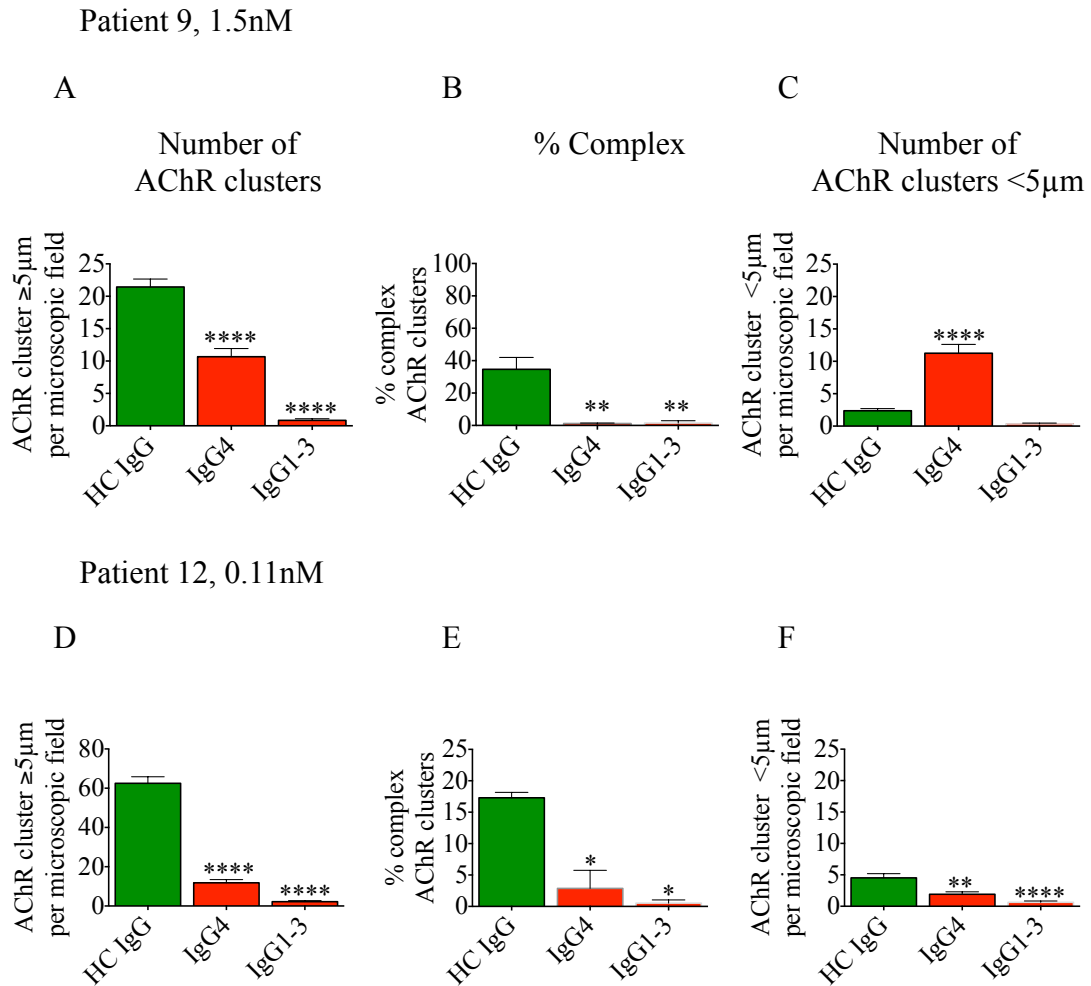


Figure 5.9: MuSK-MG patient IgG1-3 and IgG4 disrupted DOK7-induced AChR clustering on C2C12 myotubes. Myotubes were incubated overnight with 1.5nM MuSK-specific IgG4 or IgG1-3 from patient 9 (A-C) or 0.11nM MuSK specific IgG1-3 or IgG4 from patient 12 (D-F). 30 microscopic images at 20x magnification were analysed blinded for AChR numbers using ImageJ software. (A,D) Number of AChR clusters $\geq 5\mu\text{m}$. One way ANOVA ($p < 0.0001$) followed by Bonferroni post test. (B,E) The proportion of complex AChR clusters was calculated. (C,F) Number of AChR clusters $< 5\mu\text{m}$ was measured. Average of three (A-C) or two (D-F) experiments. One way ANOVA ($p < 0.0001$) followed by Bonferroni post test. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

well as IgG4 and IgG1-3 of patient 12, did not produce an increase in small clusters, perhaps because the IgG subclass preparations had dispersed all clusters. In conclusion, both MuSK IgG4 and IgG1-3 effectively reduced the number of DOK7-induced AChR clusters, with an increase of smaller, dimmer and less complex clusters found in some of the samples.

5.2.5 Digestion of Fab fragments from human IgG

IgG4 is a subclass that partly occurs in a functional monovalent form (van der Neut Kolfshoten et al., 2007). IgG4 subclass antibodies were found to affect both agrin- and DOK7-induced AChR clustering, and it was interesting to ask whether divalent binding was necessary for these effects. Fab fragments were generated from patient derived IgG to analyse their effect on agrin- and DOK7-induced AChR clustering.

Purified whole IgG was digested using papain coupled to sepharose beads. Protein G and Protein A were tested for their ability to deplete Fc regions from the Fab fragments, but they bound to Fc regions and to Fab fragments. To overcome this problem, incubation time and temperature were varied, and protein A and protein G coupled beads were compared. Protein G coupled beads were found to bind slightly less to Fab fragments than protein A coupled beads, and therefore these beads were selected for Fab purification, applying an incubation time of one hour at 4°C. At each step of the preparation, aliquots were acquired for analysis. The expected products at each step in the protocol, according to the manufacturer, are shown in table 5.5, which also depicts the range of total protein content obtained at each step.

Table 5.5: Expected contents of the aliquots collected during Fab fragment production

Aliquot	Content of the aliquots according to the manufacturers instructions	Range of total protein content obtained during Fab preparation (mg)
IgG	Whole, undigested IgG	8.82-17.92
Digest (from papain beads)	Fab fragments, Fc region, undigested IgG	4.69-10.63
Fab	Purified Fab fragments depleted of IgG and Fc	1.68-3.34
Wash 1 (of protein G)	Fab fragments	0.31-0.67
Wash 2 (of protein G)	Fab fragments	0.23-0.29
Elution 1 (of protein G)	whole IgG, Fc region	0.18-0.49
Elution 2 (of protein G)	whole IgG, Fc region	0.19-1.17
Elution 3 (of protein G)	whole IgG, Fc region	0.08-1.73

The samples were subjected to SDS-PAGE using non-reducing conditions and the proteins visualized by Coomassie blue staining (figure 5.10). Dark red arrows indicate the band sizes that would correspond to whole IgG (above 97kDa), heavy chain (approximately 50kDa), Fc region (approximately 45kDa), non-reduced Fab fragments (approximately 45kDa), light chain (25kDa) and reduced Fab fragments (below 25kDa). Bands for IgG were found in the IgG, IgG digest and elutions 1-3, but were absent from the Fabs and the washes, indicating that the Fabs were depleted of divalent IgG, and can be used to study the effects of monovalent binding. However, the presence of IgG in the digest shows that not all IgG had been digested. The presence of Fabs in the three elution fractions shows that some Fabs bound to protein G and were therefore lost during the purification. Despite these observations, since Fab fragments could be detected in the Fabs fraction and the washes, these were pooled and used in subsequent experiments.

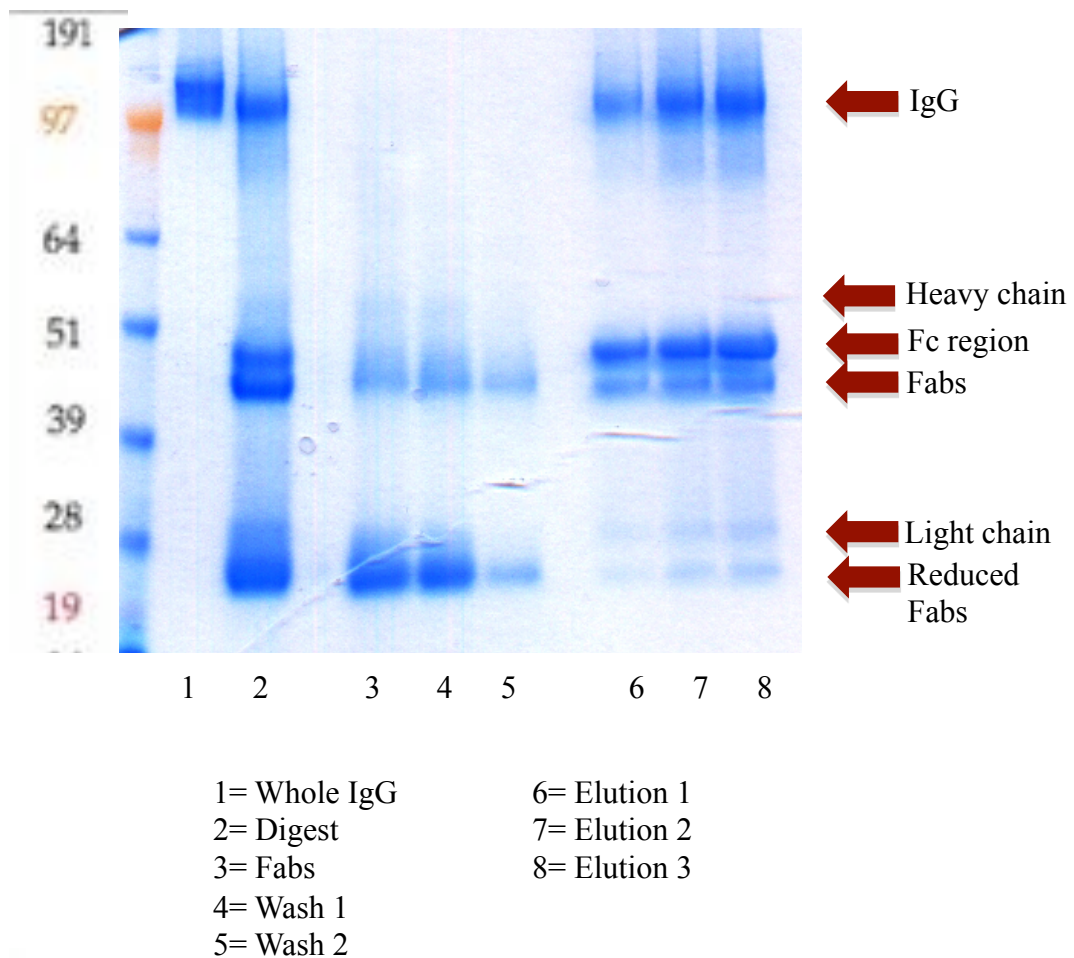


Figure 5.10: Purified Fabs were depleted of whole IgG. Purified IgG (1) was digested with immobilized papain (2), then whole IgG and Fc region were depleted from the Fabs (3) by protein G sepharose. After two washing steps (4,5), IgG and Fc region were eluted from the sepharose with glycine buffer pH 2.3 (6-8). Aliquots from each step were subjected to SDS-PAGE and proteins were visualised with coomassie blue.

In order to quantify the yield and any potential loss, protein concentrations were determined by a nanodrop (figure 5.11A). Elution 1-3 aliquots gave a characteristic peak in protein concentration and, as expected, protein concentration of the Fab aliquot was considerably lower than of the digest aliquot and whole IgG.

During the process of Fab preparation, sample volumes and thus protein concentrations changed, and therefore the total protein content was calculated (figure 5.11B). Without any loss of protein during the preparation, total protein content of the Fabs would be predicted as 66.66% of the total protein content of whole IgG, because whole IgG (150kDa) consists of two Fab fragments (2x50kDa) and one Fc region (50kDa). The total protein content decreased with each step, and total protein content of Fabs was reduced to approximately 25% (3.13mg) of total protein of IgG used at the beginning (12.76mg). This suggests a loss of approximately 63% of Fabs during the preparation.

The yield of MuSK-specific Fabs varied markedly between patients (table 5.6), while the protein concentration of all preparations was relatively similar (approximately 0.5mg/ml). Thus, protein concentration and concentration of MuSK specific Fabs did not correlate. This was not unexpected, since MuSK specific IgG and protein concentration did not correlate in purified IgG (e.g. table 5.1), due to individually different levels of total IgG in the patients. It was not possible to determine the amount of total IgG directly in the patient plasma, because of the presence of other proteins.

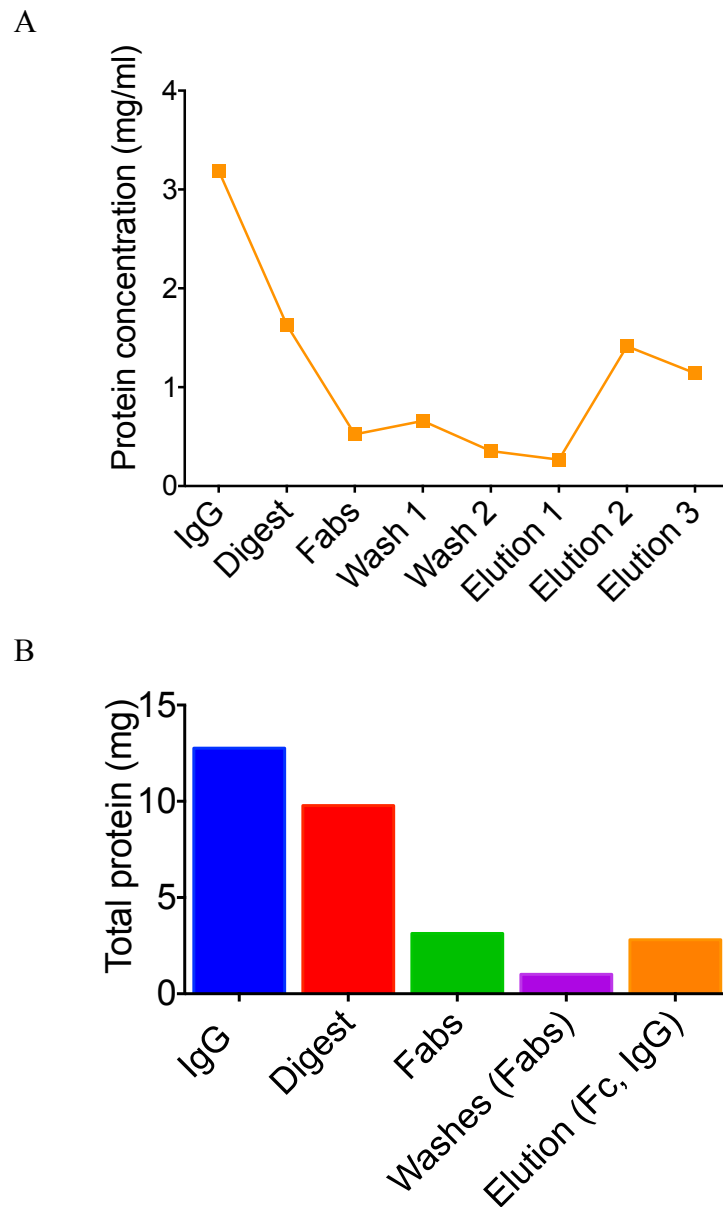


Figure 5.11: Protein loss during the production of Fabs. (A) Protein concentrations of the aliquots (see figure 5.10) were determined. (B) The differences in sample volume during the process were taken into account to calculate total protein content at each step of the process.

Table 5.6: Concentrations of Fab fragments used in the AChR assay

Patient	MuSK Fab (nM)	Protein concentration (mg/ml)
2	0.81	0.44
3	4.57	0.53
6	1.37	0.28
8	0.75	0.44
9	1.13	0.55
10	0.21	0.50
11	0.11	0.48
HC 1	0	0.50
HC 2	0	0.34

5.2.6 MuSK specific Fab fragment concentrations can be assessed with RIA

First it was determined whether RIA could be used to quantify Fab fragments. It was desirable to be able to use the polyclonal antibody used for the IgG RIA, in order to have similar conditions for quantification of all antibody preparations.

A three-fold dilution series of Fab fragments was used to test 50µl anti-human IgG or 50µl Fab-specific anti-human IgG (figure 5.12A). Similar levels of radiolabelled MuSK were precipitated with both antibodies, resulting in MuSK Fab concentrations of 0.36nM (anti-human IgG) or 0.44nM (anti-Fab). This suggests that the polyclonal anti-human IgG antibody contained sufficient Fab-specific antibodies to efficiently precipitate Fab fragments.

