Author: Huijbers, Maartje
Title: The pathophysiology of MuSK myasthenia gravis
Issue Date: 2016-07-06

CHAPTER 3

MuSK IgG4 auto-antibodies cause myasthenia gravis by inhibiting binding between MuSK and LRP4

Maartje G. Huijbers*, Wei Zhang*, Rinse Klooster, Erik H. Niks, Matthew B. Friese, Kirsten R. Straasheijm, Peter E. Thijssen, Hans Vrolijk, Jaap J. Plomp, Pauline Vogels, Mario Losen, Silvère M. Van der Maarel, Steven J. Burden, and Jan J. Verschuuren

*Both authors contributed equally

Proceedings of the National Academy of Sciences.
2013 Dec 17;110 (51):20783-8.

ABSTRACT

Myasthenia gravis (MG) is a severely debilitating autoimmune disease that is due to a decrease in the efficiency of synaptic transmission at neuromuscular synapses. MG is caused by antibodies against postsynaptic proteins, including (1) acetylcholine receptors (AChRs), the neurotransmitter receptor, (2) muscle specific kinase (MuSK), a receptor tyrosine kinase essential for the formation and maintenance of neuromuscular synapses and (3) low-density lipoprotein receptor-related protein 4 (LRP4), which responds to neural Agrin by binding and stimulating MuSK. Passive transfer studies in mice have shown that IgG4 antibodies from MuSK MG patients cause disease without requiring complement or other immune components, suggesting that these MuSK antibodies cause disease by directly interfering with MuSK function. Here, we show that pathogenic IgG4 antibodies to MuSK bind to a structural epitope in the first Ig-like domain of MuSK, prevent binding between MuSK and LRP4 and inhibit Agrin-stimulated MuSK phosphorylation. In contrast, these IgG4 antibodies have no direct effect on MuSK dimerization or MuSK internalization. These results provide insight into the unique pathogenesis of MuSK MG and provide clues toward development of specific treatment options.

INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease, caused by auto-antibodies to proteins in the postsynaptic membrane at neuromuscular synapses. Most MG patients carry antibodies to acetylcholine receptors (AChRs), the neurotransmitter receptor at vertebrate neuromuscular synapses (1,2). Auto-antibodies to AChRs are largely of the IgG1 and IgG3 subclass (3), which cause muscle weakness by three mechanisms: 1) complement-mediated membrane lysis (4), 2) crosslinking and depletion of cell surface AChRs (5) and 3) to a lesser extent, functional blocking of the ACh-binding site (6). The ability of antibodies to AChRs to recruit complement, dimerize and modulate AChR expression is an important component of their pathogenic mechanism, as animals with experimental autoimmune MG (EAMG) can be rescued from disease with monovalent Fab fragments generated from AChR IgG antibodies and complement-deficient mice are protected against EAMG (7,5,8).

Approximately 20% of patients with MG lack antibodies to AChRs, and approximately 40% of these AChR-negative patients carry auto-antibodies to muscle specific kinase (MuSK), a receptor tyrosine kinase that is essential for all aspects of synaptic differentiation and maintenance (9,10,11). The synaptic defects in MuSK MG overlap with those in AChR MG, including a reduction in the number of functional AChRs at synapses and unreliable synaptic transmission, resulting in muscle fatigue and weakness. In contrast to AChR MG, MuSK MG is caused in large part by IgG4 antibodies (12,13,14) that fail to engage complement and are considered functionally monovalent (12,13,14,15). Consequently, the accumulation of complement and muscle membrane damage, hallmark pathological features of AChR MG, appear insignificant in MuSK MG (12,16). Despite the paucity or absence of complement and cell damage in MuSK MG, the structural and functional deficits of synapses are extensive in MuSK MG, which highlights the key role that MuSK plays in organizing all aspects of synaptic differentiation (9,17).

AChR clustering and synapse formation are orchestrated by neuronally released Agrin, which binds to LRP4, a member of the lipoprotein receptor-related protein family, causing LRP4 to bind and activate MuSK (18,19,20). Once tyrosine-phosphorylated, MuSK recruits Dok-7, an adaptor protein that becomes phosphorylated and recruits additional signaling molecules essential for synapse formation (21,22,23).

The extracellular region of MuSK contains three Ig-like domains and a Frizzled-like domain (9). The first Ig-like domain in MuSK is required for MuSK to bind LRP4. Mutation of a single residue, I96, on a solvent exposed surface of the first Ig-like domain, prevents MuSK from binding LRP4 and responding to Agrin (24,20). A hydrophobic surface on the opposite side of the first Ig-like domain mediates MuSK homodimerization, which is essential for MuSK trans-phosphorylation (24). Although MuSK is expressed by muscle and not by motor neurons, MuSK is critical for presynaptic as well as postsynaptic differentiation (9). In mice lacking MuSK, motor axons fail to stop and differentiate and instead wander aimlessly throughout the muscle (10). MuSK regulates presynaptic differentiation, at least in part, by clustering LRP4 in muscle, which functions bi-directionally by serving not only as a receptor for Agrin and as a ligand for MuSK, but also as a direct retrograde signal for presynaptic differentiation (25). In addition to its role during synapse formation, MuSK is also required to maintain adult synapses, as inhibition of MuSK expression in adult muscle leads to profound defects in presynaptic and postsynaptic differentiation (26,27).

Because IgG4 antibodies do not engage complement and are thought to be incapable of cross-linking and modulating expression of cell surface antigens, we reasoned that pathogenic IgG4 auto-antibodies to MuSK may directly interfere with MuSK function.

Here, we demonstrate that human IgG4 MuSK antibodies bind to the first Ig-like domain in MuSK and prevent LRP4 from binding MuSK, thereby inhibiting Agrin-stimulated MuSK phosphorylation. We show that inhibiting the association between LRP4 and MuSK appears to be the major mechanism by which the MuSK IgG4 antibodies disrupt MuSK signaling and cause MG, as these antibodies neither modulate MuSK surface expression nor have a direct effect on MuSK dimerization.

MATERIALS AND METHODS

Patients

Twenty-five Dutch MuSK MG patients and 18 controls were included for these studies (Table 1). All patients gave written consent according to the Declaration of Helsinki, and the study was approved by the Leiden University Medical Centre ethics committee. The patients were diagnosed based on the presence of fatigable muscle weakness with electrophysiological evidence of decrementing compound muscle action potentials in response to low-rate repetitive nerve stimulation or increased jitter on single-fiber electromyography. Furthermore, they tested positive for MuSK antibodies in a standard commercial radioimmunoprecipitation assay (RIA) from RSR (RSR, Cardiff, UK). Eighteen controls included five patients with AChR MG, one with Lambert-Eaton myasthenic syndrome (LEMS) and twelve healthy controls. Plasmapheresis material from 7 patients became available during regular treatment for MuSK MG. This material was stored at -80°C until it was further processed for IgG purification. Plasmapheresis material was affinity purified for IgG4 and IgG1-3 as described previously (Supplemental Methods) (12).

