The pathophysiology of MuSK myasthenia gravis

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This thesis was supported by ArgenX, Prinses Beatrix Spierfonds and L'Association Française contre les myopathies

Cover design: Bureau Kleur door Putri

Layout & Printing: Off Page, Amsterdam

ISBN: 978-94-6182-681-7

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof.mr.C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op 6 juli 2016
klokke 13.45

Door

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Geboren te Eindhoven
in 1985

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Aan mijn ouders

CHAPTER 1

General introduction

Adapted from: Pathogenic immune mechanisms at the neuromuscular synapse: the role of specific antibody-binding epitopes in myasthenia gravis

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Journal of Internal Medicine 2014; 275(1):12-26.

ABSTRACT

Myasthenia gravis (MG) is a heterogeneous disease hallmarked by fatigable muscle weakness. Auto-antibodies against three different postsynaptic antigens and one presynaptic antigen at the neuromuscular junction are known to cause myasthenic syndromes. Diagnosing the antigen involved in the autoimmune response is essential as treatment responsiveness varies between myasthenic syndromes. Moreover, the mechanisms by which the auto-antibodies cause muscle weakness varies from antigenic modulation and complement mediated membrane damage, to inhibition of endogenous ligand binding and blocking of essential protein-protein interactions. These mechanisms are related to the auto-antibody titre, specific epitopes on the target proteins and IgG auto-antibody subclass. These characteristics guide the development of more specific treatment strategies for each of the myasthenic syndromes.

We review here the role of specific auto-antibody-binding epitopes in the different myasthenic syndromes, their possible relevance to the pathophysiology of the disease, and potential implications of epitope mapping knowledge for new therapeutic strategies.

INTRODUCTION

Myasthenia gravis (MG) is one of the best-characterized autoimmune diseases and is a direct consequence of autoimmunity at the neuromuscular junction (Fig. 1). To date, this disease has been associated with antibodies against four postsynaptic proteins at the neuromuscular synapse, including the acetylcholine (ACh) receptor (AChR), muscle-specific kinase (MuSK), low-density lipoprotein receptor-related protein 4 (LRP4) and agrin. Autoimmunity against the presynaptic Cav2.1 voltage-gated calcium channels (VGCCs) causes a distinct myasthenic syndrome, Lambert–Eaton myasthenic syndrome (LEMS). There are multiple mechanisms by which these serum auto-antibodies may interfere with the function of these proteins, including (i) complement-mediated destruction of the membrane, (ii) antigenic modulation by crosslinking of the target antigen, (iii) competition at ligand-binding sites, and (iv) steric hindrance that inhibits conformational changes or interactions with associated molecules.

These mechanisms are not equally important for the pathophysiology of all forms of MG and depend on the epitope specificity of the auto-antibody and its immunoglobulin subclass, as will be discussed below. An overview of these myasthenic syndromes is shown in Table 1.

MG associated with antibodies against AChR (AChR MG)

Although the existence of acetylcholine receptor (AChR) auto-antibodies had been postulated for many years, it was not until the mid-1970s that Lindstrom, Lennon et al. demonstrated their presence in 87% of MG patients (1,2,3). MG caused by AChR auto-antibodies is characterized by fluctuating muscle weakness, often starting with extraocular muscle weakness progressing in a craniocaudal direction. Epidemiological analysis of patients with AChR MG revealed a bimodal distribution with a female predominance in early-onset MG (at 20– 40 years of age) (4). Late-onset MG (above the age of 60 years of age) is more common in men, and there is a higher rate of association with thymoma in this age group.

Several lines of evidence suggest thymic involvement in the aetiology of AChR MG: 10% of patients have a thymoma (5), thymic lymphocytes can spontaneously produce AChR auto-antibodies (6), thymus epithelium expresses AChRs, and some patients benefit from thymectomy (7,8). There are indications of dysfunction of the autoimmune regulator protein and the interferon signalling pathway causing a disturbance of the quantitative expression of the AChR in the thymus, thereby lowering the threshold for AChR autoimmunity (9). However, the exact role of the thymus in the pathogenesis of AChR MG is not yet understood.