Further, it was investigated whether Fabs and whole IgG of the same patient had similar amounts of MuSK binding per µg protein. Fab fragments, (50kDa) as

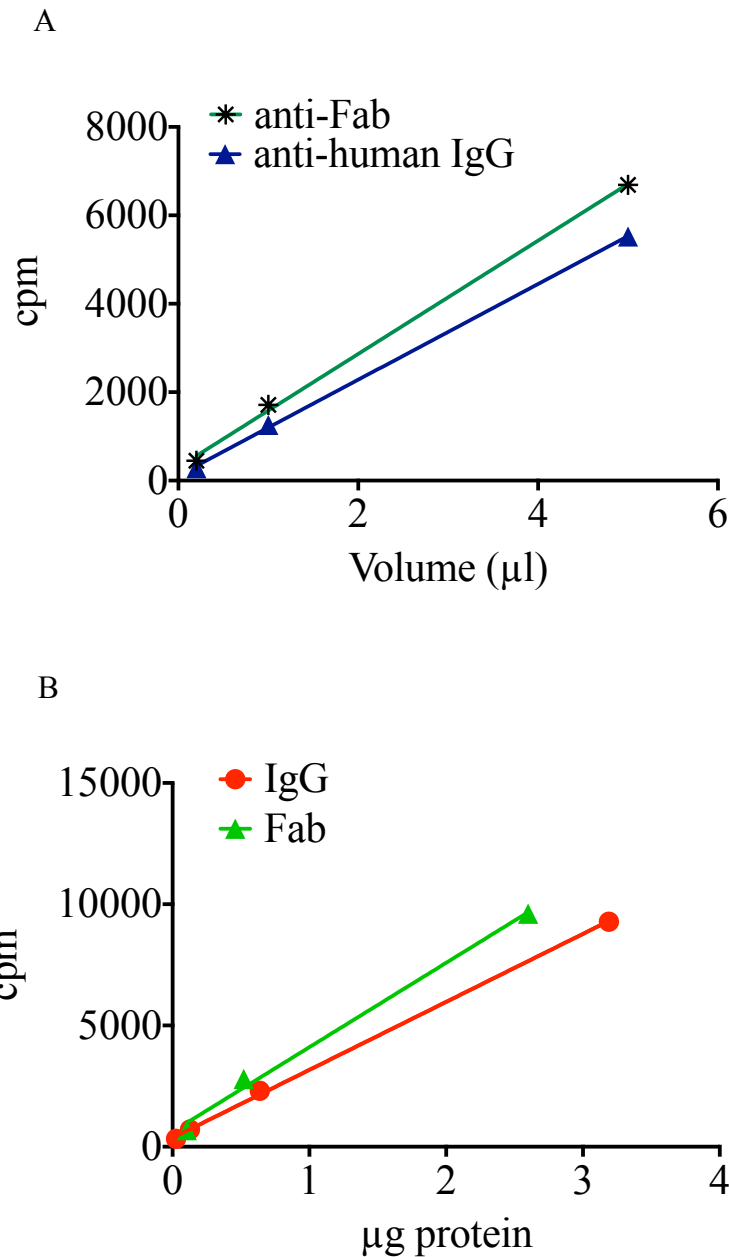


Figure 5.12: Fabs bound to ^{125}I MuSK at a similar efficacy as whole IgG in RIA. (A) Comparison of two different secondary antibodies for precipitating Fabs by RIA. 50 μl of anti-human IgG or Fab specific anti-human IgG were used in RIA with increasing volumes of Fabs (0.2, 1, 5 μl) of patient 10. Similar levels of ^{125}I MuSK were precipitated with both secondary antibodies. (B) Increasing amounts (μg protein) of whole IgG or Fabs from one patient were incubated with constant volumes of ^{125}I MuSK. Bound Fabs or IgG were precipitated using anti-human IgG, and levels of bound ^{125}I MuSK were quantified (cpm).

they are monovalent, are only able to bind to one MuSK molecule, while IgG (150kDa) are mostly divalent and therefore capable of binding two MuSK molecules simultaneously. Therefore it would be expected that a Fab fragment is 50% more efficient in binding and precipitating MuSK per μg antibody protein than a whole IgG. The Fab fragments precipitated slightly more MuSK molecules per μg antibody protein than IgG (figure 5.12B), confirming that Fab fragments were efficiently purified with little loss of functional binding capability.

5.2.7 Fab fragments reduce AChR clustering

Fab fragments from seven MuSK patients and two healthy controls, at the same concentrations as their whole IgG counterparts (figure 5.5, table 5.6) were used in the AChR clustering assay. 30 microscopic images at 20x magnification were acquired (figure 5.13A), and AChR cluster numbers were analysed as before. Data from three experiments were pooled and analysed (figure 5.13B,C). MuSK patient derived Fab fragments were sufficient to reduce agrin-induced AChR clustering (one-way ANOVA, $p < 0.0001$, Bonferroni post-test). In the absence of agrin, MuSK patient Fab treated samples did not induce AChR clustering, but reduced the number of spontaneous AChR clusters per microscopic field from averagely 2.87 with healthy control Fabs to 0.28-0.86 AChR with patient Fabs (table 5.8). Fab fragments were as efficient at reducing agrin-induced AChR clustering as whole IgG. Patient 3, 6 and 10 Fabs reduced AChR cluster numbers to a similar degree as the whole IgG of these patients. Patient 2, 8, 9 and 11 Fabs reduced AChR clustering even more efficiently than whole IgG of these patients (figure 5.5, figure 5.13).

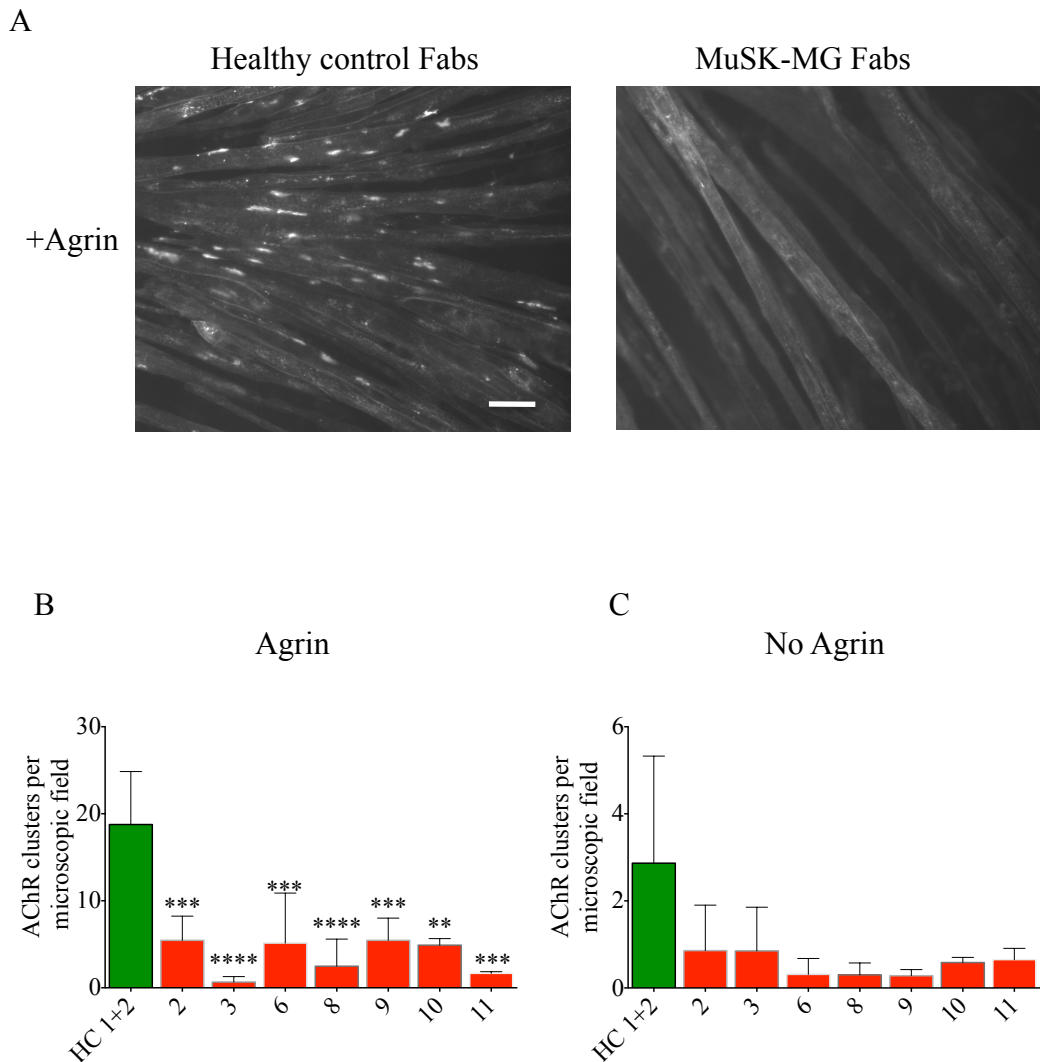


Figure 5.13: Fabs from MuSK-MG patients reduced agrin-induced AChR clustering in C2C12 myotubes. C2C12 myotubes were incubated with healthy control Fabs or MuSK-MG patient Fabs in the presence or absence of agrin over night. AChR clusters were visualised and analysed as described above (figure 5.2). (A) Example images of C2C12 myotubes incubated with healthy control or patient 3 Fabs in the presence of agrin. Scale bar = 50 μ m. (B) AChR cluster numbers in MuSK-MG patient-treated samples in the presence of agrin were significantly reduced compared to pooled healthy control samples ($p < 0.0001$, one way ANOVA, Bonferroni post-test, $n=3$). (C) No significant difference between MuSK-MG patient-treated samples and healthy controls in the absence of agrin ($p=0.0891$, one way ANOVA, Bonferroni post-test, $n=3$). ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

5.2.8 Fab fragments reduce DOK7-induced AChR clustering

To assess whether the Fab fragments were also able to disperse DOK7-induced AChR clusters, Fab fragments from five patients at the same concentrations as in the previous assay (table 5.6) were tested and analysed as above. All patient derived Fab preparations were able to reduce DOK7-induced AChR clustering (one-way ANOVA; Bonferroni post-test, figure 5.14A,B). The patient Fab treated AChR clusters showed a trend towards reduced complexity, which did not reach significance (figure 5.14C). Accordingly, when the number of AChR clusters below 5µm was analysed (figure 5.14D), no significant increase in small AChR clusters could be observed. Interestingly, two Fab preparations (patient 6, 14) led to a significant reduction in AChR clusters below 5µm.

5.2.9 LRP4 antibodies from one patient do not affect AChR clustering

Autoantibodies to LRP4 present in some MG patients were reported to affect AChR clustering in C2C12 myotubes (Zhang et al., 2012, Pevzner et al., 2012). One serum, found to be positive for LRP4 antibodies (experiments by Dr Judith Cossins), was tested for effects on agrin-induced AChR clustering.

The LRP4 patient serum, two healthy control plasmas and one plasma from MuSK patient 2 were used at 1:10 dilutions. Data of two experiments were pooled (figure 5.15). No significant difference in the number of AChR clusters was found between healthy control and LRP4 patient treated samples, either with or without agrin. The MuSK patient plasma, as expected, reduced agrin-induced AChR clustering, and also led to a moderate induction of AChR clustering. This suggests

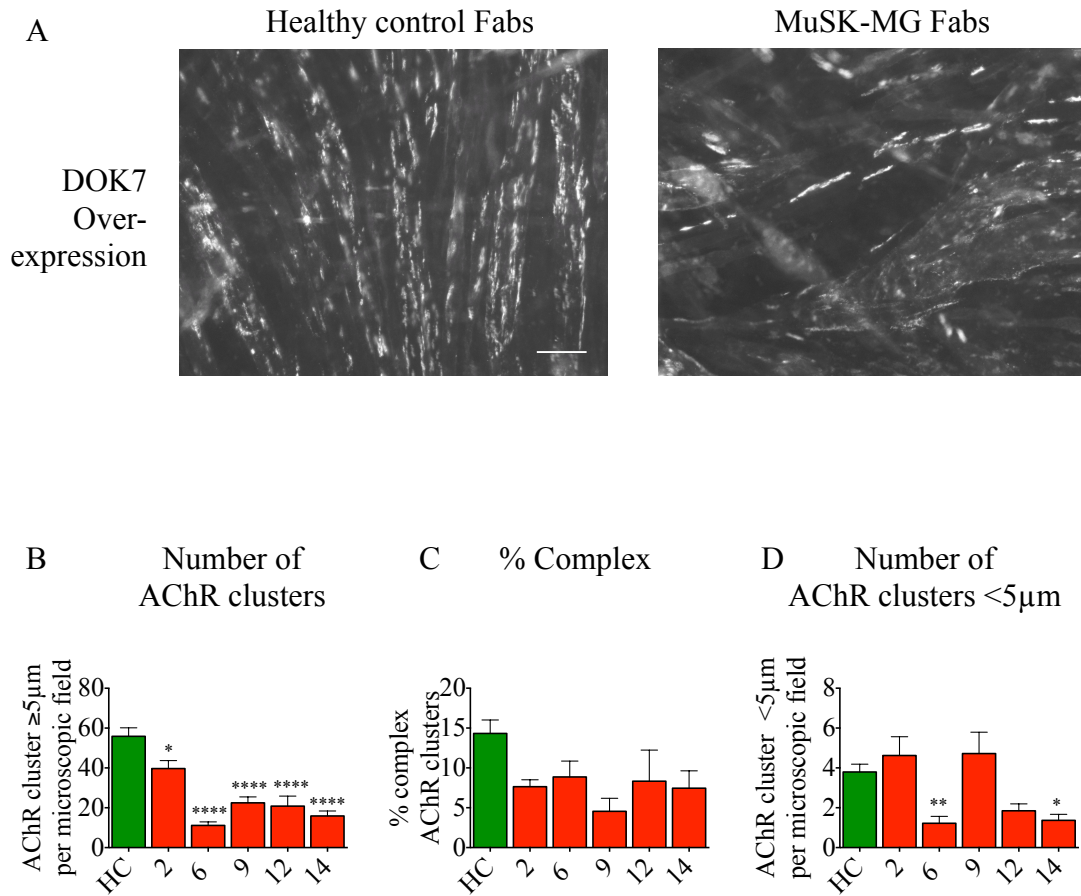


Figure 5.14: MuSK-MG Fabs disrupted DOK7-induced AChR clustering on C2C12 myotubes. (A) Myotubes were incubated overnight with Fabs from healthy controls or MuSK-MG patients. Scale bar = 50 μm . (B) Number of AChR clusters $\geq 5\mu\text{m}$ was quantitated, average of three experiments. One way ANOVA ($p < 0.0001$) followed by Bonferroni post test. (C) The percentage of complex AChR clusters was calculated. (D) Number of AChR clusters $< 5\mu\text{m}$ was assessed. One way ANOVA ($p < 0.0001$) followed by Bonferroni post test, $n = 3$. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$

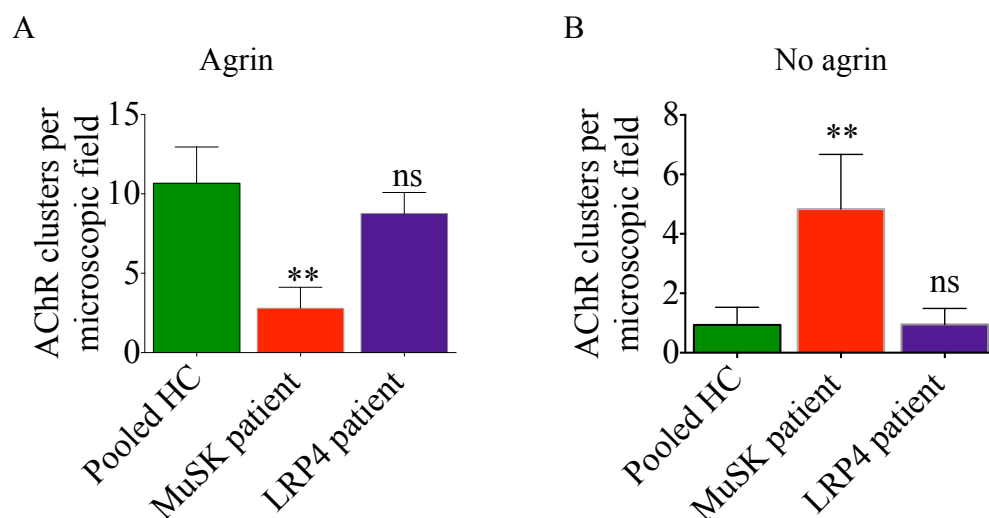


Figure 5.15: LRP4 positive MG serum did not affect AChR clustering. C2C12 myotubes were incubated with 1:10 dilution of healthy control plasma, MuSK-MG plasma (patient 2) or LRP4 MG plasma over night in the presence or absence of agrin. AChR clusters were visualised and analysed as described above (figure 5.2). (A) LRP4 MG plasma did not affect AChR clustering in the presence of agrin. MuSK-MG patient plasma reduced AChR clustering significantly. ($p=0.0099$, one-way ANOVA, Bonferroni post-test) (B) LRP4 MG plasma did not induce spontaneous AChR clustering, however MuSK-MG plasma induced AChR clustering to a modest degree ($p=0.0056$, one-way ANOVA, Bonferroni post-test). Average of two experiments. ** $p \leq 0.01$.

that the serum from this particular LRP4 antibody positive patient did not affect AChR clustering.

5.3 Discussion

Plasmas, purified IgG, IgG4 and Fab fragments derived from MuSK-MG patients all reduced AChR clustering efficiently (table 5.7), but, unexpectedly, IgG1-3 subclass antibodies were also able to reduce AChR clustering (table 5.9). Furthermore, AChR clusters induced by DOK7 overexpression were efficiently dispersed by IgG4 and IgG1-3 subclass antibodies and by Fab fragments. By contrast, plasma from an LRP4 antibody positive patient did not influence agrin-induced or spontaneous AChR clustering.

Two potential concerns regarding the AChR clustering assays are the need for manual analysis of the results, and avoidance of bias. C2C12 myotubes were variable in shape and size, and some areas of the plate could contain undifferentiated myoblasts or no cells. Furthermore, the number of AChR clusters on the C2C12 myotubes varied in different areas of the plate. AChR clusters on C2C12 myotubes also vary in size and shape. In addition, the myotubes occasionally contracted, and the ends would then become stained non-specifically with fluorescent-labelled bungarotoxin. In order to minimise these difficulties, the AChR clusters on every image had to be counted and measured manually to exclude non-specific binding, and the choice of clusters was therefore subjective. To overcome bias, the samples were blinded prior to their application to the myotubes, and an adequate number of images of microscopic fields covering the whole plate were acquired to account for the variation in AChR cluster numbers. The microscopic fields were selected in bright field, to ensure the presence of C2C12 myotubes without selecting for areas

with AChR clusters, before images of red fluorescence were taken to visualize the AChR clusters. Analysis of the images was also carried out blinded.

Table 5.7: Average AChR cluster numbers in C2C12 myotubes per microscopic field at 20x magnification

Patient	Plasma + agrin	IgG + agrin	Fabs + agrin	Fabs + DOK7 overexpression
1	2.83	-	-	-
2	8.43	11.04	5.45	39.7
3	5.13	1.68	0.65	-
4	3.13	-	-	-
5	5.93	-	-	-
6	1.67	7.12	5.14	11.14
7	2.27	-	-	-
8	3.10	11.17	2.48	-
9	6.57	2.26	5.46	22.50
10	8.90	5.83	4.90	-
11	6.59	7.57	1.67	-
12	12.13	-	-	20.81
13	3.43	-	-	-
14	6.77	-	-	15.88
Pooled HC	23.32	20.73	18.76	55.88

Purified IgG, as expected, reduced agrin-induced AChR clustering significantly, which verifies that it is the patient IgG, and not a plasma component, that is responsible for this effect. Furthermore, IgG4, which had been suggested as the main pathogenic entity by passive transfer experiments (Klooster et al., 2012), was able to reduce agrin-induced AChR clustering and to affect DOK7-induced AChR clustering. There was no evident correlation between the reduction of agrin-induced AChR clusters and MuSK antibody concentration, but the number and length of plasma-induced AChR clusters negatively correlated with the number and length of agrin-induced AChR clusters. Thus the reduction in AChR clustering by plasmas might be the result of a combination of inducing and inhibiting effects of MuSK.