Binding assays

Recombinant proteins were generated to cover the complete extracellular region of MuSK or domains of MuSK (Supplemental Methods). We identified the epitopes recognized by the MuSK MG patient antibodies using an ELISA and by a competition ELISA, using twenty amino-acid overlapping peptide fragments (LUMC peptide facility) that cover the first Ig-like domain (Table S1). We measured binding between patient antibodies, bound to a Protein A plate, and AP-ecto MuSK or AP-ecto MuSK I96A, in triplicate, using an ELISA. Binding between MuSK and LRP4 was measured using a solid-phase binding assay, as described previously (20), except that we used human rather than rat MuSK (Supplemental Methods). To determine the effect of IgG on MuSK-LRP4 binding, 10nM Agrin and 10nM LRP4-AP were co-incubated with 8.3 μM purified IgG, which is within the reported normal range for IgG4 (0.05 to – 9μM); moreover, IgG4 levels can be elevated more than 20-fold in IgG4 auto-immune diseases (53)The average value from three independent experiments for each patient was calculated.

Table 1. Demographical and clinical characteristics of 25 MuSK MG patients

Women, n (%)	15 (60)
Age at onset , y, median (range)	38.5 (2–80)
Follow-up, y, median (range)	5.8 (0.5–33)
Predominant weakness, n (%)	
Bulbar	9 (36)
Oculobulbar	12 (48)
Generalized	4 (16)
MGFA* at maximum	
II, n (b)	6 (6)
III, n (b)	4 (2)
IV, n (b)	8 (8)
V, n (%)	7 (28)
Immunosuppressive treatment at serum sampling, n (%)	16 (64)

*Myasthenia Gravis Foundation of America score is a quantitative assessment of muscle weakness.

One-way ANOVA analysis with Bonferroni's correction was used to compare differences in MuSK-LRP4 binding in the presence of IgG4 or IgG1-3 and between MuSK MG patients and controls. The values were considered statistically different if $p<0.05$.

Tyrosine phosphorylation assays

MuSK L746M/S747T was generated by site-directed mutagenesis and transfected into 3T3 cells with Lipofectamine 2000 (Invitrogen) (Supplemental Methods). 3T3 cells were treated with 40 μg/ml IgG4 from MuSK MG patients, or controls from 12 to 36 hr after transfection; cell surface proteins from triplicate samples were digested by trypsin (0.05%) for 5 minutes at 37°C. Myotubes were stimulated with 0.4nM neural Agrin or Agrin together with 40 μg/ml IgG4 from MuSK MG patients, or controls for 30 minutes at 37°C. MuSK tyrosine phosphorylation in duplicate samples was measured as described previously (54). PJ69-4A yeast were transformed with plasmids encoding the GAL4 DNA binding domain fused to wild-type rat MuSK,

MuSK D753A, MuSK L745M, S746T or the insulin receptor. Fusions proteins were immunoprecipitated with antibodies to GAL4 and Western blots were probed with antibodies to phosphotyrosine (Supplemental Methods).

Immunostaining

U2O cells were transfected with human MuSK-GFP or ΔIg-like1-MuSK-GFP, and fixed cells were stained with patient antibodies, followed by Alexa 594 conjugated-mouse anti-human IgG (Invitrogen). Stained cells were viewed with a Leica DM 5500B microscope, and images were analyzed with LAS AF software (Supplemental Methods).

RESULTS

The main immunogenic region (MIR) includes structural epitopes contained in the first Ig-like domain of MuSK

The earliest available serum samples from 25 MuSK MG patients and sera from 18 controls, were tested for immunoreactivity against human MuSK recombinant proteins using an ELISA (Fig 1a). All patients, but no controls had high

immunoreactivity against the Ig-like domain 1, although the level of binding varied among patients (Fig 1a, Figure S1). This variation likely reflects differences in antibody titre and/or affinity for MuSK. For 20 patients the immunoreactivity was limited to the first Ig-like domain (Figure 1a). Five patients showed additional immunoreactivity against the Ig-like domain 2 (Figure 1a). We did not detect reactivity to the Ig-like domain 3 or the Frizzled-like domain, although we cannot exclude the possibility that this is due to the experimental conditions (Figure 1a).

In addition, we expressed full length MuSK-GFP or a mutant form of MuSK-GFP, lacking the first Ig-like domain (ΔIg-like1 MuSK::GFP) in non-muscle cells and stained transfected cells with patient sera. Antibodies that bound selectively to the first Ig-like domain stained cells expressing wild-type MuSK but not the mutant form lacking the first Ig-like domain (Figure 1b). In contrast, antibodies that showed reactivity to the second Ig-like domain stained cells expressing wild-type MuSK as well as cells expressing MuSK lacking the first Ig-like domain (Figure 1c). In conclusion, all patients in this Dutch cohort harbor antibodies against the first Ig-like domain in MuSK at disease onset. Therefore, this region is likely to represent the MIR of MuSK. A small group of patients have additional auto-antibodies against the second Ig-like domain.

In AChR MG and other autoimmune diseases auto-antibodies often require a discontinuous stretch of amino acids that comprise a structural epitope (28,29,30,31,32). In order to determine whether the auto-antibodies to MuSK recognize a linear epitope in the first Ig-like domain of MuSK, we used a competition ELISA with overlapping 20-mer peptides from the first Ig-like domain (Figure 1d, Table S1). Pre-incubation of patient antibodies with the complete Ig-like domain 1 inhibited binding of the IgG4 fractions to full-length recombinant MuSK. Inhibition was nearly complete for the patient 1 and 5 that harbored antibodies that bind exclusively to