Pathogenesis of AChR MG

The pathogenesis of AChR MG is directly linked to AChR auto-antibodies of the IgG1 and IgG3 subtypes (10,11). These antibodies induce myasthenia through

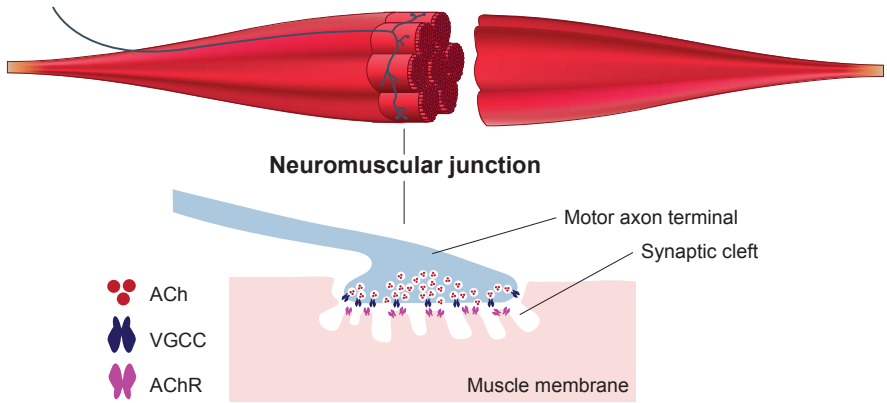

Figure 1. Schematic representation of the neuromuscular junction and its localization within the muscle. Neuromuscular junctions are located in the central part of the muscle. Each muscle fibre possesses one neuromuscular junction. The detailed image of the neuromuscular junction shows the presynaptic motor nerve terminal, where upon depolarization the voltage-gated calcium channels (VGCCs) open. This results in Ca^{2+} influx that activates the release of acetylcholine (ACh) from synaptic vesicles through exocytosis. The postsynaptic apparatus contains ACh receptors (AChRs) which are ligand-gated ion channels that open in response to ACh binding. The resulting ion fluxes depolarize the membrane which leads to a cascade of events that eventually cause muscle fibre contraction.

three distinct mechanisms: complement-mediated postsynaptic membrane damage (12); cross-linking by bivalent IgG1 and IgG3 molecules (antigenic modulation), causing internalization of AChRs and depletion of its surface pools (13); and competition with ACh on binding sites of AChRs, preventing activation and opening of the ion channel (14). These effects hamper neuromuscular transmission and ultimately result in fatigable muscle weakness. The importance of blocking of the ACh-binding site is controversial. Several groups have described blocking antibodies in varying proportions of their cohorts and showed that they were heterogeneous in their subclass distribution (15,16,17,18). In one study, it was found that there was no correlation between the titre of these ligand-blocking antibodies and clinical weakness (19). Others found significant correlations between the degree of ligand-binding block and muscle weakness (14). ACh-binding site antibodies may play an important pathogenic role in some, but not all AChR MG patients.

In addition, it has been hypothesized that some auto-antibodies might physically block the ion channel pore of the AChR; however, this has not been supported by experimental evidence. In fact, in an elegant study in 1995, Beroukhim and Unwin showed that auto-antibodies against the main immunogenic region (MIR) do not block the ion channel, but are located at the outer side of the subunit and extend away from the channel pore (20).