The induction of AChR clustering by patient antibodies could be caused by increased MuSK phosphorylation and activation (Shigemoto et al., 2006, Cole et al., 2010). This suggests the presence of a divalent antibody capable of MuSK dimerisation, as MuSK dimerisation and activation can be induced by divalent antibody binding (Hopf and Hoch, 1998a). However, the induction of AChR clusters by patient plasma in the absence of agrin, first described by Hoch et al. 2001, was variable and could not be reproduced in a reliable manner when purified IgG was used (table 5.8).

Interestingly, divalent IgG1-3, when used at MuSK Ab concentrations correlating to a 1:10 plasma dilution (figure 5.7), induced AChR clustering, although the increase reflected the presence of approximately one more AChR cluster per field, which is a very small effect. This suggests that induction of spontaneous AChR clustering, in contrast to the inhibition of agrin-induced clustering, may be largely due to plasma components rather than IgG, and may not be relevant for pathogenicity as it was only found in a subset of patient plasmas.

Table 5.8: Average number of spontaneous AChR clusters in C2C12 myotubes per microscopic field at 20x magnification

Patient	Plasma	IgG	Fabs
1	2.43	-	-
2	9.20	6.08	0.86
3	6.60	1.53	0.85
4	2.23	-	-
5	4.20	-	-
6	1.23	2.01	0.31
7	3.77	-	-
8	3.43	1.51	0.30
9	7.53	1.93	0.28
10	5.20	5.07	0.58
11	8.44	6.15	0.65
12	11.97	-	-
13	1.97	-	-
14	1.70	-	-
Pooled HC	2.30	3.81	2.87

Forced expression of DOK7 in C2C12 myotubes induces MuSK phosphorylation and activation and induces the clustering of AChR in the absence of agrin (Okada et al., 2006), perhaps by increasing MuSK dimerisation by abundant DOK7 (Bergamin et al., 2010). The AChR clusters are usually of higher structural complexity, such as perforated, c-shaped or branched (Beeson et al., 2006, Cossins et al., 2012b), than agrin-induced AChR clusters, and resemble different stages of

neuromuscular junctions during development *in vivo* (Marques et al., 2000). Thus the observed dispersal of pre-formed DOK7-induced AChR clusters by patient antibodies may reflect better the situation in the patient; the onset of MuSK-MG is after birth, when complex neuromuscular junctions are already formed. The observed dimness of DOK7-induced AChR clusters in the presence of patient antibodies was thought to derive from a reduced AChR density. This is in accordance with findings of electrophysiological studies that suggested a reduced density of AChR at the postsynaptic membrane as a cause for reduced MEPPs in MuSK-MG patients (Selcen et al., 2004, Nix et al., 2010) and experimental animals (Viegas et al., 2012, Morsch et al., 2012, Mori et al., 2012a).

Some variation in the dispersal of DOK7-induced AChR clusters was found between the two patients that were investigated. IgG4 of patient 9 led to a less pronounced dispersal of DOK7-induced AChR clusters than that of patient 12, but showed an increase in small clusters below 5µm. This suggests that MuSK antibodies break down large, complex clusters into smaller, less complex clusters, until the clusters disappear. However, the two patients were not tested with the same batch of DOK7-infected cells, and there was variation in AChR cluster number between batches (approximately 20-25 AChR clusters per microscopic field in the first batch compared with approximately 60-65 AChR clusters per microscopic field in the second). Furthermore, the results from patient 12 derive from two pooled experiments, while the results for patient 9 were pooled from three experiments. It would be interesting to investigate a larger cohort of MuSK-MG patients for dispersal of DOK7-induced AChR clusters in future experiments.

An important observation was that not only IgG4 subclass antibodies, which have been suggested to be pathogenic, but also IgG1-3 subclass antibodies from MuSK-MG patients, affected both agrin- and DOK7-induced AChR clustering.

There was some variation in the effects of IgG1-3 on agrin-induced AChR clustering between patients 9 and 12, but this might be because patient 9 subclasses were used at a higher MuSK antibody concentration (1.5nM) than patient 12 subclasses (0.7nM). These observations suggest that the IgG1-3 subclass antibodies may have a pathogenic role as well, which is important as current therapy strategies, such as the use of anti-CD20 therapeutic antibody (rituximab), appear to be more effective against IgG4 specific B-cell populations (Khosroshahi et al., 2010, Khosroshahi et al., 2012).

Since higher concentrations of MuSK IgG4 and IgG1-3 clearly reduced the number of AChR clusters, this suggests a dose-dependent effect of the MuSK IgG subclasses on agrin-induced AChR clustering. However, when they were used at MuSK-Ab concentrations found in 1:10 dilutions of patient plasmas (figure 5.7), no significant reduction in agrin-induced AChR clustering was observed, although a trend for fewer AChR clusters in the presence of IgG4 was found. This was unexpected, since a 1:10 dilution of patient plasma had previously been found able to reduce agrin-induced AChR clustering significantly. It is possible that the calculated percent of IgG4 might have been inaccurate. The concentration of MuSK IgG4 and IgG1-3 used in the AChR clustering assay at 1:10 dilution (figure 5.7) had been calculated using a percentage of IgG4 derived from a preliminary IgG subclass profiling experiment (patient 9: 88.5%, patient 12: 83.8%, data not shown), which harboured lower percentages for IgG4 than later IgG subclass profiling experiments (patient 9: 91.7%, patient 12: 94.2 %, table 3.2). A reason for the variation in IgG4 percentage in the two different IgG subclass profiling experiments was the inaccurate measurement of low values in flow cytometry (discussed in Chapter 3).

Table 5.9: Effect of purified patient IgG4 and IgG1-3 on AChR cluster numbers in C2C12 myotubes

Sample	Spontaneous AChR clusters with max. sample concentration	Agrin induced AChR clusters with max. sample concentration	Spontaneous AChR clusters with 1:10 sample concentration	Agrin induced AChR clusters with 1:10 sample concentration	DOK7 induced AChR clusters with max. sample concentration cell batch 1	DOK7 induced AChR clusters with max. sample concentration cell batch 2
Patient 9 IgG4	3.53	5.71	0.92	15.87	10.66	-
Patient 9 IgG1-3	0.84	0.73	1.93	25.85	0.84	-
Patient 12 IgG4	2.90	11.77	0.86	14.22	-	11.80
Patient 12 IgG1-3	3.50	16.21	2.59	36.41	-	2.22
Pooled HC IgG	5.85	27.08	0.22	29.82	21.44	62.52

The higher concentration of patient 12 MuSK IgG4 may have been sufficient to give a significant reduction of agrin-induced AChR clustering. Nevertheless, the difference would be minimal for patient 9 IgG4 which would change from 88.5% to 91.70%.

IgG4 subclass antibodies are thought to occur at least partly in a functionally monovalent form by a process called Fab arm exchange (van der Neut Kolfshoten et al., 2007). Thus it was interesting to ask whether Fabs were able to cause the effects described for patients' whole IgG by generating Fab fragments from patient IgG. Fab fragments were sufficient to reduce agrin-induced AChR clustering in C2C12 myotubes (table 5.7). This suggests that divalent binding of MuSK IgG and the presence of the Fc were not required for pathogenicity *in vitro*. Similar results were found in a study that used Fab fragments generated from IgG from immunized animals (Mori et al., 2012b). Fab fragments were also capable of reducing DOK7-induced AChR clustering, but there was no significant reduction of complex AChR clusters. It may be that Fab fragments reduce AChR clusters in a different way than whole IgG, perhaps by an all-or-none response where Fab fragment-challenged AChR clusters become more efficiently and more quickly dispersed without leaving traces of smaller and less complex AChR cluster fragments behind. Interestingly, whereas IgG-induced AChR clusters were found with some IgG preparations, the Fabs did not induce, but even reduced, spontaneous AChR clustering (table 5.8). This suggests that divalent binding of whole IgG, or the presence of other plasma components, are required for induction of spontaneous AChR clustering, and that the MuSK Fabs interfered with signal transduction pathways responsible for the induction and maintenance of spontaneous AChR clusters.

It would be interesting to investigate the effect of Fabs derived from IgG1-3 and IgG4 on AChR clustering. The low yield of Fabs, that was largely caused by

non-specific binding to protein G sepharose, was a reason why the production of IgG4 and IgG1-3 specific Fabs was not undertaken. Protein G and protein A sepharose had been tested at different incubation times and temperatures, but it was not possible to prevent non-specific binding of Fabs to either protein G or A, resulting in a loss of approximately 63% of the Fabs during the production. In addition, purified IgG4 was very low in protein concentration, and purified IgG1-3 was very low in MuSK Abs. In future experiments, it would be desirable to optimize both the production of Fabs and of IgG4 and IgG1-3, to increase the yields and enable the production of IgG4 and IgG1-3 specific Fabs. Then, Fabs from IgG4 and IgG1-3 could be further investigated, for example to determine the relevance of divalent binding for MuSK IgG1-3 in their reduction of AChR clusters.

In all AChR cluster assays, healthy control plasma, Fabs or IgG were used at the same volumes as the highest volume of the corresponding patient samples, because purified IgG and Fabs were contained in Hartmann's buffer (compound sodium lactate). This buffer was considered as ideal for storage of IgG and Fabs, but had detrimental effects on cells when used at low dilutions in preliminary experiments (data not shown). In retrospect it may have been better to adjust healthy control samples to the same protein concentration in every experiment, followed by dialysis against medium to remove the buffer. Fortunately, the healthy control samples had similar, or higher, protein concentrations than the patient samples.

Furthermore, it was decided to use MuSK-MG Ab preparations, such as IgG, Fab and the IgG subclasses, at specific MuSK Ab concentrations, and not at specific protein concentrations. The protein concentration was not related to MuSK Ab concentration in purified IgG1-3 and IgG4 from MuSK-MG patients. MuSK IgG4, albeit markedly lower in protein concentration than IgG1-3, had higher MuSK Ab concentrations than IgG1-3 (table 5.2). In addition, the levels of MuSK specific Fabs

varied between patients, independent of the plasma MuSK antibody concentration, although protein concentrations were similar. These observations had determined the decision to conduct the experiments with different MuSK Ab species using the same MuSK Ab concentrations, and not the same overall protein concentrations.

Chapter 6: Investigating the effect of MuSK-MG autoantibodies on LRP4-MuSK interaction by co-immunoprecipitation

6.1 Introduction

As described in chapter 5, MuSK antibodies, and also monovalent Fabs, reduced AChR clustering in C2C12 myotubes. The pathogenic mechanism underlying this effect should therefore not rely on the function of the Fc region or on the occurrence of divalent binding, because the Fc region is absent in Fabs. LRP4 is crucial for neuromuscular junction development, and both LRP4 and MuSK knockout mice have a lack of neuromuscular junctions and die at birth (Weatherbee et al., 2006, DeChiara et al., 1996). During neuromuscular junction development, agrin binds to LRP4, which in turn binds and activates MuSK (Zhang et al., 2008, Kim et al., 2008). The binding sites for MuSK antibodies are mainly the Ig-like domains of MuSK (McConville et al., 2004) and the first Ig-like domain of MuSK is responsible for agrin-induced MuSK activation (Zhou et al., 1999, Stiegler et al., 2006), and is also the site where LRP4 binds to MuSK (Stiegler et al., 2006, Zhang et al., 2011).

One potential pathogenic mechanism in MuSK-MG is that MuSK antibodies might interfere with the interaction between LRP4 and MuSK. Only one study had explored this, and reported that LRP4-MuSK interaction was not disrupted by MuSK antibody binding. However that study used recombinant LRP4 and MuSK constructs in an ELISA-like approach with Maxisorp-plates and only showed data using antibodies from one patient (Ohno, 2012).

Therefore, to study interaction between MuSK and LRP4 under more physiological conditions, a co-immunoprecipitation experiment was established. Full length human MuSK and full length human LRP4 were expressed in HEK293 cells which were incubated with healthy or patient plasma or IgG preparations. MuSK was

then immunoprecipitated from the cell surface of the living cells, using the patient antibody or a commercial MuSK antibody. Immunoprecipitated MuSK and co-precipitated LRP4 levels were quantified by western blot. If patient IgG disrupted the interaction between LRP4 and MuSK, it would be expected that reduced levels of LRP4 would co-immunoprecipitate when compared with co-immunoprecipitation using a commercial MuSK antibody.

6.2 Results

6.2.1 Detection of MuSK and LRP4 expressed in HEK293 cells by western blot

To establish a co-immunoprecipitation experiment, it was first necessary to identify antibodies that could detect MuSK and LRP4 on western blots. The most convenient way was to fuse each protein with a different tag and to use antibodies against those tags. Fluorescent tags were selected, giving the added advantage that efficient transfection of HEK293 cells could be validated by fluorescence microscopy. Figure 6.1 shows fluorescent images of HEK293 cells co-transfected with plasmids expressing MuSK-mCherry and LRP4-EGFP (figure 2.2). Whole cell lysate (WCL) of cells transfected with MuSK-mCherry and/or LRP4-EGFP was subjected to SDS-PAGE followed by western blot to test the commercial antibodies against mCherry or EGFP (figure 6.2). An antibody against MuSK was also used to enable identification of the correct size for MuSK-mCherry. The commercial antibodies against MuSK and mCherry both reliably detected a band at a size of approximately 120kDa, which corresponds with the size of full length human MuSK (approximately 95kDa) conjugated to mCherry (approximately 28kDa). The band was only found in WCL from MuSK-mCherry transfected cells. Thus, the commercial anti-mCherry antibody was selected for detection of MuSK-mCherry in subsequent experiments.

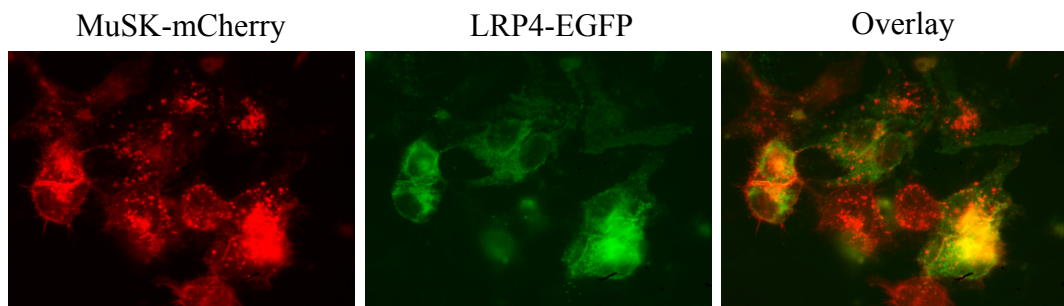


Figure 6.1: MuSK-mCherry and LRP4-EGFP were expressed in HEK293 cells. HEK293 cells were transfected with MuSK-mCherry and LRP4-EGFP. After 24 hours, cells were analysed by fluorescence microscopy for the expression of MuSK-mCherry (red) and LRP4-EGFP (green).

An additional band of approximately 55kDa was also detected with the mCherry antibody, but this was likely to be nonspecific binding of the secondary antibody, as it was also observed with long film exposures when the same secondary antibody was used with other primary antibodies (data not shown). The commercial antibody to EGFP detected a band of approximately 240kDa, which corresponds to LRP4-EGFP (210kDa) conjugated to EGFP (27kDa), in WCLs from LRP4-EGFP transfected cells.

6.2.2 MuSK was immunoprecipitated by commercial and patient derived antibodies

Next, several antibodies to MuSK were tested for their ability to bind to cell surface-expressed MuSK-mCherry. MuSK-mCherry-transfected HEK293 cells were incubated with 1:50 dilutions of three commercial antibodies from two different companies and one MuSK antibody (83033) developed by Steve Burden (Herbst et al., 2002), or with a 1:50 dilution of patient 2 plasma, which had been shown to bind to MuSK-mCherry in preliminary experiments, as a positive control. Binding of primary antibody was visualized using an appropriate green fluorescent-labelled secondary antibody (figure 6.3). Patient 2 plasma, an R&D systems antibody (AF562, goat anti-rMuSK) and a Santa Cruz Biotechnology, Inc antibody (N19, sc-6010) were found to bind specifically to MuSK-mCherry transfected cells, but not to mock-transfected cells. The H300 antibody by Santa Cruz Biotechnology, Inc (sc-33204) did not specifically bind to MuSK. The MuSK antibody (83033) developed by Steve Burden (Herbst et al., 2002) bound HEK293 cells non-specifically. The goat anti-rMuSK antibody from R&D systems (AF562) was selected for MuSK immunoprecipitation from MuSK-mCherry transfected HEK293 cells. The Sc-6010 antibody by Santa Cruz Biotechnology, Inc would also have been suitable.

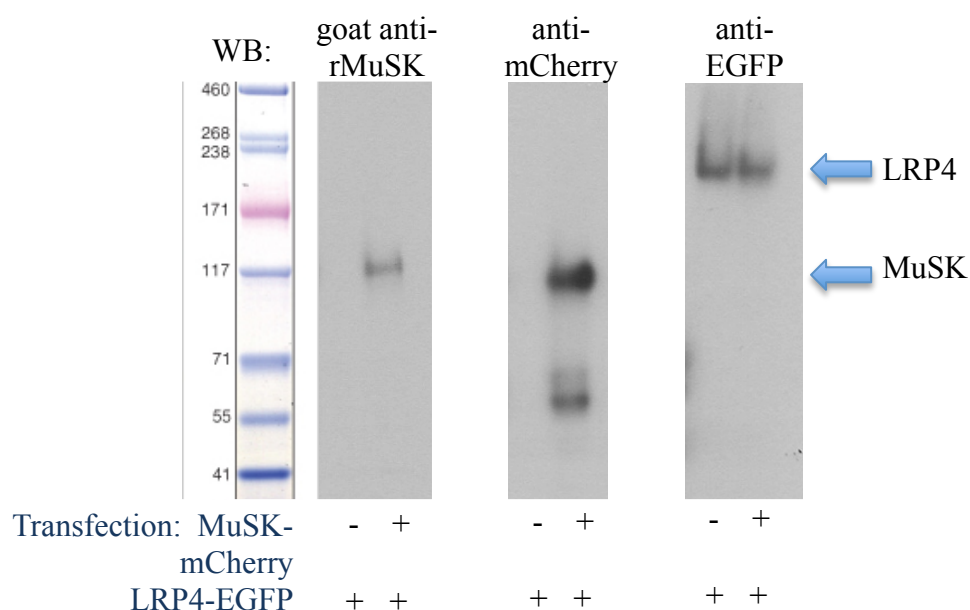


Figure 6.2: Test of different commercial antibodies in western blot. HEK293 cells were transfected with MuSK-mCherry and/or LRP4-EGFP. Whole cell lysates (WCL) were subjected to SDS PAGE and western blot using commercial antibodies against rMuSK (goat anti-rMuSK, R&D systems), mCherry (Biovision) or EGFP (Abcam). Bands corresponding to MuSK-mCherry (120kDa) or LRP4-EGFP (240kDa) could be detected exclusively in samples that were transfected with the corresponding plasmids.