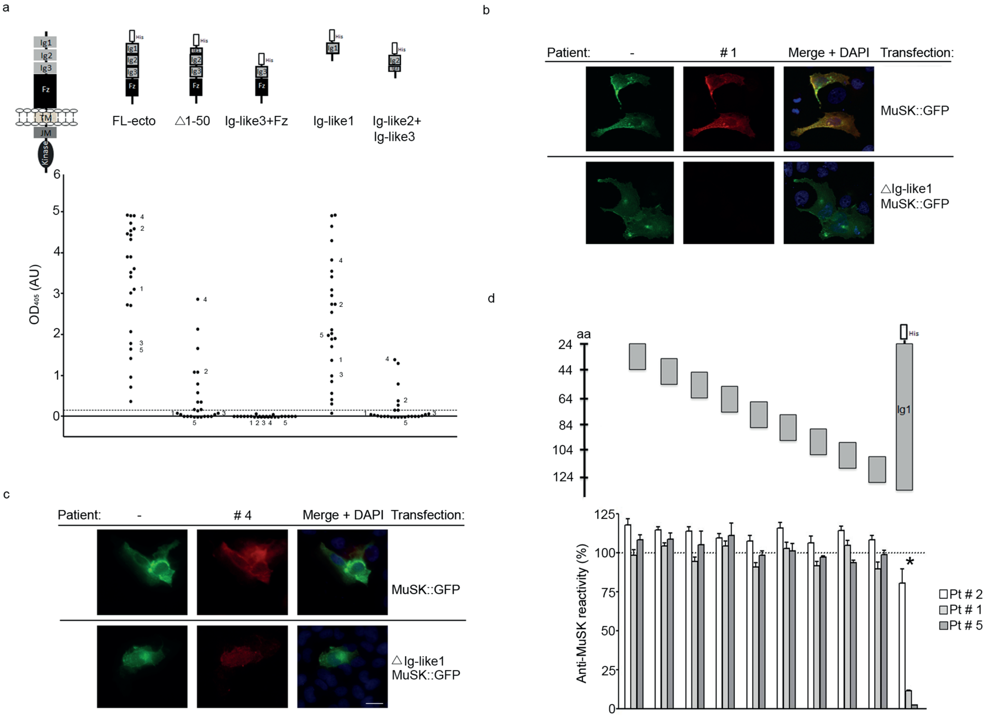


Figure 1. MuSK MG IgG4 antibodies bind predominantly to the first Ig-like domain in MuSK. (a) An ELISA shows that antibodies from all 25 patients bind to the extracellular region of MuSK. The predominant binding sites reside in the first Ig-like domain, as antibodies bind to this domain nearly as well as the entire extracellular region. Moreover, deletion of the N-terminal half of the first Ig-like domain substantially reduces antibody-binding. Antibodies from five patients have additional reactivity to the second Ig-like domain. Data shown reflects the average binding per patient determined in three independent assays (Figure S1) (b,c) Antibodies that bind selectively to the first Ig-like domain stain cells expressing full-length MuSK-GFP but not ΔIg-like1-MuSK-GFP, whereas antibodies with additional reactivity bind to cells expressing either construct. (d) An ELISA shows that antibody-binding to the extracellular region of MuSK is strongly inhibited by the first Ig-like domain but poorly by 20-mer overlapping peptides from this domain.

the first Ig-like domain, whereas competition was incomplete for patient 2 with reactivity to the second Ig-like domain, confirming our findings from the direct ELISA. Pre-incubation of the patient antibodies with 20-mer peptides, covering the first Ig-like domain, was without effect (Figure 1d, Table S2). These findings indicate that the antibodies bind to a structural epitope, formed either by non-contiguous sequences within the first Ig-like domain or folding of a linear amino acid sequence, which is poorly represented in short peptides. Thus, similar to antibodies in AChR MG, antibodies to MuSK recognize linear sequences poorly, if at all.

MuSK patient IgG4 antibodies interfere with Agrin-dependent association between MuSK and LRP4

One face of the first Ig-like domain in MuSK is solvent-exposed and binds LRP4. Because pathogenic IgG4 antibodies to MuSK bind the first Ig-like domain we asked whether the auto-antibodies interfered with the association between LRP4 and MuSK. We measured binding between LRP4 and MuSK using a solid phase binding assay in which the extracellular region of MuSK, fused to Fc (ecto-MuSK-Fc) was adsorbed to protein A plates. The extracellular region of LRP4 (ecto-LRP4), fused to human alkaline phosphatase (AP), binds specifically but weakly to ecto-MuSK in the absence of neural Agrin; neural Agrin binds LRP4 and stimulates strong and specific binding of AP-ecto-LRP4 to ecto-MuSK (20). We tested IgG4 as well as IgG1-3 antibodies from 7 MuSK MG patients and found that the IgG4 auto-antibodies from all MuSK MG patients strongly inhibited binding between LRP4 and MuSK, reducing binding by as much as 80-100%, in a dose-dependent manner (Figure 2a, Figure S2), whereas IgG1-3 patient antibodies had little effect, similar to IgG4 antibodies from healthy controls (Figure 2a). The patient antibodies that were the most effective inhibitors of MuSK-LRP4 association were the most potent inducers of myasthenia in vivo in a passive transfer model (12). Because the association between MuSK and LRP4 is crucial for maintaining neuromuscular synapses, these findings raise the possibility that the auto-antibodies cause myasthenia by interfering with binding between MuSK and LRP4.

Given these findings, we wondered whether binding of patient IgG4 antibodies to MuSK required MuSK I96, which is required for MuSK to bind LRP4. We used an ELISA, in which patient antibodies were attached to a Protein-A plate, which was probed with alkaline phosphatase (AP)-MuSK fusion proteins, encoding either the entire extracellular region from wild-type MuSK or MuSK I96A. Figure 2b shows that mutation of MuSK I96 had no significant effect on antibody binding. These findings demonstrate that the patient antibodies and LRP4 bind distinctly to the first Ig-like domain in MuSK.

Because binding between LRP4 and MuSK is essential for Agrin to stimulate MuSK phosphorylation, we asked whether the pathogenic IgG4 auto-antibodies to MuSK prevented Agrin-induced MuSK phosphorylation. We added IgG4 antibodies from patients with MuSK MG to cultured C2 myotubes, together with neural Agrin, and measured MuSK phosphorylation. Patient IgG4 antibodies to MuSK blocked MuSK phosphorylation (Figure. 2c,d). Together, these data indicate that the MuSK antibodies cause disease by preventing LRP4 from binding MuSK and blocking MuSK phosphorylation.

MuSK patient IgG4 antibodies do not inhibit MuSK dimerization

Because the first Ig-like domain of MuSK also contains a hydrophobic surface that functions as a dimerization interface, which is important for Agrin to induce MuSK