Epitope mapping in AChR MG

The effector functions of the auto-antibodies are highly correlated with their IgG subclass and the epitopes they recognize. The 'muscle type' nicotinic AChR is

a transmembrane protein comprising five subunits with a stoichiometry of α2βγδ (an overview of the AChR structure and MIR localization is shown in Fig. 2). In the adult AChR, the γ subunit is replaced by the ε subunit. Auto-antibodies against the AChR recognize a large variety of epitopes on the receptor (21). Epitope mapping in 86 patients revealed that, depending on disease severity, more than half of the different AChR auto-antibodies bind to a distinct part of the AChR α-subunit, thus termed the MIR (21,22). Parts of the β and γ subunits adjacent to the MIR were also found to be immunogenic (23). Auto-antibodies against the ε subunit have been reported to cause acquired slow channel myasthenia, which is characterized by delayed closure of the AChR ion channel (24). A study amongst 102 patients with either ocular or generalized AChR MG showed that anti-MIR antibodies are significantly more common in the patients with generalized disease and that the titre and presence of these antibodies exclusively correlate with disease severity (25). The authors of this study therefore concluded that MIR auto-antibodies play a crucial role in the pathogenesis, can be useful in predicting disease severity and may form a promising target for future therapies (26, 27, 28).

The AChR MIR is composed of a set of (overlapping) epitopes mainly located around a loop of amino acids 66–76 on the α1 subunit (21, 26). Interaction between the α1 66–76 loop and the N- terminal α1 1–14 amino acids creates a three-dimensional structure that is essential for making the MIR even more myasthenogenic (29,30) The structural integrity of this epitope is required for most auto-antibodies (31). When antibodies bind to the MIR, they often prevent binding of other antibodies against the MIR to the entire region (21). Amino acids 68 and 71 are of particular importance for monoclonal antibody binding to the MIR. Mutating these residues resulted in almost complete loss of antibody binding (32). Immunization of rats with a chimera of the human α1-MIR and ACh-binding protein from Aplysia induced experimental autoimmune myasthenia gravis (EAMG), suggesting that the MIR sequence alone is enough to trigger antibodies that cause clinical myasthenia (33, 34). Surprisingly, the ACh-binding protein alone also induced EAMG in some rats. This is most likely to be due to sequence homology (20%) between the ACh-binding protein and the human AChR α1 subunit. Thus, in AChR-associated EAMG, antibodies against the human AChR MIR are sufficient to induce myasthenia, whilst structural integrity and the presence of additional epitopes of other AChR regions are enhancing factors. This is in agreement with observations in passive transfer MG rat models in which monoclonal antibodies against the MIR alone had less myasthenogenic potency than polyclonal serum antibodies obtained from rats with EAMG induced by active immunization (2,35). These results emphasize the important role of epitope spreading in the development of autoimmune diseases.

Epitope spreading in AChR MG

The epitope spreading hypothesis proposes that initial epitope targets of auto-antibodies do not remain fixed, but extend to other epitopes within the same protein and/or even to other, closely associated, proteins. This phenomenon has been

described for a wide variety of autoimmune diseases (reviewed by Vanderlugt and Miller (36)). In EAMG, epitope spreading may be responsible not only for maintaining but also for enhancing myasthenia by potentiating antigenic modulation and surface depletion of the receptors.

For AChR MG, epitope spreading has been described in several EAMG animal models (34, 37,38,39). Vincent et al. (37) immunized rabbits with a mixture of synthetic peptides including amino acids 138–199 of the human α1 AChR subunit. This resulted in a broad immune response against epitopes outside of this region of the rabbit AChR (including the MIR). A more recent study demonstrated a similar effect in rats immunized with parts of the extracellular domain of the AChR α subunit. After 3 weeks, auto-antibodies against the cytoplasmic domain could be detected and they seemed to correlate with the onset of clinical weakness in the animals (39). Investigating epitope spreading in myasthenic humans is difficult as there is usually a delay between the onset of the disease and the first visit to the clinic. However, in addition to the MIR antibodies, auto-antibodies against the cytoplasmic domain of the α1 AChR subunit have been detected in sera from MG patients, suggesting that epitope spreading occurs in humans as well (40).