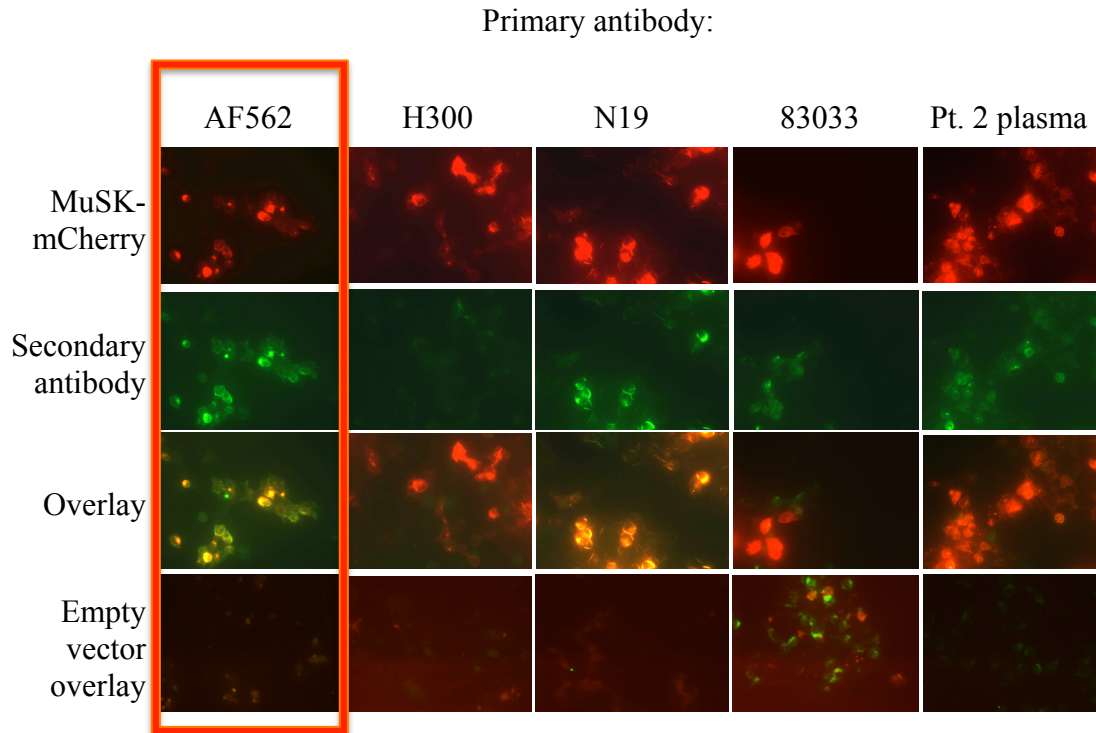


Figure 6.3: Test of commercial and patient derived MuSK antibodies for binding to MuSK-mCherry at the cell surface of living cells. HEK293 cells were transfected with MuSK-mCherry (red) or empty vector (mock). The cells were incubated with MuSK specific antibodies from R&D systems (AF562, also goat anti-rMuSK), Santa Cruz Biotechnology, Inc. (H300, N19) or from Steve Burden (83033), or patient 2 plasma at 1:50 dilution. Then the cells were incubated with Alexa Fluor 488 conjugated secondary antibody diluted 1:750 (green). Specific binding of antibody to MuSK could be determined by overlay of green and red fluorescence (overlay). The red box marks the antibody selected for further experiments (AF562) which is further on named “goat anti-rMuSK”.

It was then tested whether the goat anti-rMuSK antibody and patient plasma containing MuSK autoantibodies were able to immunoprecipitate MuSK from the cell surface of transfected HEK293 cells. HEK293 cells transfected with MuSK-mCherry and LRP4-EGFP or with LRP4-EGFP only were incubated with the goat anti-rMuSK antibody in the presence or absence of plasma from patient 2 or healthy control serum. Cells were lysed, and the lysate incubated with protein G conjugated magnetic beads. After washing, immunoprecipitated proteins were eluted from the beads by addition of reducing buffer and heating for either 10 min at 70°C or 5 min at 95°C, and subjected to SDS-PAGE and western blotting (figure 6.4). Bands of approximately 120kDa, corresponding to MuSK-mCherry, were detected in samples from MuSK-mCherry-transfected cells incubated with patient plasma or goat anti-rMuSK antibody, but not from cells that only expressed LRP4-EGFP. No bands were found when the cells were incubated with control serum or with no antibody.

In addition to the expected size of 120kDa, MuSK-MG patient plasma immunoprecipitated additional bands of approximately 170kDa, 200kDa and above 240kDa in size. The bands were not immunoprecipitated from cells that were transfected with LRP4-EGFP only. This suggests that the additional bands contain MuSK-mCherry and this was investigated further.

6.2.3 Additional bands immunoprecipitated by patient plasma could derive from antibody fragments bound to MuSK

The sizes of the additional bands mentioned above indicated that antibody fragments, such as heavy chain (approximately 50kDa) or half-molecules of IgG4 (approximately 75kDa), or whole antibodies (approximately 150kDa), might be

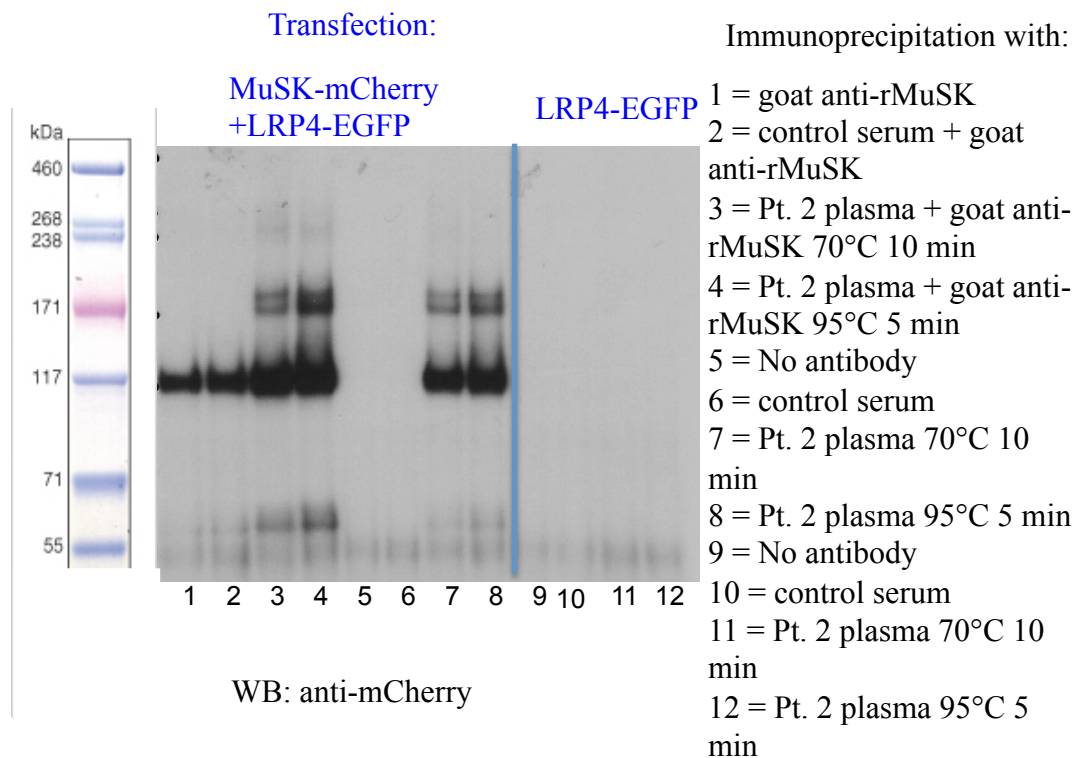


Figure 6.4: MuSK could be precipitated by commercial antibody and by MuSK-MG plasma. HEK293 cells transfected with MuSK-mCherry and LRP4-EGFP (1-8) or LRP4-EGFP only (9-12) were incubated with 1:12.5 goat anti-rMuSK (1) in the presence of 1:50 control serum (2) or patient 2 plasma (3,4). As a control, cells were incubated with no antibody (5, 9), control serum only (6, 10) or patient 2 plasma only (7, 8, 11, 12). Cells were then lysed and protein G coupled magnetic beads were used to precipitate antibody-bound protein. Precipitated protein was eluted at 70°C for 10 minutes (1, 2, 3, 5, 6, 7, 9, 10, 11) or at 95°C for 5 minutes (4, 8, 12) and subjected to SDS PAGE and western blot. Bands corresponding to MuSK-mCherry (120kDa) were detected using a mCherry specific antibody.

associated with MuSK-mCherry (120kDa). Such complexes would be consistent with the observed band shifts to 170kDa, 200kDa and above 240kDa. To investigate whether antibody fragments are present in these additional bands, MuSK-mCherry was immunoprecipitated using plasma from patient 4, and analysed by western blot using antibodies that recognise either mCherry to detect MuSK, or the Fab portion of human IgG (figure 6.5). As a control, MuSK-mCherry was immunoprecipitated with commercial goat anti-rMuSK antibody (R&D systems). The same additional bands were detected with both the anti-mCherry antibody and with the anti-human Fab antibody (see arrows), but only when the immunoprecipitation was carried out with patient plasma and not with the goat anti-rMuSK antibody. This suggests that the additional bands contained MuSK-mCherry bound to parts of human IgG. Bands of approximately 90 and of 115kDa were also found with the anti-Fab antibody and it is not clear where these derive from.

To further investigate the appearance of the additional bands at 170, 200 and >240kDa, plasmas from three patients (2, 4, 6) at three different concentrations (0.2nM, 1nM and 2nM) were used to precipitate MuSK-mCherry (figure 6.6). With increased MuSK antibody concentration, the density and number of additional bands detected with the mCherry antibody increased in all three patients. Additional bands appeared with increased concentrations, in the order 170/200kDa, then >240kDa.

If antibody fragments were bound to MuSK-mCherry after elution from protein G coupled magnetic beads, it was likely to have been a consequence of elution for 10 minutes at 70°C, which is a relatively short time and a relatively low temperature for elution. The presence of these additional bands made quantification difficult, and so in order to dissociate the antibody fragments from MuSK-mCherry, incubation temperature and time during the elution from the beads were increased. The additional bands disappeared with incubation of the immunoprecipitate for 30

minutes at 95°C. Unfortunately, no bands corresponding with LRP4 could be observed in the whole cell lysate after incubation for 30 minutes at 95°C (data not shown). This suggests that LRP4-EGFP is degraded under these conditions. Since subsequent experiments were to explore co-immunoprecipitation of LRP4-EGFP with MuSK-mCherry, an elution temperature of 70°C for 10 minutes had to be used.

Binding of antibody fragments to MuSK would be surprising, as most antibody-antigen reactions would not survive reducing SDS-PAGE, even though MuSK antibody affinity was found to be high with values in the picomolar range (figure 3.16). However, these bands could be detected on a western blot using an anti-Fab specific antibody and an anti-mCherry antibody, they disappeared with prolonged incubation at 95°C and their size corresponded with antibody fragments bound to MuSK. As it remained questionable what these additional bands contained, only the band for MuSK-mCherry at 120kDa was regarded in subsequent experiments.

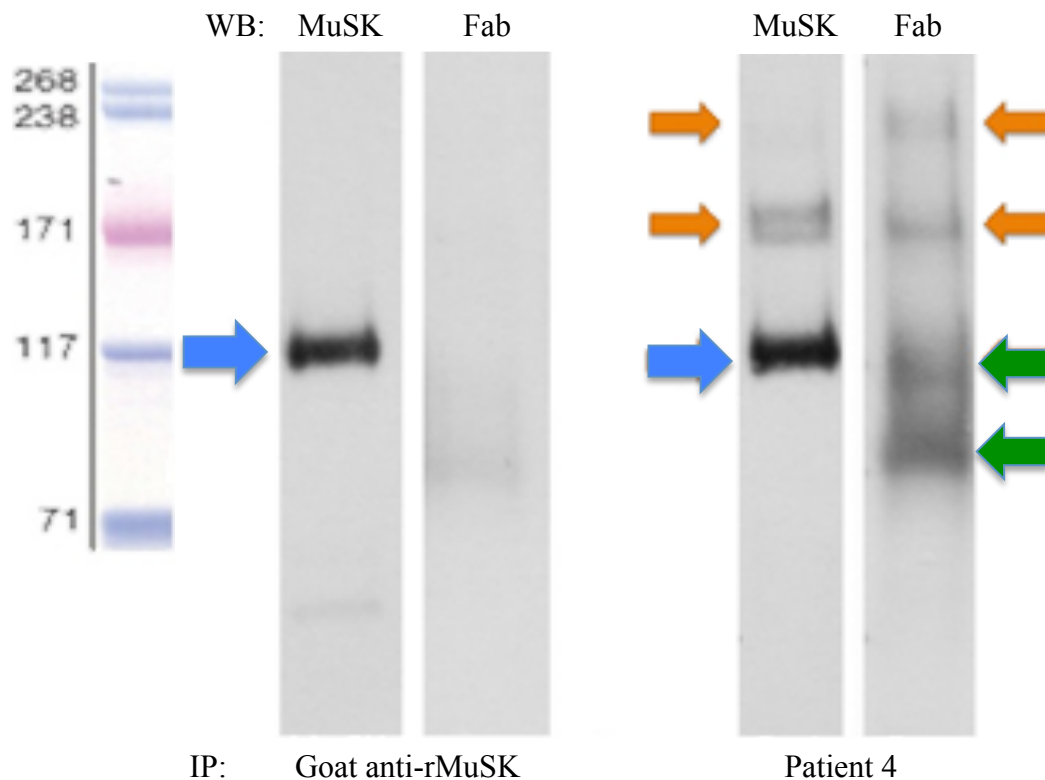
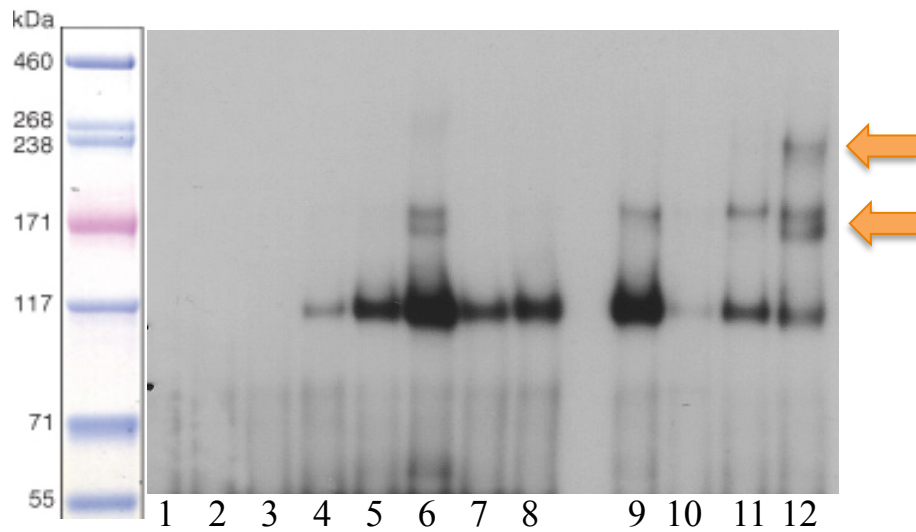


Figure 6.5: The pattern of additional bands for MuSK-mCherry in western blot could be reproduced using Fab-specific antibody. HEK293 cells transfected with MuSK-mCherry were subjected to co-immunoprecipitation with patient 4 plasma or commercial goat anti-rMuSK antibody. The immunoprecipitates were subjected to SDS-PAGE and western blot using antibodies to mCherry (MuSK) or Fab specific anti-human IgG (Fab). Bands corresponding to MuSK-mCherry (120kDa, blue arrow) and additional bands (approximately 200, 240kDa, orange arrows) were found in patient 4 immunoprecipitate both with mCherry (MuSK)- and Fab-specific commercial antibody. Fab-specific antibody also detected bands of approximately 90 and 115kDa (green arrows). Goat anti-rMuSK immunoprecipitate contained only one band corresponding to MuSK-mCherry (120kDa, blue arrow) that was detected by the mCherry (MuSK)-specific antibody, but not by the Fab-specific antibody.

WB: anti-mCherry



Immunoprecipitation with:

- | | |
|---------------------------|----------------------------|
| 1= control serum 1:2 | 7= Patient 4 plasma 0.2nM |
| 2= control serum 1:20 | 8= Patient 4 plasma 1nM |
| 3= control serum 1:200 | 9= Patient 4 plasma 2nM |
| 4= Patient 2 plasma 0.2nM | 10= Patient 6 plasma 0.2nM |
| 5= Patient 2 plasma 1nM | 11= Patient 6 plasma 1nM |
| 6= Patient 2 plasma 2nM | 12= Patient 6 plasma 2nM |

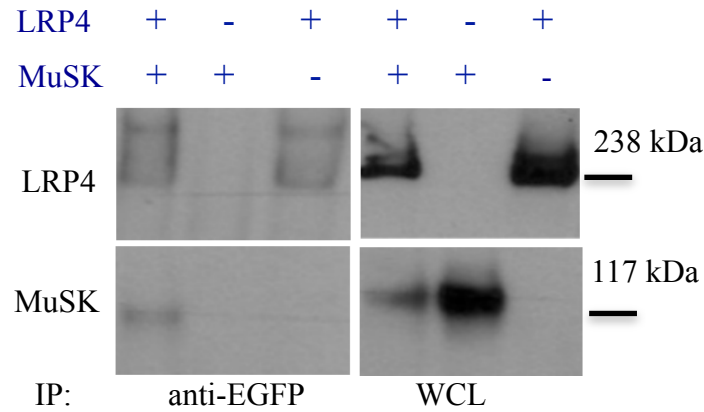
Figure 6.6: Additional bands for MuSK increased with MuSK antibody concentration. HEK293 cells expressing MuSK-mCherry were incubated with increasing concentrations of MuSK antibodies from patient 2 (4, 5, 6), patient 4 (7, 8, 9), patient 6 (10, 11, 12) at concentrations of 0.2nM (4, 7, 10), 1nM (5, 8, 11) or 2nM (6, 9, 12) or healthy control serum (1, 2, 3) at the same dilutions as the lowest dilution (1), a medium dilution (2) or the highest dilution (3). Antibody bound protein was precipitated with protein G sepharose. The western blot was incubated with an antibody to mCherry. Additional bands for MuSK-mCherry at 200 and 240kDa (orange arrows) appeared with higher concentrations of MuSK antibodies.