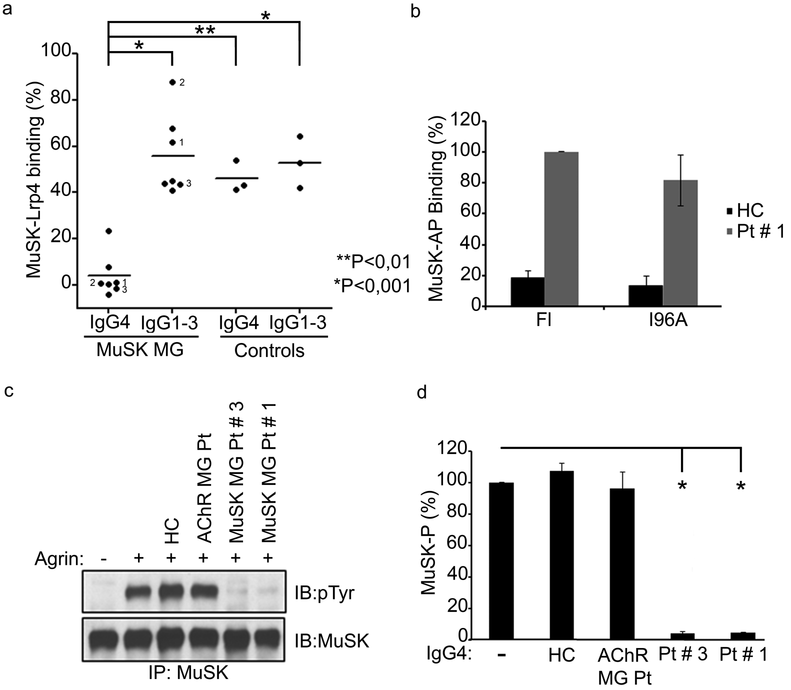


Figure 2. MuSK MG IgG4 antibodies block binding between LRP4 and MuSK and inhibit Agrin-stimulated MuSK phosphorylation in muscle. (a) IgG4 antibodies from MuSK MG patients but not IgG1-3 from the same patients significantly inhibit Agrin-dependent binding between MuSK and LRP4. IgG1-3 antibodies from MuSK MG patients and IgG4 and IgG1-3 antibodies from AChR MG and healthy controls moderately and equally reduce association between MuSK and LRP4. The values for these control groups do not differ from one another but differ significantly from those for MuSK MG patient IgG4 ($p<0.05$, n=3). (b) Mutation of MuSK I96 fails to reduce binding of AP-ecto-MuSK to IgG4 antibodies from patient 1 ($p>0.05$, n=3) (c) IgG4 antibodies from MuSK MG patients but not from an AChR MG patient or a healthy control inhibit Agrin-stimulated MuSK phosphorylation in C2 myotubes. (d) IgG4 antibodies from MuSK MG patients reduce MuSK phosphorylation (*, $p<0.01$, n=4).

phosphorylation (24), we considered the possibility that pathogenic IgG4 antibodies to MuSK might also directly interfere with MuSK homo-dimerization. We therefore sought to determine whether IgG4 antibodies to MuSK inhibit MuSK phosphorylation in fibroblasts expressing MuSK but not LRP4, a context where MuSK phosphorylation is dependent upon MuSK dimerization and not facilitated by LRP4.

Previously, we found that MuSK is poorly tyrosine phosphorylated in transfected mammalian non-muscle cells and in yeast. Because MuSK has an unusually high Km for ATP (33), similar to the ATP concentration in muscle but higher than the ATP concentration in most cell types, we considered the possibility that this high Km for ATP restrained MuSK phosphorylation in non-muscle cell types. Because the insulin receptor has a lower Km for ATP, typical for receptor tyrosine kinases, we mutated two amino acids in the activation loop of MuSK to the corresponding residues in the insulin receptor, reasoning that these substitutions might destabilize the activation loop, lower the Km for ATP and increase MuSK phosphorylation in non-muscle cells.

We generated an activation loop double mutant, MuSK L746M, S747T and found that this activation loop mutant, unlike wild-type MuSK, was efficiently tyrosine phosphorylated in non-muscle cells (Figure 3a,b,c). We transfected 3T3 cells with the activated form of MuSK and measured MuSK phosphorylation 12, 24 and 36 hours after transfection and found that MuSK phosphorylation was undetectable at 12 hr but increased in a linear manner over the next 24 hr (Figure 3d). Nearly all tyrosine phosphorylated MuSK was on the cell surface, as mild trypsin treatment degraded MuSK, leading to the disappearance of the tyrosine phosphorylated MuSK band at ~110kd (Figure 3d). We therefore treated transfected 3T3 cells with IgG4 antibodies to MuSK, beginning at 12 hr after transfection and measured MuSK phosphorylation at 24 and 36 hr. Figure 3 shows that IgG4 from MuSK MG patients failed to inhibit MuSK phosphorylation (Figure 3e,f,g). Because these antibodies completely block binding between LRP4 and MuSK, but have no detectable effect on MuSK dimerization, inhibition of MuSK dimerization likely plays little if any role in pathogenesis.

MuSK patient IgG4 antibodies do not deplete MuSK cell surface expression

Nearly all MuSK expressed in 3T3 cells was on the cell surface and removed by mild trypsin treatment (Figure 3d). Treatment of these cells with IgG4 patient antibodies did not alter the amount of MuSK expressed by 3T3 cells (Figure 3e), which was likewise found almost entirely on the cell surface and removed by mild trypsin treatment (Figure 4). In contrast, intracellular proteins, such as GAPDH, were not degraded by trypsin (Figure 4). Thus, the pathogenic IgG4 antibodies to MuSK do not modulate and reduce MuSK cell surface expression in this context.

DISCUSSION

MuSK MG is an IgG4-mediated autoimmune disease. Transfer of purified IgG4 from MuSK MG patients into immune-deficient mice causes myasthenic weakness that mimics the pathophysiology of patients with MuSK MG (13). Thus, these autoantibodies exert their pathogenic effects independent of the immune system by binding to MuSK and interfering with normal neuromuscular physiology. Our studies show that pathogenic IgG4 antibodies to MuSK prevent Agrin from stimulating MuSK by blocking association between LRP4 and MuSK. This inhibitory mechanism likely plays a key role in disrupting the structure of the synapse, compromising synaptic transmission and causing disease. Because the pathogenic antibodies neither decrease MuSK surface expression nor directly interfere with MuSK dimerization, therapeutic strategies designed to increase MuSK activity may prove effective in treating MuSK MG.