6.2.4 Co-immunoprecipitation of MuSK and LRP4 by commercial antibodies

It was then necessary to ask whether MuSK-mCherry and LRP4-EGFP expressed in HEK293 cells interacted with each other and could therefore be co-immunoprecipitated. Two separate co-immunoprecipitations were conducted for this purpose. First, LRP4-EGFP was immunoprecipitated with an EGFP-specific antibody (ab6556), to investigate whether MuSK-mCherry could be co-immunoprecipitated. As the EGFP tag of LRP4 was expressed intracellularly, the immunoprecipitation had to be conducted from the cell lysate. HEK293 cells expressing MuSK-mCherry and/or LRP4-EGFP were lysed, and the lysates were incubated with the EGFP-specific antibody (ab6556) followed by immunoprecipitation with protein G coupled magnetic beads. After washing, the immunoprecipitate was subjected to SDS-PAGE and western blotting with antibodies to mCherry and EGFP (ab6556) (figure 6.7A). MuSK-mCherry was found to co-immunoprecipitate when both LRP4-EGFP and MuSK-mCherry were expressed in the cells, but not from MuSK-mCherry only transfected lysates.

Secondly, to confirm these findings, the converse experiment was carried out. MuSK-mCherry was immunoprecipitated with goat anti-rMuSK. This antibody was raised against an extracellular epitope of rat MuSK, thus MuSK-mCherry could be immunoprecipitated from the cell surface, followed by western blotting to determine

A Transfection:



B

Transfection: MuSK+LRP4

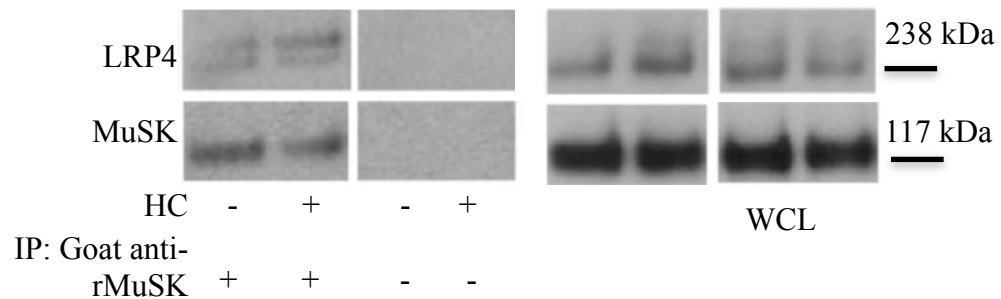


Figure 6.7: MuSK and LRP4 interacted and could be co-immunoprecipitated. HEK293 cells expressing MuSK-mCherry and/or LRP4-EGFP were used to study MuSK-LRP4 binding using co-immunoprecipitation. (A) An EGFP-specific antibody was able to co-immunoprecipitate LRP4-EGFP (detected with an EGFP-specific antibody) and MuSK-mCherry (detected with a mCherry-specific antibody) only from the whole cell lysate of cells that were transfected with both LRP4-EGFP and MuSK-mCherry. (B) Immunoprecipitation of MuSK-mCherry from the cell surface of transfected HEK2932 cells. Goat anti-rMuSK antibody could only co-immunoprecipitate MuSK-mCherry and LRP4-EGFP from the surface of cells transfected with both proteins. Healthy control serum (HC) did not influence this co-immunoprecipitation.

whether LRP4-EGFP was in the co-immunoprecipitate (figure 6.7B). It was found that LRP4-EGFP specifically co-immunoprecipitated with MuSK-mCherry when cells were transfected with both LRP4-EGFP and MuSK-mCherry, but not from singly-transfected cells. As additional controls, samples were incubated with or without healthy control serum, and MuSK-mCherry was immunoprecipitated from the cell surface with goat anti-rMuSK. The presence of healthy control serum did not impair the immunoprecipitation of MuSK-mCherry or co-immunoprecipitation of LRP4-EGFP from the cell surface. Hence MuSK-mCherry and LRP4-EGFP interact on the surface of transfected HEK cells and can be co-immunoprecipitated with the commercial antibodies.

6.2.5 MuSK-MG patient IgG inhibited LRP4-MuSK binding

To test whether the interaction between LRP4-EGFP and MuSK-mCherry was affected by MuSK-MG patient antibodies, MuSK-mCherry was immunoprecipitated from the cell surface of MuSK-mCherry and LRP4-EGFP transfected HEK293 cells with either goat anti-rMuSK antibody or with MuSK-MG patient plasma. Figure 6.8A shows an initial experiment. Similar levels of MuSK-mCherry were immunoprecipitated by goat anti-rMuSK (1) and plasma from patient 4 (2), but LRP4-EGFP was only co-immunoprecipitated by goat anti-rMuSK (1) and not by the patient derived antibody (2). This suggested that LRP4-EGFP was not bound to MuSK-mCherry when MuSK-MG patient antibody was bound to MuSK. As expected, no MuSK-mCherry was immunoprecipitated from LRP4-EGFP transfected cells, and only MuSK-mCherry, but not LRP4-EGFP, was immunoprecipitated from MuSK-mCherry transfected cells.

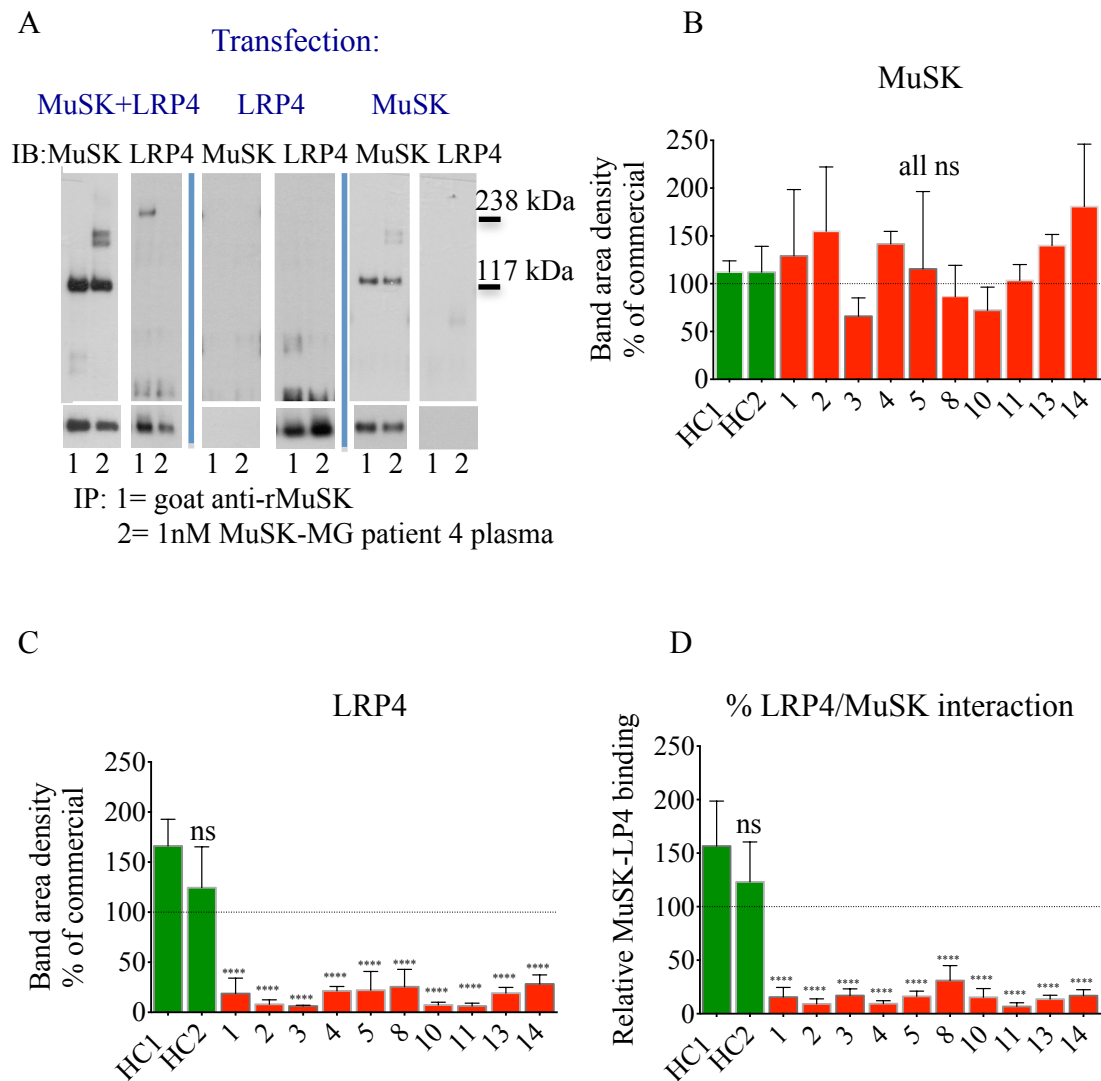


Figure 6.8: MuSK antibodies blocked binding between LRP4 and MuSK. (A) MuSK-MG patient plasma was able to immunoprecipitate MuSK-mCherry from the cell surface of transfected HEK293 cells, but prevented LRP4-EGFP from being co-immunoprecipitated. In the same experiments, goat anti-rMuSK antibody could co-immunoprecipitate LRP4-EGFP, but only in the presence of MuSK-mCherry. (B, C and D) The experimental set-up shown in (A) was carried out with plasma from 10 MuSK-MG patients, diluted to final MuSK antibody concentration of 1nM ($n \geq 3$). Control immunoprecipitations were carried out with goat anti-rMuSK in the presence of plasma from two healthy individuals (HC1 and HC2). All results were normalised to those obtained when the immunoprecipitation was carried out with only goat anti-rMuSK antibody (shown as a dotted line). (B) Relative area density of bands for MuSK-mCherry and (C) for LRP4-EGFP were used to calculate the relative LRP4/MuSK binding strength (D). Statistical analysis: one-way ANOVA with Bonferroni post-test, $p=0.8273$ (B), $p<0.0001$, (C), $p<0.0001$ (D). **** $p \leq 0.0001$

Preliminary experiments were then performed in order to determine the volumes of patient plasmas that would immunoprecipitate similar amounts of MuSK-mCherry when compared with goat anti-rMuSK antibody. This value was 1nM MuSK antibody final concentration for each patient plasma.

The experiment was conducted three to five times for each patient. Immunoprecipitation was also carried out using goat anti-rMuSK antibody in the absence or presence of healthy control serum. Area band densities of the MuSK-mCherry bands immunoprecipitated with either patient plasma or with goat anti-rMuSK antibody in the presence of healthy serum were measured. These were normalised to the area density of the band immunoprecipitated using goat anti-rMuSK with no human plasma or serum, which is represented by a dashed line at 100% in figure 6.8B. Similarly, area band density of LRP4-EGFP co-immunoprecipitated with patient plasma or with goat anti-rMuSK antibody and healthy control serum was normalised to area band density of LRP4-EGFP immunoprecipitated by goat anti-rMuSK antibody, also represented by a dashed line at 100% (figure 6.8C). The normalisation was intended to overcome variability in the precipitations. Patient plasma (red columns), or commercial antibody in the presence of healthy control serum (green columns), immunoprecipitated similar levels of MuSK-mCherry (figure 6.8B), with some variation that did not reach significance. Levels of co-immunoprecipitated LRP4-EGFP were considerably reduced in all patient samples (figure 6.8C), compared to healthy control 1 sample (HC1, one-way ANOVA, Bonferroni post-test). The same result was found when the analysis was conducted by comparison of all samples to healthy control 2 sample (HC2).

To overcome the variation in immunoprecipitation in each experiment, and to gain a measure for relative LRP4-MuSK interaction, the ratio between the area density of LRP4-EGFP and of MuSK-mCherry was calculated and multiplied by 100

for each experiment and the results were pooled (figure 6.8D). The dashed line represents the relative LRP4-MuSK interaction in cells incubated only with goat-anti-rMuSK antibody. While healthy control serum incubated samples had a relative LRP4-MuSK binding of above 100, all patient samples had significantly reduced relative LRP4-MuSK binding compared to healthy control 1 sample (one-way ANOVA, Bonferroni post-test). The same result was found when all samples were compared to healthy control 2 sample. This suggests that all investigated patient plasmas interfered with LRP4-MuSK interaction.

To gain more insight in the antibody mediated block in LRP4-MuSK binding, the influence of patient antibodies on LRP4-MuSK interaction over time was investigated. A time course experiment was devised, in which HEK293 cells expressing MuSK-mCherry and LRP4-EGFP were incubated with goat anti-rMuSK antibody or plasma from patient 4 for 1, 2 or 4 hours (figure 6.9). With longer incubation time, the amount of immunoprecipitated MuSK-mCherry increased markedly in commercial antibody incubated samples, and to a lesser degree in patient incubated samples. The amount of co-immunoprecipitated LRP4-EGFP increased in the commercial antibody incubated samples, but not in the patient incubated samples. This suggests that the block of LRP4-MuSK interaction by the patient antibody did not change over time.

It was also important to look at overall protein expression levels, because it was possible that patient antibody binding to MuSK may alter protein expression. Changes in protein expression as a result of MuSK-MG patient serum had been observed previously *in vitro* (Benveniste et al., 2005, Boneva et al., 2006). To see whether overall protein expression changed when MuSK-mCherry and LRP4-EGFP transfected HEK293 cells were incubated with patient plasma or healthy control

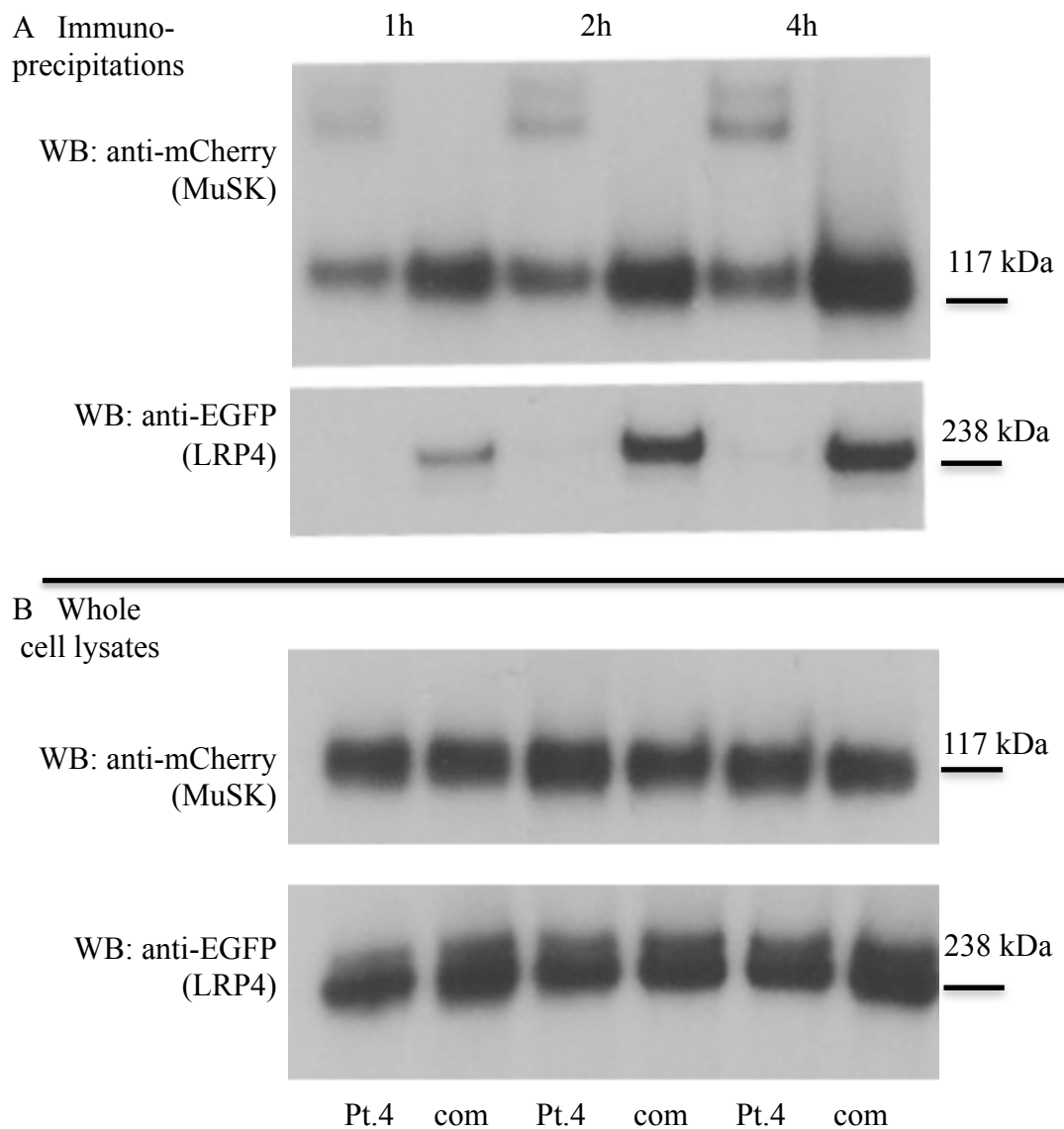


Figure 6.9: Time course of MuSK and LRP4 co-immunoprecipitation. MuSK-mCherry and LRP4-EGFP transfected HEK293 cells were incubated with patient 4 plasma (Pt.4) or commercial goat anti-rMuSK antibody (com) for 1, 2 or 4 hours. MuSK was then immunoprecipitated as described and the immunoprecipitate (A) as well as the whole cell lysate (B) subjected to SDS-PAGE and western blot with antibodies against EGFP or mCherry. Over time, the amount of immunoprecipitated MuSK and LRP4 increased in the immunoprecipitate of commercial goat anti-rMuSK antibody, while band sizes for WCL remained similar. Patient 4 immunoprecipitate did not show an increase in LRP4 over time.

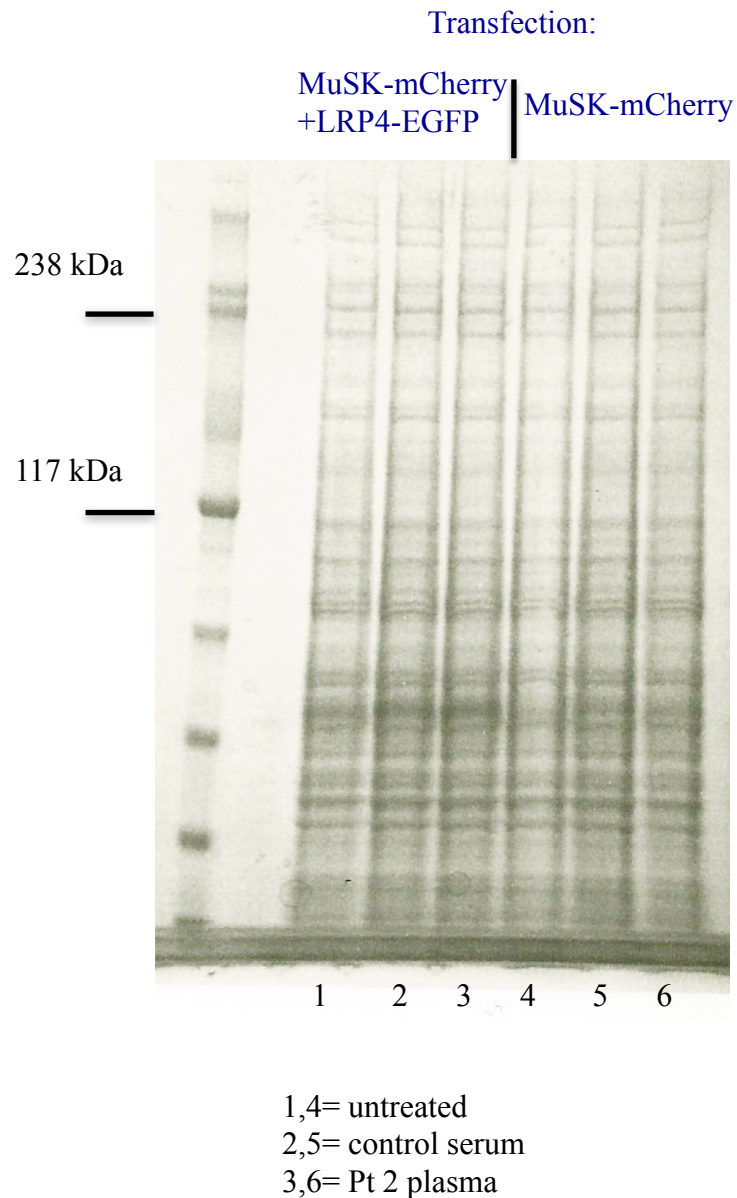


Figure 6.10: No differential protein expression in the presence of MuSK-MG patient plasma could be detected using silver stain. HEK293 cells transfected with MuSK-mCherry and LRP4-EGFP (1-3) or MuSK-mCherry only (4-6) were incubated with medium only (1, 4), 1:50 dilution of healthy control serum (2, 5) or patient 2 plasma (3 ,6) for 60 minutes. Cells were then lysed and subjected to SDS-PAGE followed by silver stain of total protein in the gel. No difference in band pattern could be observed.

serum, cells were lysed and subjected to SDS-PAGE and silver stain (figure 6.10). No changes in the appearance of the bands were observed, suggesting that if patient plasma changed protein expression levels, these changes had to be subtle and were not detectable by silver stain.

6.2.6 Patient derived IgG4 inhibited LRP4-MuSK binding

To assess whether the observed block in LRP4-MuSK interaction was due to MuSK IgG of a specific subclass, the co-immunoprecipitation was performed with purified IgG4 and IgG1-3 from two patients, or as a control with commercial goat anti-rMuSK antibody (figure 6.11A). IgG4, but not IgG1-3 subclass antibodies, co-precipitated reduced levels of LRP4. There was considerable variation in band area density between the immunoprecipitation experiments, and therefore the experiment was conducted five times. Area band densities were quantified and relative LRP4-MuSK binding was calculated (figure 6.11B). The relative binding between LRP4-EGFP and MuSK-mCherry was significantly reduced in IgG4 incubated samples, but not in IgG1-3 incubated samples (one-way ANOVA, Bonferroni post-test). Taken together, this suggests that only the MuSK specific IgG4 subclass interfered with the LRP4-MuSK interaction. Furthermore, the additional bands for MuSK, observed at approximately 200kDa, were only observed in samples that were incubated with IgG4 antibodies.

As a control, the co-immunoprecipitation was conducted with commercial goat anti-rMuSK antibody in the presence of healthy control IgG (figure 6.13). Healthy control IgG were used at the same volume as the highest concentration of patient IgG subclass that was necessary to obtain a concentration of 0.5nM MuSK IgG. Protein concentrations of patient IgG4 were considerably lower than those of

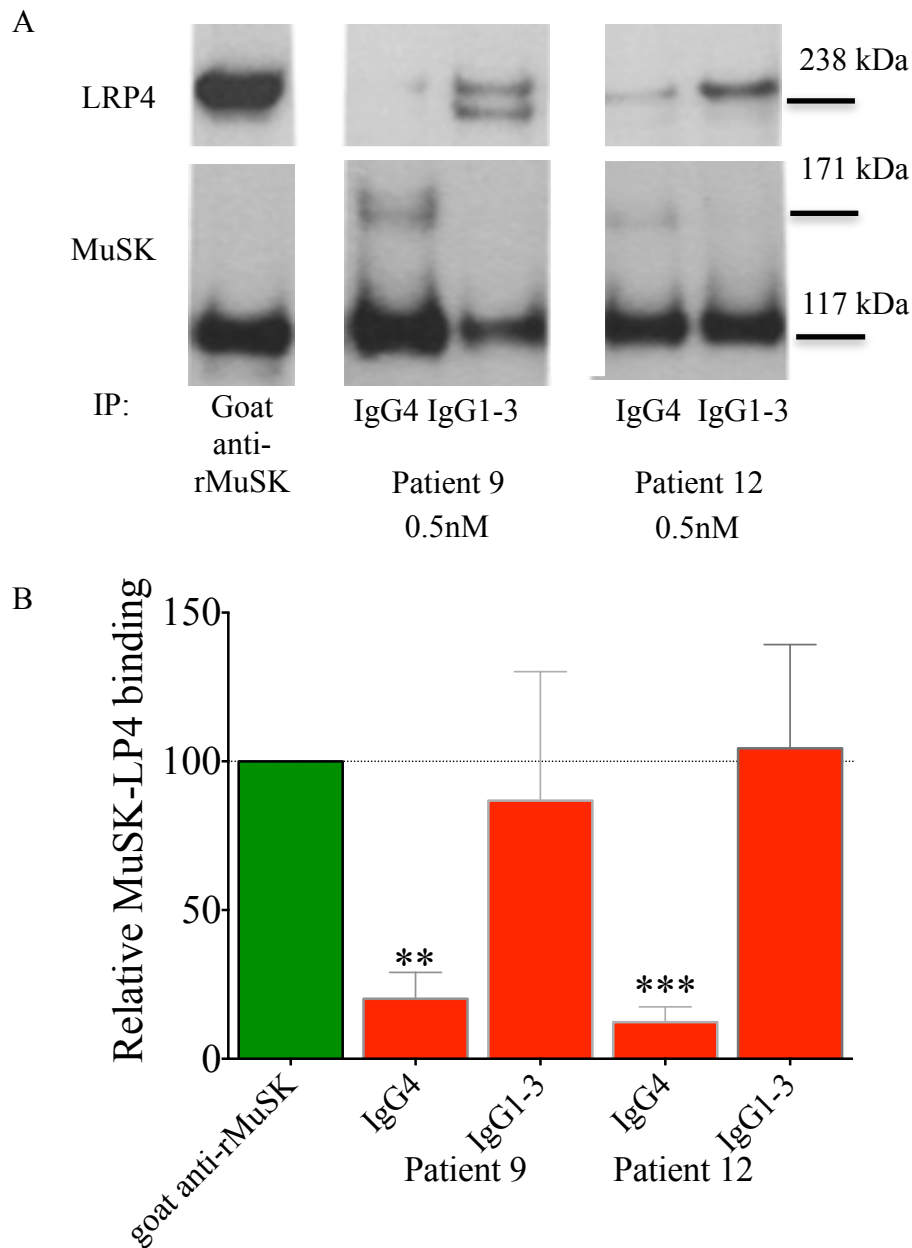


Figure 6.11: MuSK IgG4 exclusively blocked LRP4-MuSK interaction. Purified IgG4 or IgG1-3 from patients 9 and 12 diluted to MuSK antibody concentration of 0.5nM, or goat anti-rMuSK, were used to co-immunoprecipitate MuSK-mCherry and LRP4-EGFP from the cell surface of transfected HEK293 cells. (A) Example western blots probed with antibodies against MuSK-mCherry or LRP4-EGFP as indicated. (B) Quantification of relative MuSK-LRP4 binding strength, which was calculated the same way as for Figure 6.8D. Statistical analysis with one way ANOVA ($p=0.0952$) followed by Bonferroni post test, $n=5$, comparing data from patient samples with data using goat anti-rMuSK antibody for co-IP. ** $p \leq 0.01$, *** $p \leq 0.001$.

IgG1-3. Protein concentrations of healthy control IgG was slightly higher than protein concentrations of IgG1-3 and thus notably higher than that of patient IgG4 (table 6.1). Healthy control IgG did not reduce LRP4-MuSK binding.

Table 6.1: Protein concentrations of IgG subclass preparations used in co-immunoprecipitation

Sample	Protein concentration (mg/ml)
Patient 9 IgG4	0.41
Patient 9 IgG1-3	2.59
Patient 12 IgG4	0.26
Patient 12 IgG1-3	2.08
HC 1 IgG	4.48
HC 2 IgG	3.23

6.2.7 Fab fragments were sufficient to block LRP4-MuSK interaction

To investigate the relevance of the Fc region and of divalent binding for the block of LRP4-MuSK interaction, the co-immunoprecipitation experiment was conducted using patient derived Fab fragments and Fab-specific anti-human IgG (figure 6.12A). MuSK-mCherry was efficiently immunoprecipitated by the Fab fragments, but LRP4-EGFP was only co-precipitated at very low levels. Data from three experiments were pooled (figure 6.12B) and a significant reduction in relative LRP4-MuSK binding was found (one-way ANOVA, Bonferroni post-test). This suggests that the Fc region and divalent binding were not required for the block of LRP4-MuSK interaction.

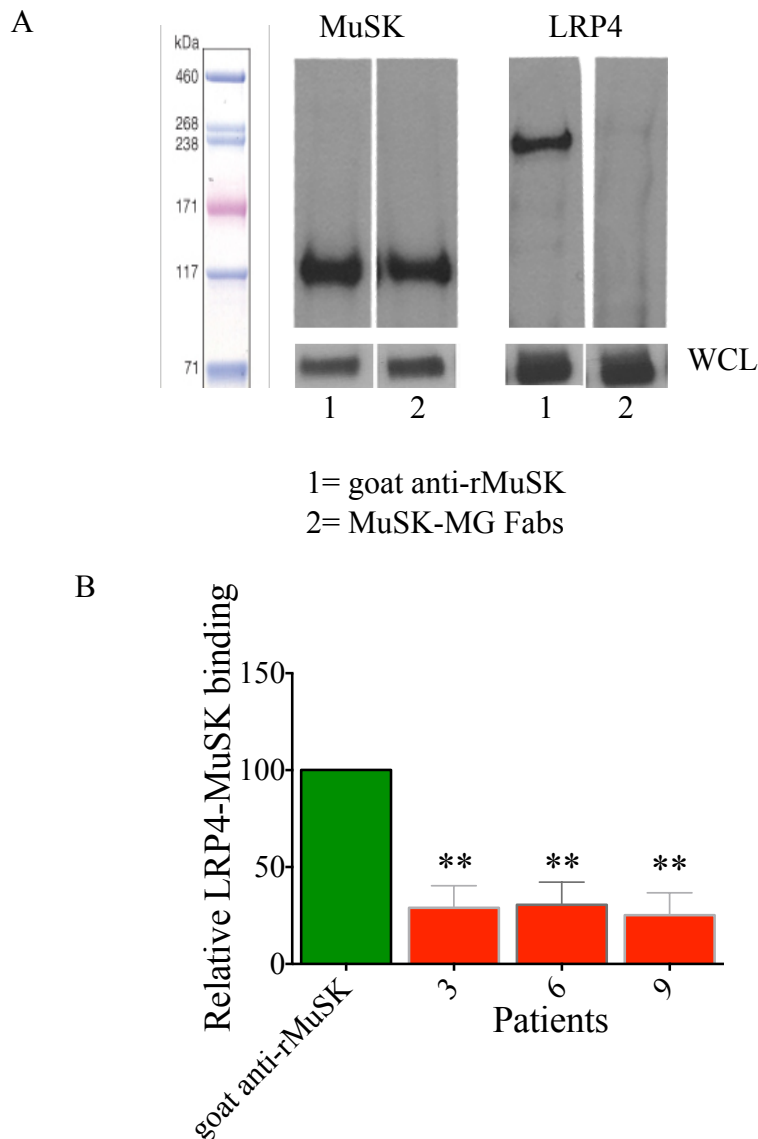
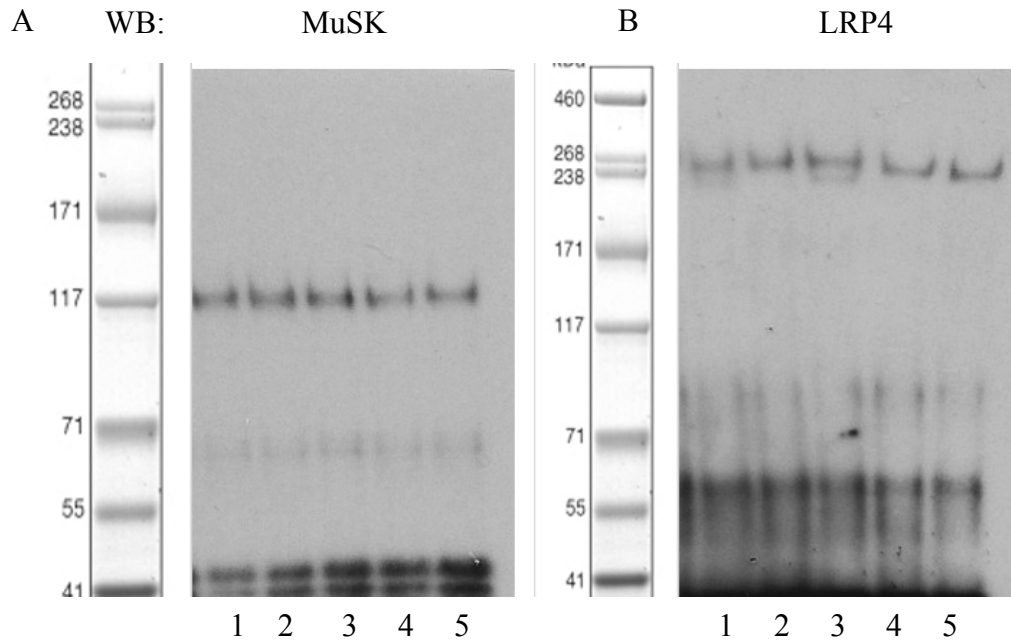


Figure 6.12: Fab fragments of MuSK-MG patient IgG were sufficient to block LRP4-MuSK interaction. (A) 0.25nM MuSK specific Fab fragments from patients 3, 6 or 9 as well as a Fab-specific secondary antibody or goat anti-rMuSK antibody were used for co-immunoprecipitation. The precipitate and the WCL were subjected to SDS-PAGE and western blotting, which were probed with mCherry- specific antibody (MuSK) or EGFP-specific antibody (LRP4). (B) The relative MuSK-LRP4 binding strength was calculated as in figure 6.8D. Statistical analysis: one way ANOVA ($p=0.0021$) followed by Bonferroni post test, $n=3$, comparing each MuSK-MG patient column (red) to the commercial antibody column (green). ** $p\leq 0.01$

As a further control, the co-immunoprecipitation was conducted with commercial goat anti-rMuSK antibody in the presence of healthy control Fabs (figure 6.13). Healthy control Fabs were used at the same volume as the highest volume of patient Fabs that was necessary to obtain a concentration of 0.25nM MuSK Fabs. The protein concentrations of healthy control and patient Fabs was very similar (table 6.2). Healthy control Fab fragments did not reduce LRP4-MuSK binding.

Table 6.2: Protein concentrations of Fab preparations used in co-immunoprecipitation

Sample	Protein concentration (mg/ml)
Patient 3 Fabs	0.53
Patient 6 Fabs	0.28
Patient 9 Fabs	0.55
HC 1 Fabs	0.50
HC 2 Fabs	0.34



IP with commercial antibody against MuSK in the presence of:

- 1= HC 1 IgG
- 2= HC 2 IgG
- 3= untreated
- 4= HC 1 Fab fragments
- 5= HC 2 Fab fragments

Figure 6.13: Healthy control IgG and Fab fragments did not interfere with LRP4-MuSK binding. MuSK-mCherry was immunoprecipitated from the cell surface of HEK293 cells transfected with MuSK-mCherry and LRP4-EGFP using goat anti-rMuSK antibody alone (3), or in the presence of healthy control IgG (1, 2) or healthy control Fab fragments (4, 5) at volumes equal to those of patient samples used in experiments. (A) Western blot using an anti-mCherry antibody to detect MuSK-mCherry, and (B) western blot with anti-EGFP antibody to detect LRP4-EGFP. Similar amounts of MuSK-mCherry and LRP4-EGFP were precipitated in all samples.

6.3 Discussion

One possible mechanism of MuSK antibodies is the interference with LRP4-MuSK binding. Using co-immunoprecipitation, the interaction between LRP4 and MuSK was found to be inhibited by whole plasma, IgG4 and monovalent Fab fragments from MuSK-MG patients. On the other hand IgG1-3 subclasses from the same patients did not interfere with LRP4-MuSK interaction.