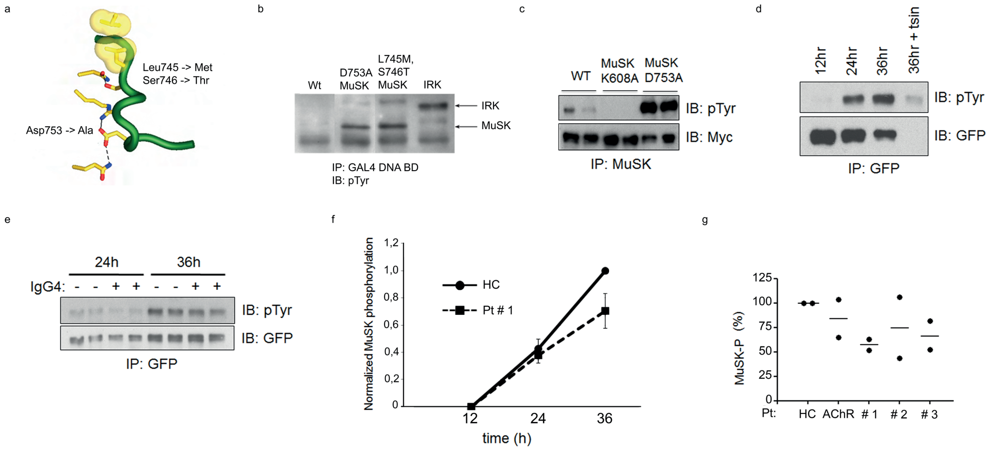


Figure 3. IgG4 antibodies from MuSK MG patients fail to reduce MuSK phosphorylation in 3T3 cells transfected with MuSK but not LRP4. (a) The diagram of the MuSK activation loop shows the substitutions in rodent MuSK that increase MuSK kinase activity. (b) MuSK tyrosine phosphorylation in yeast is enhanced to levels that are comparable to the insulin receptor (IRK) by mutation of D753 or L745/S746 in rodent MuSK (L746/S747 in human MuSK). Yeast were transformed with plasmids encoding fusion proteins between the DNA binding domain (BD) of GAL4 and MuSK or IRK. (c) MuSK tyrosine phosphorylation in 293 cells is enhanced 50-fold by mutation of D753. 293 cells were transfected with wild-type Myc-MuSK (21), Myc-MuSK K608A, a kinase-inactive form of MuSK, or Myc-MuSK D753A. (d) Tyrosine phosphorylation of MuSK L746M/S747T-GFP is detectable 24 h after transfection and increases over the next 12 h. Nearly all tyrosine phosphorylated MuSK is on the cell surface, as it is removed by digestion of cell surface proteins with trypsin. (e,f,g) IgG4 antibodies from MuSK MG patients, a healthy control (HC) or an AChR MG patient were added to cells 12 h following transfection. (f) Differences in MuSK phosphorylation in cells treated with patient 1 or control antibodies are not significant (*p*>0.05) at 24 or 36 h (n=4). (g) The level of MuSK phosphorylation in cells treated with patient or control antibodies (●, value from separate experiments; -, mean value).

All Dutch MuSK MG patients tested had strong immunoreactivity against the first Ig-like domain of MuSK. Five patients harbored additional reactivity against the Ig-like domain 2 in the ELISA. Others have reported that patients harbor auto-antibodies outside of the Ig-like domains, including the Frizzled-like domain (34,13,35). These differences might be caused by racial differences, and/or different disease states of the patients.

Other studies have explored an active immunization model of MuSK MG instead of a passive transfer model with patient auto-antibodies. These studies have shown that bivalent MuSK antibodies activate MuSK phosphorylation and inhibit Agrin-dependent AChR clustering (16,36), whereas monovalent Fab fragments, generated from these antibodies, inhibit MuSK phosphorylation and AChR clustering (37). Because rabbits lack the equivalent of human IgG4 antibodies, and mouse IgG binds complement (38,39), the active immunization models lead to the production of classic, bivalent antibodies that cross-link antigens, deplete cell surface expression and engage complement. In addition, the MuSK epitopes recognized by these polyclonal antibody responses are unknown. As such, the nature of disease in the active immunization model is distinct from MuSK MG caused by human IgG4 antibodies.

Passive transfer of total IgG from MuSK MG patients into mice can stimulate rather than inhibit MuSK phosphorylation, and in contrast to our findings, induce MuSK internalization (40). Given the structure and function of IgG4 antibodies, as well as our findings showing that IgG4 antibodies inhibit MuSK phosphorylation, it seems likely that stimulation of MuSK phosphorylation was due to IgG1-3 rather than IgG4 in these passive transfer experiments. Combining the IgG1-3 and IgG4 fractions might mask individual effects of the different anti-MuSK IgG subclasses. Because IgG4 rather than IgG1-3 antibodies are pathogenic in Dutch MuSK MG patients (12), these findings, taken together, raise the possibility that different ethnic groups generate different immune responses to MuSK and that some MG patients carry IgG1-3 antibodies to MuSK that cause disease by other mechanisms. As such, identifying the optimal therapy for MuSK MG may require an understanding of the mechanism by which MuSK antibodies cause disease in individual MuSK MG patients.

MuSK is essential for all known aspects of presynaptic and postsynaptic differentiation (9,10,17). As such, antibodies that inhibit MuSK function would be expected to disrupt the architecture of the neuromuscular synapse as well as perturb neurotransmitter release and reception (41,9,42,34,18,43,44,19). Because the synaptic accumulation of acetylcholinesterase (AChE), like all other postsynaptic proteins, depends upon MuSK (10,42), IgG4 antibodies to MuSK are likely to lower AChE expression at the synapse, which may explain the hypersensitivity of MuSK MG patients to AChE inhibitors.

In AChR MG, the decrease in AChR expression and function leads to a compensatory increase in neurotransmitter release, termed quantal content. An increase in quantal content, however, is not evident in MuSK MG (12,16,45).

These findings suggest that MuSK plays an important role in this homeostatic response. Because muscle inactivity increases MuSK expression (46), antibodies to AChRs may stimulate MuSK expression and MuSK-dependent retrograde signaling, thereby increasing quantal content. Because MuSK signaling is required to cluster LRP4, which serves as a retrograde signal for presynaptic differentiation (25), antibodies that inhibit MuSK are likely to compromise presynaptic differentiation and prevent a compensatory increase in transmitter release. If so, auto-antibodies to LRP4 may likewise perturb presynaptic and postsynaptic differentiation (40,47,12,48,49).

Traditionally, IgG4 antibodies have been considered to have an anti-inflammatory role, as they can compete with IgG1-3, thereby attenuating antigen down-regulation, complement-mediated cell damage and inflammation.

There is growing evidence, however, that IgG4 antibodies can be pathogenic, as IgG4 antibodies against desmoglein cause a skin-blistering disease, termed pemphigus (50), and IgG4 antibodies to the phospholipase A2 receptor are thought to

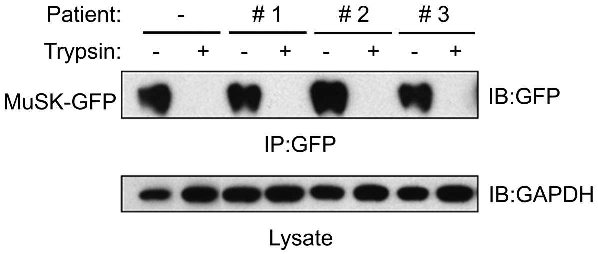


Figure 4. IgG4 antibodies from MuSK MG patients do not reduce MuSK cell surface expression. 3T3 cells, which were transiently transfected with MuSK-GFP, were treated with MuSK MG IgG4 antibodies for 24 h (Figure 3). Cells were harvested, or treated with trypsin prior to harvesting. MuSK-GFP was immunoprecipitated from lysates and detected in Western blots using antibodies to GFP, and the level of GAPDH in lysates was determined by Western blotting.

contribute to membranous nephropathy (51). Moreover, IgG4 auto-antibodies to Leucine-rich glioma inactivated 1 (Lgi1), a regulator of the voltage-gated potassium channel, are thought to be responsible for limbic encephalitis (52). The mechanisms by which these IgG4 antibodies disrupt function and cause disease, however, are not understood. Our studies provide the first mechanistic understanding of an auto-immune disease caused by IgG4 antibodies and may shed light on the mechanisms of other IgG4 mediated auto-immune diseases.

ACKNOWLEDGEMENTS

We thank Prof. André Deelder and Carolien Koeleman for technical support in the IgG purifications and Ingrid Hegeman, Dr. Jan Kuks and Dr. Aad Verrips for a longstanding collaboration in collecting MuSK MG material. We thank Stevan Hubbard for his keen insight and suggesting substitutions that generated a more active form of MuSK. This research was supported by funding of the Prinses Beatrix Spierfonds, Association Française contre les Myopathies, a short term fellowship from the European Molecular Biology Organization to M.G.H., a research grant (NS36193) from the NIH to S.J.B. and a Veni fellowship to ML from the Netherlands Organization for Scientific Research and a fellowship of the Brain Foundation.

REFERENCES

1. Lennon VA, Lindstrom JM, Seybold ME (1975) Experimental autoimmune myasthenia: A model of myasthenia gravis in rats and guinea pigs. J Exp Med 141:1365-1375.
2. Lindstrom JM et al. (1976) Pathological mechanisms in experimental autoimmune myasthenia gravis. II. Passive transfer of experimental autoimmune myasthenia gravis in rats with anti-acetylcholine recepotr antibodies. J Exp Med 144:739-753.
3. Rodgaard A et al. (1987) Acetylcholine receptor antibody in myasthenia gravis: predominance of IgG subclasses 1 and 3. Clin Exp Immunol 67:82-88.
4. Engel AG, Arahata K (1987) The membrane attack complex of complement at the endplate in myasthenia gravis. Ann N Y Acad Sci 505:326-332.
5. Drachman DB et al. (1978) Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. N Engl J Med 298:1116-1122.
6. Drachman DB, Adams RN, Josifek LF, Self SG (1982) Functional activities of autoantibodies to acetylcholine receptors and the clinical severity of myasthenia gravis. N Engl J Med 307:769-775.
7. Christadoss P (1988) C5 gene influences the development of murine myasthenia gravis. J Immunol 140:2589-2592.
8. Papanastasiou D, Poulas K, Kokla A, Tzartos SJ (2000) Prevention of passively transferred experimental autoimmune myasthenia gravis by Fab fragments of monoclonal antibodies directed against the main immunogenic region of the acetylcholine receptor. J Neuroimmunol 104:124-132.
9. Burden SJ, Yumoto N, Zhang W (2013) The role of MuSK in synapse formation and neuromuscular disease. Cold Spring Harb Perspect Biol 5:a009167-
10. DeChiara TM et al. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85:501-512.
11. Hoch W et al. (2001) Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. Nat Med 7:365-368.
12. Klooster R et al. (2012) Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. Brain 135:1081-1101.
13. McConville J et al. (2004) Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. Ann Neurol 55:580-584.
14. Niks EH et al. (2008) Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. J Neuroimmunol 195:151-156.
15. van der Neut KM et al. (2007) Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 317:1554-1557.
16. Mori S et al. (2012) Antibodies against muscle-specific kinase impair both presynaptic and postsynaptic functions in a murine model of myasthenia gravis. Am J Pathol 180:798-810.
17. Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791-805.
18. Kim N et al. (2008) Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell 135:334-342.
19. Zhang B et al. (2008) LRP4 serves as a coreceptor of agrin. Neuron 60:285-297.
20. Zhang W, Coldefy AS, Hubbard SR, Burden SJ (2011) Agrin binds to the N-terminal region of Lrp4 protein and stimulates association between Lrp4 and the first immunoglobulin-like domain in muscle-specific kinase (MuSK). J Biol Chem 286:40624-40630.
21. Hallock PT et al. (2010) Dok-7 regulates neuromuscular synapse formation by recruiting Crk and Crk-L. Genes Dev 24:2451-2461.

22. Hamuro J et al. (2008) Mutations causing DOK7 congenital myasthenia ablate functional motifs in Dok-7. J Biol Chem 283:5518-5524.

23. Okada K et al. (2006) The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science 312:1802-1805.

24. Stiegler AL, Burden SJ, Hubbard SR (2006) Crystal structure of the agrin-responsive immunoglobulin-like domains 1 and 2 of the receptor tyrosine kinase MuSK. J Mol Biol 364:424-433.

25. Yumoto N, Kim N, Burden SJ (2012) Lrp4 is a retrograde signal for presynaptic differentiation at neuromuscular synapses. Nature 489:438-442.

26. Hesser BA, Henschel O, Witzemann V (2006) Synapse disassembly and formation of new synapses in postnatal muscle upon conditional inactivation of MuSK. Mol Cell Neurosci 31:470-480.

27. Kong XC, Barzaghi P, Ruegg MA (2004) Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. EMBO Rep 5:183-188.

28. Im SH, Barchan D, Fuchs S, Souroujon MC (2000) Mechanism of nasal tolerance induced by a recombinant fragment of acetylcholine receptor for treatment of experimental myasthenia gravis. J Neuroimmunol 111:161-168.

29. Lennon VA et al. (1991) Recombinant human acetylcholine receptor alpha-subunit induces chronic experimental autoimmune myasthenia gravis. J Immunol 146:2245-2248.

30. Lindstrom J, Einarson B (1979) Antigenic modulation and receptor loss in experimental autoimmune myasthenia gravis. Muscle Nerve 2:173-179.

31. Luo J et al. (2009) Main immunogenic region structure promotes binding of conformation-dependent myasthenia gravis autoantibodies, nicotinic acetylcholine receptor conformation maturation, and agonist sensitivity. J Neurosci 29:13898-13908.

32. Mahler M, Fritzler MJ (2010) Epitope specificity and significance in systemic autoimmune diseases. Ann N Y Acad Sci 1183:267-287.

33. Till JH et al. (2002) Crystal structure of the MuSK tyrosine kinase: insights into receptor autoregulation. Structure 10:1187-1196.

34. Kawakami Y et al. (2011) Anti-MuSK autoantibodies block binding of collagen Q to MuSK. Neurology 77:1819-1826.

35. Takamori M, Nakamura T, Motomura M (2013) Antibodies against Wnt receptor of muscle-specific tyrosine kinase in myasthenia gravis. J Neuroimmunol 254:183-186.

36. Shigemoto K et al. (2006) Induction of myasthenia by immunization against muscle-specific kinase. J Clin Invest 116:1016-1024.

37. Mori S et al. (2012) Divalent and monovalent autoantibodies cause dysfunction of MuSK by distinct mechanisms in a rabbit model of myasthenia gravis. J Neuroimmunol 244:1-7.