Setting up the co-immunoprecipitation experiments was technically challenging for several reasons. The first obstacle was the identification of an antibody that could detect MuSK-mCherry in a western blot. In addition to the antibodies shown in figure 6.2, further antibodies had been tested prior to this experiment, but were unable to detect MuSK-mCherry. Among these were commercial antibodies against MuSK (Santa Cruz Biotechnology, Inc H300, Abcam ab5510), as well as a MuSK-specific antibody that was custom made for the laboratory of Professor David Beeson (antibody 131; using the same MuSK epitope as the 83033 antibody used by Steve Burden, not to be confused with the actual 83033 antibody, which was kindly provided by Steve Burden and used for the cell based assay shown in figure 6.3). Patient 2 plasma did not detect MuSK on western blot, which suggests that the antibodies from this patient do not recognize linear epitopes.

After establishing conditions and reagents for the co-immunoprecipitation experiment, previous reports that LRP4 and MuSK interacted (Kim et al., 2008, Zhang et al., 2008) could be confirmed by co-immunoprecipitation of LRP4-EGFP via MuSK-mCherry and vice versa from transfected HEK293 cells. It is important to note that MuSK-mCherry was immunoprecipitated from the cell surface of the intact, live cells in all experiments, and thus only interactions between cell surface expressed proteins were investigated in the experiments with patient or healthy

control derived antibodies. Only one control experiment precipitated LRP4-EGFP itself, which required immunoprecipitation from the lysate due to the intracellular localisation of the EGFP tag

A second challenge was the presence of additional bands in western blot that migrate at higher molecular weights than MuSK-mCherry in immunoprecipitations with patient plasma and IgG4. As IgG4 subclass antibodies are able to split into half-molecules (King et al., 1992, van der Neut Kolfshoten et al., 2007, Margni and Binaghi, 1988, van der Zee et al., 1986), it was possible that the additional bands may derive from IgG4 half-molecules that were bound to MuSK-mCherry. This would be surprising, as most antibody-antigen reactions do not survive reducing SDS-PAGE, though MuSK antibody affinity is particularly high with values in the picomolar range (figure 3.16, (McConville et al., 2004)). Nevertheless, since these bands could be detected on a western blot using an anti-Fab specific antibody and an anti-mCherry antibody, it seemed likely that they correspond with antibody fragments bound to MuSK. Although these additional bands were interesting, they also made quantification rather difficult. The denaturing conditions (70°C for 10 minutes) used in western blotting for this immunoprecipitation may not be harsh enough to dissociate MuSK from bound patient antibodies, and this is supported by the fact that the bands disappeared when harsher conditions of 95°C for 30 minutes were used. However, LRP4-EGFP also seemed to be destroyed under these conditions, and so for quantification the less harsh denaturing conditions of 70°C for 10 minutes had to be used. It is unclear why the anti-Fab antibody detected bands of approximately 90 and of 115kDa (figure 6.5). A possibility is that the patient antibody immunoprecipitated an unknown protein of this size that may have cross-reacted with the secondary antibody. Alternatively, some of the antibody fragments might have detached from the MuSK-antibody complexes during denaturation, and

appeared as these bands. Nevertheless, the molecular weights of 90 and 115 kDa do not correspond to any component of antibody, though they may for unknown reasons migrate with different molecular weights.

In order to confirm the presence of MuSK-mCherry and IgG fragments in these additional bands, preliminary experiments using mass spectrometry were conducted, but the protein content in the bands was very low, and no protein could be detected even though protein was immunoprecipitated from two T175 culture flasks containing transfected HEK293 cells (results not shown).

It is important to note that it was difficult to set up control immunoprecipitations using healthy control antibodies, because healthy control antibodies do not bind MuSK. Thus a commercial antibody against MuSK had to be included in the healthy control serum to enable immunoprecipitation of the protein. This means that the total amount of IgG (human and commercial) was increased compared to the patient samples that only contained patient antibody and no commercial antibody, and thus the experimental conditions were not identical. However, even with this increase in total IgG for the controls, it was found that the healthy control IgG (and Fabs) did not affect the interaction between LRP4 and MuSK in the co-immunoprecipitation experiment, and therefore were considered to be valid controls.

The experimental setup of the co-immunoprecipitation with the Fabs made it difficult to find appropriate control conditions. The immunoprecipitation with patient derived Fabs, in contrast to immunoprecipitation with whole human IgG or goat anti-rMuSK, required the use of a Fab specific anti-human IgG antibody, because Fabs do not contain a Fc region that could bind to protein G sepharose. Since a secondary antibody was used for the Fabs, the same secondary antibody should also be used with the goat anti-rMuSK antibody to have the conditions as similar as possible.

However, since the commercial antibody was produced in goat, it would not be recognized by a human specific secondary antibody. Thus it would not be an appropriate control. Therefore, it cannot be excluded that the Fab specific anti-human IgG antibody affected LRP4-MuSK interaction, for example by steric hindrance. In retrospect, an anti-goat secondary antibody could have been included

Table 6.3: Summary of chapter 4: LRP4-MuSK co-immunoprecipitation.

Patient	MuSK Ab conc. (nM)	[§]LRP4/MuSK Plasma (%)	[§]LRP4/MuSK Fabs (%)	[§]LRP4/MuSK IgG4 (%)	[§]LRP4/MuSK IgG1-3 (%)
1	37.57	15.52	-	-	-
2	32.46	9.19	-	-	-
3	25.65	16.99	28.95		
4	19.37	9.42	-	-	-
5	18.87	16.23	-	-	-
6	14.48	-	30.54	-	-
7	14.70	-	-	-	-
8	10.72	31.01	-	-	-
9	10.51		25.19	20.2	86.79
10	10.47	15.37	-	-	-
11	7.10	6.77	-	-	-
12	6.48	-	-	12.33	104.40
13	4.02	13.59	-	-	-
14	1.03	16.78	-	-	-
goat anti- rMuSK	-	*139.80	100.00	100.00	-

*=in the presence of healthy control plasma.

[§]=LRP4/MuSK= relative LRP4-MuSK binding.

after all as a control for steric hindrance, though it would not be the same secondary antibody as in the patient samples. Thus, as a control, goat anti-rMuSK in the presence of healthy control Fabs was used.

Despite the technically challenging experimental setup, it was clear that MuSK-MG patient antibodies interfered with MuSK and LRP4 interactions (table 6.3). Furthermore, MuSK IgG4 subclass exclusively reduced the binding between LRP4-EGFP and MuSK, which is interesting as it is the main IgG subclass found in MuSK-MG patients as shown here and elsewhere (McConville et al., 2004). This suggests that the block of LRP4-MuSK interaction by IgG4 may be a major pathogenic mechanism, but contrasts with the effects of both IgG4 and IgG1-3 shown on AChR clustering (Chapter 5), suggesting a different mechanism for MuSK IgG1-3. In addition, MuSK-MG patient derived Fabs were sufficient to block binding between MuSK-mCherry and LRP4, which suggests that the Fc region is not required for the observed block. It also demonstrates that monovalent binding, a suggested feature of the IgG4 subclass (King et al., 1992, van der Neut Kolfshoten et al., 2007, Margni and Binaghi, 1988, van der Zee et al., 1986), is sufficient to impair LRP4-MuSK interaction.

Interestingly, the amount of MuSK-mCherry that immunoprecipitated with goat anti-rMuSK increased over time, in contrast to the patient antibody immunoprecipitate that only showed a modest increase. It may be that newly synthesized MuSK-mCherry was inserted in the cell membrane over time, and thus became bound by goat anti-rMuSK antibody. This would suggest a reduced protein expression or transport of MuSK-mCherry in the presence of patient antibodies. Alternatively, perhaps only the goat anti-rMuSK antibody was present in excess and was able to bind freshly synthesized MuSK, while the patient antibodies may have been saturated at the beginning of the experiment.

It would be important to explore the block of LRP4-MuSK interaction in further experiments under more physiological conditions, for example by immunoprecipitating endogenous MuSK from C2C12 myotubes and assessing the amount of co-precipitated endogenous LRP4. To study the LRP4-MuSK interaction *in vivo* in the presence of MuSK autoantibodies, patient IgG could be passively transferred into mice. The interaction between LRP4 and MuSK could then be analysed in muscles by studying the colocalisation with immunohistochemistry, and by immunoprecipitation of MuSK and co-immunoprecipitation of LRP4 from the tissue.

Chapter 7: Discussion

7.1 Introduction

The aim of the thesis was to examine the mechanisms of MuSK antibodies. As with all antibody-mediated diseases, the mechanisms can include complement-dependent lysis of the target tissue, endocytosis of the target antigen, or inhibition of specific function of the antigen. In the case of MuSK antibodies, complement-dependent mechanisms are thought to be unlikely, as the majority of antibodies, and the apparently most pathogenic components are IgG4 antibodies, which do not activate complement. Therefore, experiments were established to look at MuSK endocytosis, the interaction between LRP4 and MuSK, and the effects of the antibodies on agrin-induced (and agrin-independent, DOK-7 stimulated) AChR clustering in C2C12 myotubes. To confirm others' experiments, the IgG of patients were separated into IgG4 and IgG1-3 subclass fractions, and to see whether divalent antibody binding was important for the effects, MuSK-Ab Fab fragments were also prepared.

No evidence for MuSK endocytosis was found, but patient IgG and Fabs disrupted the binding between LRP4 and MuSK. Although both purified IgG4 and IgG4-depleted IgG1-3 subclass antibodies from patients bound with high affinity to MuSK, only IgG4 subclass antibodies blocked LRP4-MuSK binding. By contrast, and unexpectedly, both IgG4 subclass and IgG1-3 subclass reduced agrin- and DOK7-induced AChR clustering in C2C12 myotubes.

7.2 The pathogenic mechanisms of MuSK IgG4

The characteristics of IgG4 suggest that the effects of MuSK antibodies will not involve endocytosis, complement deposition or cellular cytotoxicity and are more likely to involve direct effects on MuSK function. And indeed, MuSK antibodies

have been described to reduce agrin-induced AChR clustering in C2C12 mouse myotubes (Hoch et al., 2001, Shigemoto et al., 2006, Farrugia et al., 2007), which is a model that can be used to study MuSK function in the agrin-LRP4-MuSK signalling pathway.

Here, as expected, no evidence for endocytosis by MuSK IgG4 was found. Instead, antibodies from MuSK-MG patients were found to disrupt the LRP4-MuSK interaction *in vitro*. LRP4 is the agrin co-receptor (Kim et al., 2008, Zhang et al., 2008) that stimulates close binding to the first Ig-like domain of MuSK (Zhang et al., 2011), an interaction that is important for the development and maintenance of dense AChR clustering at the neuromuscular junction. Therefore, a block of LRP4-MuSK interaction would be expected to lead to reduced AChR clustering as has been described in C2C12 myotubes (Hoch et al., 2001, Shigemoto et al., 2006, Farrugia et al., 2007). The disruption of LRP4-MuSK interaction may derive either from direct block of the LRP4 binding site or indirectly by changing the conformation of MuSK.

One other study investigating LRP4-MuSK interaction in the presence of MuSK-MG antibodies has been reported, but no reduction in binding between LRP4 and MuSK was found (Ohno, 2012). However, the study by Ohno et al. used solid-phase assay and recombinant proteins, which do not provide physiological conditions. In our study, cell surface expressed full length human proteins were used, which provided a more relevant model for the neuromuscular junction. A very recent report (Huijbers et al., 2013), published after our own study (Koneczny et al., 2013), showed that experiments using Maxisorp plates and an ELISA like approach were able to detect a reduction of LRP4-MuSK interaction by patient antibodies, and supported our findings. Although our experiments provide a physiological environment, with proteins expressed on the surface of eukaryotic cells it would be interesting to explore the LRP4-MuSK interaction in the presence of MuSK

antibodies in a more tissue-specific context, for example by co-immunoprecipitation of LRP4 and MuSK from C2C12 myotubes. In addition, the relevance of LRP4-MuSK interaction *in vivo* could be explored by passive transfer of patient antibodies to mice, followed by co-immunoprecipitation of LRP4 and MuSK from mouse muscle.

There are several findings that support the notion that IgG4 block of LRP4-MuSK binding is indeed a major pathogenic mechanism in MuSK-MG. MuSK autoantibodies are predominantly of the IgG4 subclass (McConville et al., 2004), which is the least common IgG subclass (Aucouturier et al., 1984), and IgG4 subclass antibodies correlate with disease severity in individual patients (Niks et al., 2008a). IgG4 subclass antibodies were pathogenic in extensive electrophysiological studies with patient IgG4 and IgG1-3 injected mice (Klooster et al., 2012). Since IgG4 subclass antibodies, and not the IgG1-3 antibodies, blocked binding between LRP4 and MuSK (see also table 7.1), it is likely that this mechanism is very important for pathogenicity. However, some of our findings suggested that this is not the whole picture (see below).

The IgG4 subclass is thought to occur at least partly in a functionally monovalent form (van der Zee et al., 1986, Marni and Binaghi, 1988, van der Neut Kolfschoten et al., 2007). Here, monovalent binding of patient derived Fab fragments was sufficient to block binding between LRP4 and MuSK, as well as to reduce both agrin- and DOK7-induced AChR clustering. Hence monovalent Fab fragments were shown to be pathogenic *in vitro*. A similar observation had been made previously, when Fab fragments, that were derived from an animal model of MuSK-MG, were found to reduce agrin-induced AChR clustering (Mori et al., 2012b). Taken together,

Table 7.1: Summarised results of purified IgG4 and IgG1-3 subclass antibodies

	IgG4		IgG1-3	
Patient	9	12	9	12
MuSK Ab conc.(nM)	6.47	5.77	1.5	0.51
Protein conc. (mg/ml)	0.41	0.26	2.59	2.08
MuSK Ab conc.(nmol/g)	15.78	22.19	0.57	0.24
Kd (pM)	15.31	11.56	2.79	20.27
IgG4 Δ mfi	1576.77	1468.13	-4.65	15.87
Evidence for endocytosis	no	no	no	no
Agrin-induced AChR clusters, % of HC	21.09	43.46	2.70	59.90
DOK7-induced AChR clusters,% of HC	49.72	18.87	0.84	3.55
LRP4/MuSK binding, % of control	20.02	12.33	86.79	104.40

these results imply that monovalent binding, as is suggested for IgG4 subclass antibodies, was sufficient for this pathogenic mechanism and the Fc region may not be required. On the other hand, since IgG4 and Fabs had similar effects, our findings do not address the question of whether MuSK IgG4 antibodies are monovalent or divalent.

MuSK antibodies have been suggested to bind mainly to the first two Ig-like domains of MuSK (McConville et al., 2004, Hoch et al., 2001) and a recent publication narrows the epitope down to the first Ig-like domain (Huijbers et al., 2013). Interestingly, the first Ig-like domain of MuSK, and specifically one amino acid residue, Ile96, has been shown to be essential for LRP4 binding (Zhang et al., 2011). Perhaps MuSK antibodies that bind to the Ig1-like domain can alter the accessibility of the Ile96 to LRP4 either directly (by direct block) or indirectly (by conformational changes), and thereby prevent binding between LRP4 and MuSK. Epitope mapping studies are necessary to explore this possibility.

Taken together, a block of LRP4-MuSK interaction by IgG4 subclass antibodies is likely to be a major pathogenic mechanism. However, IgG4 subclass antibodies were able to disperse agrin-independent, DOK7-induced AChR clusters (see also table 7.1). This was an unexpected finding, because the LRP4-MuSK interaction is thought to play its main role in agrin-induced AChR clustering (Kim et al., 2008, Zhang et al., 2008, Zong et al., 2012). DOK7-induced AChR clustering does not require the addition of agrin. As a logical consequence, we thought it would be independent of LRP4, and unlikely to be affected by a block of LRP4-MuSK interaction. However, the results imply that LRP4-MuSK interaction is also important in signalling processes down-stream of MuSK. The extracellular domain of LRP4 is essential to bind agrin and MuSK (Zhang et al., 2011). Furthermore, deletion of the transmembrane region leads to reduced NMJ development in mice, which suggests a role for LRP4 membrane anchoring in NMJ development (Choi et al., 2013). However, deletion of the intracellular region of LRP4 does not affect the NMJ (Gomez and Burden, 2011). It may be that the transmembrane domains of LRP4 and MuSK may be important for the signalling cascade. Maybe DOK7 induces a conformational change in MuSK to promote interaction between the transmembrane domains.

Another possibility is that MuSK antibodies interfere with MuSK dimerisation and activation. It may be that a proportion of MuSK antibodies bind to the hydrophobic residues around Leu38 in the Ig1-like domain of MuSK. This region is important for dimerisation of MuSK (Stiegler et al., 2006), which is thought to be essential for MuSK activation (Hopf and Hoch, 1998a, Hopf and Hoch, 1998b). By binding to this hydrophobic residue, the MuSK antibodies could interfere with MuSK dimerisation. The work by Huijbers et al. suggests that MuSK IgG4 inhibits agrin-induced MuSK phosphorylation, probably caused by the blocking of LRP4-

MuSK interaction, but do not interfere with MuSK phosphorylation in mutant MuSK that is constitutively active (Huijbers et al., 2013). Since MuSK phosphorylation is a consequence of MuSK dimerisation (Hopf and Hoch, 1998a), these findings suggest that IgG4 does not affect MuSK dimerisation, but block LRP4-MuSK interaction. On the other hand, MuSK IgG1-3 might affect dimerisation, and this could be explored by size exclusion chromatography. The effect of IgG1-3 on AChR clustering is discussed below.

7.3 Pathogenicity of MuSK IgG1-3

It could be argued that IgG4 antibodies are the predominant antibody in MuSK-MG (McConville et al., 2004, Niks et al., 2008a) and likely to be the most pathogenic. However, the presence of high concentrations of serum IgG4 antibodies does not necessarily mean that they are the only pathogenic moiety. For instance, antigen specific IgG4 levels increase after long term stimulation with the antigen (Aalberse et al., 1983), but in this context, IgG4 is not considered as pathogenic, but protective (Lessof et al., 1978, van der Neut Kolfshoten et al., 2007). Furthermore, a whole group of diseases, IgG4-related diseases, is defined by an abundance of IgG4 subclass antibodies, and it remains unknown whether these antibodies are the cause of disease or just a side effect of persistent inflammation (Stone, 2012). Therefore it is difficult to dismiss the possibility that other IgG subclasses are also pathogenic, even though their concentration is low (McConville et al., 2004).