38. Dangl JL et al. (1988) Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. EMBO J 7:1989-1994.

39. Oi VT et al. (1984) Correlation between segmental flexibility and effector function of antibodies. Nature 307:136-140.

40. Cole RN et al. (2010) Patient autoantibodies deplete postsynaptic muscle-specific kinase leading to disassembly of the ACh receptor scaffold and myasthenia gravis in mice. J Physiol 588:3217-3229.

41. Amenta AR et al. (2012) Biglycan is an extracellular MuSK binding protein important for synapse stability. J Neurosci 32:2324-2334.

42. Cartaud A et al. (2004) MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. J Cell Biol 165:505-515.

43. Luo ZG et al. (2002) Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. Neuron 35:489-505.

44. Ngo ST et al. (2012) Neuregulin-1 potentiates agrin-induced acetylcholine receptor clustering through muscle-specific kinase phosphorylation. J Cell Sci 125:1531-1543.

45. Viegas S et al. (2012) Passive and active immunization models of MuSK-Ab positive myasthenia: electrophysiological evidence for pre and postsynaptic defects. Exp Neurol 234:506-512.

46. Valenzuela DM et al. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron 15:573-584.

47. Higuchi O, Hamuro J, Motomura M, Yamanashi Y (2011) Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. Ann Neurol 69:418-422.

48. Pevzner A et al. (2012) Anti-LRP4 autoantibodies in AChR- and MuSK-antibody-negative myasthenia gravis. J Neurol 259:427-435.

49. Zhang B et al. (2012) Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. Arch Neurol 69:445-451.

50. Di ZG et al. (2012) Pemphigus autoantibodies generated through somatic mutations target the desmoglein-3 cis-interface. J Clin Invest 122:3781-3790.

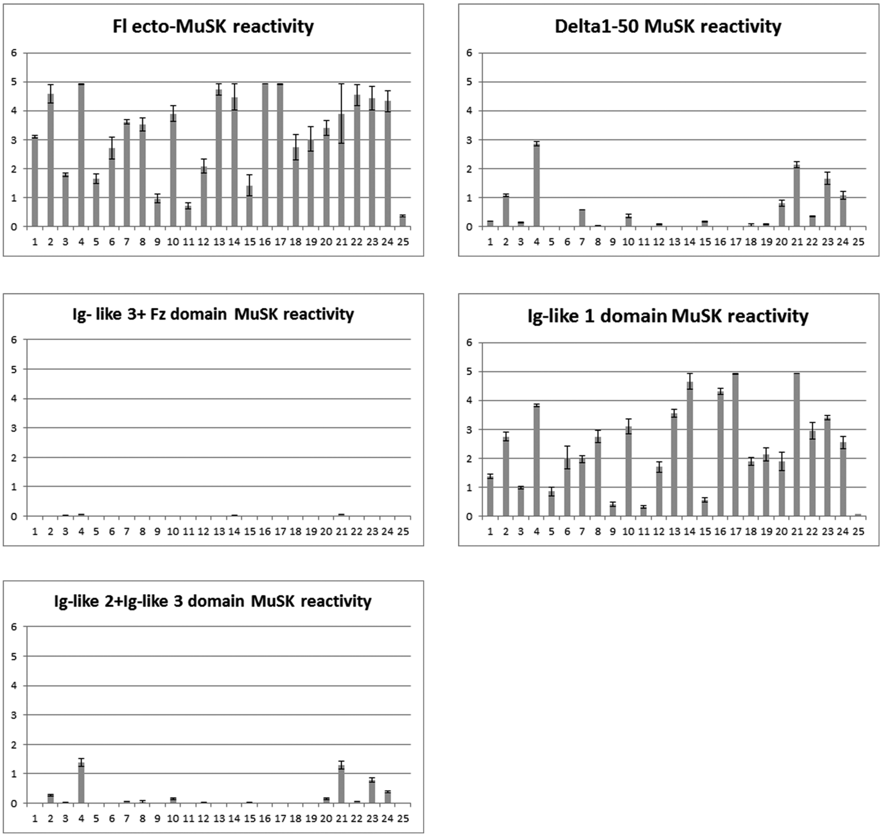
51. Beck LH, Jr. et al. (2009) M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med 361:11-21.

52. Irani SR et al. (2012) Morvan syndrome: clinical and serological observations in 29 cases. Ann Neurol 72:241-255.

53. Khosroshahi A, Bloch DB, Deshpande V, Stone JH (2010) Rituximab therapy leads to rapid decline of serum IgG4 levels and prompt clinical improvement in IgG4-related systemic disease. Arthritis Rheum 62:1755-1762.

54. Herbst R, Burden SJ (2000) The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling. EMBO J 19:67-77.

SUPPLEMENTARY DATA


Fl ecto-MuSK reactivity

Delta1-50 MuSK reactivity

Ig- like 3+ Fz domain MuSK reactivity

Ig-like 1 domain MuSK reactivity

Ig-like 2+Ig-like 3 domain MuSK reactivity

Figure S1 Overview of individual patient antibody reactivity to the full length extracellular domain or truncated versions of recombinant MuSK proteins. The graph represents average reactivity based on three independent experiments with error bars depicting the SEM values.

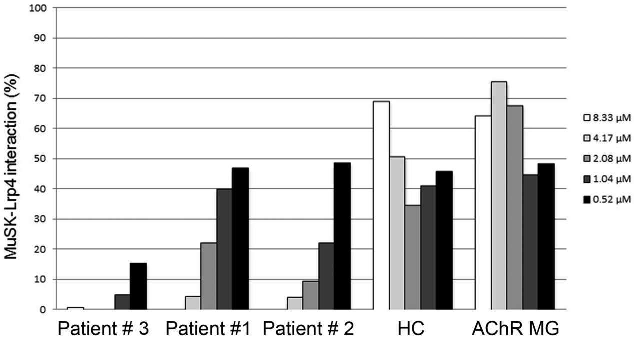


Figure S2 MuSK MG IgG4 antibodies inhibit MuSK-LRP4 interaction in a dose-dependent manner.