AChR-MG is mediated by IgG1 and IgG3, which are the predominant IgG subclasses in that disease (Rodgaard et al., 1987, Vincent and Newsom-Davis, 1982, Lefvert et al., 1981). MuSK IgG1-3 was suggested to be non-pathogenic in MuSK-MG using passive transfer experiments in mice (Klooster et al., 2012); but in that

study very low amounts of MuSK IgG1-3 were injected and they were undetectable in the sera of the injected mice. This is not representative of the situation in MuSK-MG patients where these subclasses can be easily measured, as shown here and elsewhere (McConville et al., 2004, Niks et al., 2008a). It could be that passive transfer of a larger amount of IgG1-3 might have caused disease. We therefore investigated whether MuSK-MG IgG1-3 subclass antibodies have the potential to be pathogenic.

IgG1-3 subclass antibodies did not reduce the LRP4-MuSK interaction, which is interesting. Why do only antibodies of the IgG4 subclass block the binding between LRP4 and MuSK? One explanation could be that MuSK antibodies belong initially to the IgG1-3 subclasses that do not compete effectively with LRP4 to achieve inhibition of MuSK function. By sequential class switch from IgG1-3 towards the IgG4 subclass and antigenic drift the antibodies may then compete successfully with LRP4 for binding to MuSK. Another explanation is that LRP4 and MuSK tightly interact, and that the epitope that is bound by the blocking monovalent IgG4 antibodies is not accessible for divalent IgG1-3 subclass antibodies. IgG4 has unique structural features that allow for the splitting up of the immunoglobulin into half-molecules (reviewed by (Nirula et al., 2011, Aalberse et al., 2009)), and IgG4 has a more flexible structure, allowing for more compact conformations (reviewed by (Aalberse et al., 2009)). This may enable IgG4 to bind to a less accessible epitope than IgG1-3.

Another possibility is, that for unknown reasons, only immunoglobulins that are specific for a certain epitope undergo class switch towards IgG4. Detailed epitope mapping studies and structural studies with IgG4 and IgG1-3 subclass antibodies will be necessary to address these questions.

Since MuSK IgG1-3 antibodies did not block LRP4-MuSK interaction, it was unexpected that MuSK IgG1-3 subclass antibodies were able to reduce agrin-induced AChR clustering although only at the concentrations similar to those found in patient plasmas (it should be appreciated that these would be lower than those present in the untreated patient's serum). Thus it could be that IgG1-3 subclass antibodies play a pathogenic role *in vivo*. Interestingly, MuSK IgG1-3 were also able to completely disperse pre-formed DOK7-induced AChR clusters. It seems that these antibodies affect MuSK function in a manner that is independent of agrin binding to LRP4.

There are other potential effects of IgG1-3 antibodies that might even override the effects of IgG4. IgG1-3 subclass antibodies are divalent and they should be able to cross-link and internalise their target, in a similar manner to autoantibodies to AChR that lead to endocytosis of AChR (Drachman et al., 1978). Indeed one study showed data suggesting that MuSK-MG patient IgG-induced endocytosis of MuSK in MuSK-EGFP transfected C2 myoblast cells (Cole et al., 2010). However, no evidence for endocytosis could be found in this study, neither for IgG4 subclass antibodies, which would be unlikely due to their proposed functional monovalency, nor for IgG1-3 subclass antibodies, purified IgG or plasma from MuSK-MG patients. MuSK IgG4 did not reduce MuSK expression at the cell surface (Huijbers et al., 2013), thus supporting the results described here.

Perhaps there are additional muscle factors that are required for MuSK endocytosis. However, this seems unlikely because auto-antibodies that cause several different diseases are able to internalise their antigen from the surface of HEK cells (e.g. antibodies to AQP4 (Hinson et al., 2008), and glycine receptors (A Vincent, P. Waters, A. Carvajal, personal communication)). Further endocytosis experiments using a more muscle cell-like environment were set up. For example MuSK-mCherry

transfected C2C12 myoblasts and HEK293 cells co-transfected with LRP4 and MuSK, were carried out but did not yield different results.

Why did divalent IgG1-3 subclass antibodies not induce cross-linking and internalisation? It may be that antibodies bind in a monovalent manner that does not allow for inter-MuSK cross-linking. Both IgG4 and IgG1-3 had similar binding affinities with K_d values in the picomolar range. The remarkably high affinity of these antibodies, similar to that of AChR antibodies (Vincent and Newsom-Davis, 1982), is intriguing.

A further potential mechanism for MuSK IgG1-3 could be the interference of MuSK and ColQ binding, which has been described as a mechanism for MuSK autoantibodies (Ohno, 2012). ColQ, which is up-regulated in C2C12 upon differentiation to myotubes (Ting et al., 2005), is bound to MuSK and anchors AChE, which is expressed at the NMJ (Cartaud et al., 2004, Krejci et al., 1997) and at low levels in C2C12 myotubes (Legay et al., 1995). AChE is required to recycle acetylcholine, therefore a loss of AChE at the NMJ due to a block of binding between ColQ and MuSK may interfere with acetylcholine recycling and affect AChR clustering, as acetylcholine is also a negative regulator of AChR clustering (Lin et al., 2005, Misgeld et al., 2005). This possibility could be explored by co-immunoprecipitation of MuSK and ColQ in the presence or absence of IgG1-3 subclass antibodies. However, it is unclear why MuSK antibodies, if they act by blocking ColQ-MuSK interaction, affect AChR clustering in C2C12 myotubes, where acetylcholine is not present.

A more likely pathogenic mechanism for IgG1-3 antibodies would be the interference with MuSK dimerisation via the hydrophobic patch in the Ig1-like domain (Stiegler et al., 2006, Hopf and Hoch, 1998b, Hopf and Hoch, 1998a), or activation of the complement system. Total IgG from MuSK patients has been

demonstrated to induce MuSK phosphorylation in the absence of agrin (Shigemoto et al., 2006, Cole et al., 2010), and since MuSK IgG4 does not induce phosphorylation (Huijbers et al., 2013), it is likely by exclusion, that MuSK IgG1-3 is responsible for induction of MuSK phosphorylation, but would suggest that MuSK IgG1-3 induce MuSK dimerisation rather than prevent it.

This leads to the question how induction of MuSK dimerisation and phosphorylation by MuSK IgG1-3 leads to such a marked reduction in agrin- and DOK7-induced AChR clustering. MuSK activation requires phosphorylation of four phosphotyrosine residues to become fully activated (Herbst and Burden, 2000), and MuSK IgG1-3 may only induce initial phosphorylation of MuSK, thus locking MuSK in a half-activated state that is not able to promote AChR clustering. It would be interesting to explore this possibility further, for example with antibodies raised against the four different phosphotyrosines of MuSK to analyse different levels of phosphorylation in the presence of IgG4 or IgG1-3 from MuSK-MG patients.

Complement has not been detected in mouse models of MuSK-MG, but this may be because mouse complement is not well activated by human IgG (Saadoun et al., 2010). Complement depositions have not been found in patient biopsies from limb muscle (biceps brachii) (Shiraishi et al., 2005a), but cranial muscles are often more severely affected than limb muscles (Oh, 2009, Yanagawa et al., 2009), and biopsies cannot be taken from cranial muscles. The expression levels of MuSK mRNA varies in different muscles (Punga et al., 2011, Viegas et al., 2012), thus it may be possible that cranial muscles have higher levels of MuSK, which may lead to more efficient complement activation in these muscles. Indeed one study described increased levels of complement breakdown products in MuSK-MG patients (Tuzun et al., 2011), and MuSK antibodies from sera of some MuSK-MG patients were

found to activate complement on transfected HEK293 cells *in vitro* (Leite et al., 2008).

Although data presented here suggests that IgG1-3 can affect AChR clustering *in vitro*, it is still not clear whether MuSK IgG1-3 subclass antibodies are pathogenic *in vivo*, and what relevance IgG1-3 subclass antibodies have for the course of the disease. Despite their low concentrations, if MuSK IgG1-3 are pathogenic *in vivo*, there may be clinical implications that need to be considered in the development of novel therapies and in long-term clinical studies.

7.4 Concluding remarks

The aim of this project was to identify pathogenic mechanisms of MuSK autoantibodies.

The first hypothesis was that MuSK autoantibodies induce endocytosis of MuSK. In AChR-MG, cross-linking and endocytosis of AChRs is a key pathogenic mechanism (Drachman et al., 1978, Heineman et al., 1977). A paucity of MuSK due to endocytosis and degradation could lead to reduced signal transduction via the agrin-LRP4-MuSK pathway that is responsible for dense AChR clustering on top of the postsynaptic folds (Glass et al., 1996, DeChiara et al., 1996, Kim et al., 2008, Zhang et al., 2008). This may lead to reduced density of AChRs that were measured as reduced MEPPs in patients (Selcen et al., 2004, Niks et al., 2010) and animal models of MuSK-MG (Viegas et al., 2012, Morsch et al., 2012, Mori et al., 2012a) and explain neuromuscular transmission failure and muscle weakness. Hence endocytosis was investigated as potential mechanism. To address this possibility, similar techniques were used as in studies that demonstrated autoantibody-induced endocytosis of AChR (Drachman et al., 1978, Heineman et al., 1977), aquaporin-4

(Hinson et al., 2007) or NMDA-receptor (Dalmau et al., 2008, Hughes et al., 2010). Briefly, patient antibody binding to cell surface expressed autoantigen was investigated over time. As a positive control, endocytosis was artificially induced by cross-linking with a secondary antibody, based on experiments by Drachman (Drachman et al., 1978). No evidence for patient antibody induced endocytosis was found, unless secondary antibody was added.

The second hypothesis was that patient antibodies disrupt the close interaction between LRP4 and MuSK. This interaction is crucial for signal transduction via the agrin-LRP4-MuSK pathway (Kim et al., 2008, Zhang et al., 2008), and a disruption of this interaction would provide a plausible mechanism, since MuSK antibodies impair agrin-induced AChR clustering *in vitro* (Hoch et al., 2001, Farrugia et al., 2007) and are mainly directed against the first two Ig-like domains of MuSK (Hoch et al., 2001, McConville et al., 2004, Oh et al., 2009), of which the first Ig like domain is essential for binding of LRP4 (Zhang et al., 2011). The interaction between LRP4 and MuSK was studied using co-immunoprecipitation. Here in this project it was described for the first time that MuSK antibodies indeed block binding between LRP4 and MuSK, a finding that was confirmed later on in an independent study (Huijbers et al., 2013) published after our own (Koneczny et al., 2013). This finding is thus novel, and significant to the field, because it unravels for the first time a key pathogenic mechanism of MuSK autoantibodies.

As MuSK antibodies belong mainly to the IgG4 subclass (McConville et al., 2004) that were described to be functionally monovalent (reviewed by Nirula et al., 2011), it was then investigated whether monovalent binding was sufficient for pathogenicity *in vitro*. Patient and healthy control IgG were digested with papain to obtain Fab fragments that are monovalent. The purity and protein concentration of the Fabs was verified, and functional binding to MuSK could be verified by RIA.

Patient and healthy control Fabs were tested for their effect on AChR clustering in C2C12 myotubes *in vitro*, and indeed it was found that Fabs were sufficient to reduce agrin-induced AChR clustering efficiently. DOK7-induced AChR clustering, which harbours complex AChR clusters that resemble more the adult NMJ (Okada et al., 2007), was also efficiently reduced by Fabs. Furthermore, Fabs were sufficient to block LRP4-MuSK interaction. These findings are all novel, and are relevant to the field because they demonstrate that monovalent binding, as it is proposed for IgG4, is sufficient for pathogenicity *in vitro*.

Finally, patient IgG4 and IgG1-3 subclasses were isolated and characterised to study how they contribute to pathogenicity *in vitro*, individually or together. IgG4 is the main subclass in MuSK-MG (McConville et al., 2004), and a recent publication suggests that MuSK IgG4 are pathogenic by passive transfer into experimental animals (Klooster et al., 2012). IgG4 was isolated from patient plasma using an IgG4 affinity matrix, and IgG4 depleted IgG1-3 were purified with protein G sepharose. As expected, IgG4, although low in protein concentration, showed high concentrations of MuSK antibodies, in contrast to IgG1-3, which harboured only low MuSK antibody concentrations despite relatively high protein concentrations. The binding affinities for IgG4 and IgG1-3 were calculated individually for the first time, and harboured both very high estimated binding affinities in picomolar range, similar to the K_{ds} of total MuSK antibodies from a previous study (McConville et al., 2004). This finding is relevant for the field because it suggests that IgG4 and IgG1-3 have similar binding affinities.

Importantly, it was described for the first time that IgG4 subclass antibodies exclusively block the binding between LRP4 and MuSK, suggesting that this is likely a major pathogenic mechanism, because IgG4 is the main subclass of MuSK autoantibodies (McConville et al., 2004). Here it was also described for the first time

that both IgG4 and IgG1-3 subclass antibodies derived from MuSK-MG patients reduced agrin- and DOK7-induced AChR clustering *in vitro*. This is relevant both to the field and clinically, as IgG1-3 antibodies were previously considered as non-pathogenic (Klooster et al., 2012), but may yet contribute to the disease.

Taking all findings into account, MuSK-MG is a distinct disease that differs from AChR-MG regarding clinical, immunological, genetic, therapeutic and, as demonstrated in this study, pathomechanistic aspects. The IgG4 antibodies appear to block the activation of MuSK, but IgG1-3 antibodies also affect the down-stream AChR clustering, which is the essential final pathway of MuSK activation. The differences in their pathogenic mechanisms may be important for therapy. The different outcomes of anti-CD20 therapeutic antibody (Diaz-Manera et al., 2012, Illa et al., 2008), which is markedly better in MuSK-MG than AChR-MG, together with the difference in IgG subclass, suggests the involvement of different plasma cells in these two distinct pathologies. It will be important to identify and characterise the cell types that are responsible for antibody production in AChR- and MuSK-MG. This may in the future allow for tailored therapies for these two distinct diseases, that target only the pathogenic cells.

The finding of this study may have wider implications outside the pathology of myasthenia gravis. First, our findings suggest that even low concentrations of antibody species, such as the IgG1-3 MuSK-Abs, should not be dismissed but need to be studied in parallel. Second, the functional block by IgG4 that interferes with signal transduction pathways, may be relevant in other IgG4 mediated diseases. In addition to MuSK antibodies, desmoglein antibodies in pemphigus (Kricheli et al., 2000, Futei et al., 2001, Ayatollahi et al., 2004), antibodies against components of voltage-gated potassium channel complexes (VGKCs), Leucine-rich, glioma inactivated 1 (LGI1) and Contactin-associated protein-2 (CASPR2), were found to

be variably, but mainly IgG4 subclass (reviewed by (Vincent et al., 2011)). It may be that more autoimmune diseases harbouring IgG4 subclass antibodies, that do not activate complement, are yet to be discovered, and that these antibodies act more by interfering with function than by traditional immunological mechanisms. Finally, both IgG1-3 and IgG4 antibodies dispersed DOK7-induced AChR clusters, that are not thought to be dependent on agrin/LRP4 activation of the MuSK pathway. Further investigation of this phenomenon may lead to a greater understanding of the agrin-LRP4-MuSK pathway.

Chapter 8: Bibliography

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Chapter 9: Appendix

Abbreviations

Ab	Antibody
AcGFP	<i>Aequorea coerulescens</i> derived green fluorescent protein
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AChR-MG	Myasthenia gravis with antibodies to the AChR
APC	Allophycocyanin
BSA	Bovine serum albumin
CASPR2	Contactin-associated protein 2
CBA	Cell based assay
Co-IP	Co-immunoprecipitation
DMEM	Dulbecco's Modified Eagle Medium
DOK7	Docking protein 7
EAMG	Experimental Autoimmune Myasthenia gravis
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EOMG	Early onset myasthenia gravis
EPP	End-plate potential
Fab	Fragment, antibody binding
Fc	Fragment, crystallisable region
FCS	Fetal calf serum
HC	Healthy control
HEK293 cells	Human embryonic kidney cells
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid
HRP	Horse-radish peroxidase
IgG	Immunoglobulin G
IgG1-3	Immunoglobulin G subclasses 1, 2 and 3
IgG4	Immunoglobulin subclass 4
IgG4-RLD	IgG4-related disease

IP	Immunoprecipitation
IVIG	Intravenous Immunoglobulin
LGi1	Leucine glioma inactivated protein
LOMG	Late onset myasthenia gravis
LRP4	Low density lipoprotein receptor-related protein 4
MEPP	Miniature end-plate potential
Mfi	Median fluorescence intensity
MG	Myasthenia gravis
MuSK	Muscle specific tyrosine kinase
MuSK-MG	Myasthenia gravis with antibodies to MuSK
NMDA	N-methyl-d-aspartate
NMJ	Neuromuscular junction
NMT	Neuromuscular transmission
OD	Optical density
PBS	Phosphate buffered saline
PEI	Polyethylenimine
PEX	Plasma exchange
PIC	Protease inhibitor cocktail
PLL	Poly-l-lysine
PTX	PBS containing triton-X
RIA	Radioimmunoprecipitation assay
SD	Standard deviation
SEM	Standard error of the mean
SNMG	Seronegative myasthenia gravis
VGKC	Voltage-gated potassium channel complex
WCL	Whole cell lysate

Publications generated within the scope of this thesis

Data from this thesis (Chapters 3-6) was summarised in one publication:

KONECZNY, I., COSSINS, J., WATERS, P., BEESON, D. & VINCENT, A. 2013. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1-3 can disperse preformed agrin-independent AChR clusters. *PLoS One*, 8, e80695.

A review about MuSK-MG was published in the Journal of Anatomy:

KONECZNY, I., COSSINS, J. & VINCENT, A. 2014. The role of muscle-specific tyrosine kinase (MuSK) and mystery of MuSK myasthenia gravis. *J Anat*, 224, 29-35.

Furthermore, data from Chapter 3 and data produced within the scope of Chapter 5 contributed to two reviews:

COSSINS, J., BELAYA, K., ZOLTOWSKA, K., KONECZNY, I., MAXWELL, S., JACOBSON, L., LEITE, M. I., WATERS, P., VINCENT, A. & BEESON, D. 2012. The search for new antigenic targets in myasthenia gravis. *Ann N Y Acad Sci*, 1275, 123-8.

VINCENT, A., WATERS, P., LEITE, M. I., JACOBSON, L., KONECZNY, I., COSSINS, J. & BEESON, D. 2012. Antibodies identified by cell-based assays in myasthenia gravis and associated diseases. *Ann N Y Acad Sci*, 1274, 92-8.