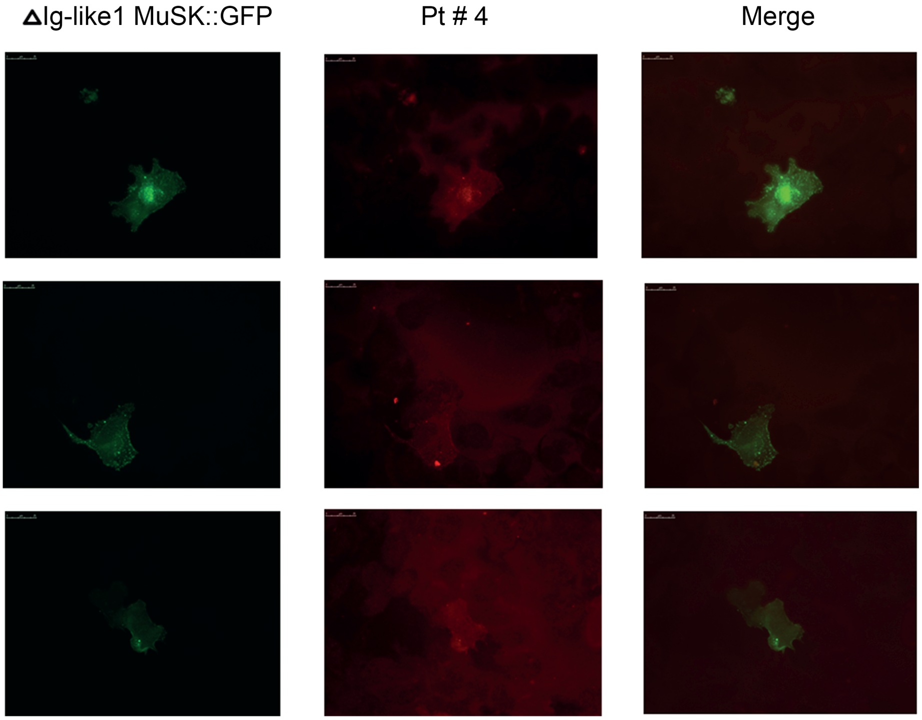


Figure S3 Examples of MuSK MG pt # 4 antibody staining of cells expressing ΔIg-like 1 MuSK::GFP.

Table S1 Peptide sequences from the first MuSK Ig-like domain. The overlap between consecutive peptides is underlined. The first Ig-like domain in MuSK extends from amino acid 25 to 119 (Stiegler et al., 2006).

Peptide #	**Peptide sequences**	**Position of peptide**
1	TEKLPKAPVITTPLETVDAL	24-44
2	TTPLETVDALVEEVATFMCA	34-54
3	VEEVATFMCAVESYPQPEIS	44-64
4	VESYPQPEISWTRNKILIKL	54-74
5	WTRNKILIKLFDTRYSIREN	64-84
6	FDTRYSIRENGQLLTILSVE	74-94
7	GQLLTILSVEDSDDGIYCCT	84-104
8	DSDDGIYCCTANNGVGGAVE	94-114
9	ANNGVGGAVESCGALQVKMK	104-124

Table S2 ONE-WAY ANOVA analysis of patient antibody reactivity to the peptide sequences and the Ig-like1 domain of MuSK (Ig1-TAP). The average reactivity of three MuSK MG patients IgG4 to the peptide sequences did not differ significantly between the different peptides covering the Ig-like 1 domain of MuSK. The reactivity to the first Ig-like domain significantly reduced MuSK reactivity in these IgG4 samples compared to the peptides. IgG4 MuSK MG patient antibodies do not bind to linear sequences covering the first Ig-like domain of MuSK.

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
1 vs 2	-1,135	0,08892	P > 0.05	-49.69 to 47.42
1 vs 3	3,545	0,2777	P > 0.05	-45.01 to 52.10
1 vs 4	-0,2572	0,02015	P > 0.05	-48.81 to 48.30
1 vs 5	9,087	0,712	P > 0.05	-39.47 to 57.64
1 vs 6	1,361	0,1066	P > 0.05	-47.20 to 49.92
1 vs 7	9,586	0,751	P > 0.05	-38.97 to 58.14
1 vs 8	3,913	0,3066	P > 0.05	-44.64 to 52.47
1 vs 9	9,166	0,7182	P > 0.05	-39.39 to 57.72
1 vs Ig1-TAP	76,59	6,001	P < 0.001	28.03 to 125.1
2 vs 3	4,68	0,3666	P > 0.05	-43.88 to 53.24
2 vs 4	0,8777	0,06877	P > 0.05	-47.68 to 49.43
2 vs 5	10,22	0,8009	P > 0.05	-38.33 to 58.78
2 vs 6	2,495	0,1955	P > 0.05	-46.06 to 51.05
2 vs 7	10,72	0,8399	P > 0.05	-37.84 to 59.28
2 vs 8	5,048	0,3955	P > 0.05	-43.51 to 53.60
2 vs 9	10,3	0,8071	P > 0.05	-38.26 to 58.86
2 vs Ig1-TAP	77,73	6,09	P < 0.001	29.17 to 126.3
3 vs 4	-3,802	0,2979	P > 0.05	-52.36 to 44.76
3 vs 5	5,543	0,4343	P > 0.05	-43.01 to 54.10
3 vs 6	-2,184	0,1711	P > 0.05	-50.74 to 46.37
3 vs 7	6,041	0,4733	P > 0.05	-42.52 to 54.60
3 vs 8	0,3682	0,02885	P > 0.05	-48.19 to 48.93
3 vs 9	5,622	0,4405	P > 0.05	-42.94 to 54.18
3 vs Ig1-TAP	73,05	5,723	P < 0.001	24.49 to 121.6
4 vs 5	9,345	0,7321	P > 0.05	-39.21 to 57.90
4 vs 6	1,618	0,1267	P > 0.05	-46.94 to 50.17
4 vs 7	9,843	0,7712	P > 0.05	-38.71 to 58.40
4 vs 8	4,17	0,3267	P > 0.05	-44.39 to 52.73
4 vs 9	9,424	0,7383	P > 0.05	-39.13 to 57.98
4 vs Ig1-TAP	76,85	6,021	P < 0.001	28.29 to 125.4
5 vs 6	-7,727	0,6054	P > 0.05	-56.28 to 40.83
5 vs 7	0,4982	0,03903	P > 0.05	-48.06 to 49.06
5 vs 8	-5,175	0,4054	P > 0.05	-53.73 to 43.38
5 vs 9	0,07902	0,006191	P > 0.05	-48.48 to 48.64
5 vs Ig1-TAP	67,5	5,289	P < 0.01	18.95 to 116.1
6 vs 7	8,225	0,6444	P > 0.05	-40.33 to 56.78
6 vs 8	2,552	0,2	P > 0.05	-46.00 to 51.11
6 vs 9	7,806	0,6116	P > 0.05	-40.75 to 56.36
6 vs Ig1-TAP	75,23	5,894	P < 0.001	26.67 to 123.8

Table S2. (continued)

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
7 vs 8	-5,673	0,4444	P > 0.05	-54.23 to 42.88
7 vs 9	-0,4192	0,03284	P > 0.05	-48.98 to 48.14
7 vs Ig1-TAP	67,01	5,25	P < 0.01	18.45 to 115.6
8 vs 9	5,254	0,4116	P > 0.05	-43.30 to 53.81
8 vs Ig1-TAP	72,68	5,694	P < 0.001	24.12 to 121.2
9 vs Ig1-TAP	67,43	5,283	P < 0.01	18.87 to 116.0