Besides enhancing cross-linking and subsequent endocytosis of AChRs, the epitope spreading phenomenon may also facilitate complement activation because it eventually induces the build-up of a high density of antigen–antibody complexes at the postsynaptic membrane (41,42). Epitope spreading can also expand the immune response beyond the initial antigen. For AChR MG, an array of other antigenic targets are known (i.e. titin, ryanodine receptor 1 and 2, Kv 1.4 α-subunit, actin, α-actinin, tropomyosin, myosin, filamin, vinculin, rapsyn and HSP-70) (43,44,45,46,47,48). There is no evidence that these auto-antibodies induce MG by themselves, that is, without the presence of AChR antibodies. However, they are associated with a more severe clinical progression. One hypothesis is that these antibodies develop as secondary responses to muscle fibre destruction, exposing intracellular muscle proteins to an inflammatory environment, thereby facilitating the breakage of immunotolerance. Alternatively, these antibodies are often only found in the context of a thymoma, suggesting that they might result from an anti-tumour response rather than an anti-muscle response.

Various causes of epitope spreading have been suggested. It might be a useful ability of the immune system to enhance the response towards invading pathogens, such as bacteria or viruses. Another possible explanation is the opportunity for the immune system to deviate the pathogenic response into a protective immune response (49). Activating immunity against other nonpathogenic epitopes can divert the immune response away from the pathogenic response and dilute its effects (50). This theory has been applied for the development of more specific therapies for MG (see below). Understanding the basis of epitope spreading will be of importance for further development of specific treatments.

Role of IgG subclass in AChR MG

Human serum contains four different IgG subclasses, each with specific characteristics. AChR MG is caused by auto-antibodies of the IgG1 and IgG3 subclass (10,11). These auto-antibodies have high affinity for Fc receptors on immune cells and are also potent complement activators, in contrast to IgG2 and IgG4. Complement-mediated pathology in MG is strongly suggested by the deposition of complement factors at neuromuscular junctions of both humans with AChR MG and animals with EAMG (51,52). Furthermore, complement consumption increases during exacerbations of the disease; rats that are deficient in complement components C3 and C5 are protected against experimental MG after both active and passive immunization protocols, and susceptibility to EAMG is increased in mice deficient in complement inhibitor decay accelerating factor (53,54).

The other main pathological mechanism in AChR MG, antigenic modulation, is directly linked to the functional bivalency of IgG1 and IgG3 and their ability to bind two antigen molecules. Drachman and colleagues showed both *in vitro* and *in vivo* that AChR antibodies cause loss of postsynaptic AChRs (13,55). However, monovalent Fab fragments (produced from these antibodies by papain digestion) failed to produce such AChR depletion. Crosslinking of these Fab fragments restored their AChR-reducing ability. These results demonstrated that AChR auto-antibodies deplete surface AChRs through crosslinking and internalization. Therefore, the specific characteristics of the IgG subclasses involved are key determinants in the pathophysiology of AChR MG.

Therapeutic strategies evolved from epitope mapping in AChR MG

Currently, symptomatic treatment of AChR MG mainly consists of acetylcholinesterase (AChE) inhibition. Furthermore, the immune system is often (non-specifically) suppressed by plasmapheresis, intravenous administration of immunoglobulins and/or immunosuppressive drugs, or thymectomy. For successful treatment, these therapies often need to be continued for several years to prevent return of the symptoms. Specific treatment, which ideally would block the production and/or effects of only the pathogenic auto-antibodies in one therapeutic session, is lacking. Epitope mapping has provided clues to the development of more specific treatment options; some of the approaches are discussed below.

Several groups demonstrated that nasal and oral tolerization with either recombinant fragments of the AChR, synthetic peptide sequences or native AChRs can prevent the onset of EAMG in animals (56,57,58,59,60,61). Active EAMG could be modestly inhibited in rats by oral administration of recombinant α1 subunit extracellular domain (62). However, there are concerns about the risk of intensifying the autoimmune response rather than suppressing it (63, 64). Luo et al. (50) developed an immunotherapy to divert the immune response away from the AChR MIR in EAMG by administering a mixture of cytoplasmic domains of human AChR subunits. This type of epitope spreading successfully suppressed ongoing EAMG in rats by shifting

Table 1. Summary of the features of myasthenia subtypes

	AChR MG	MuSK MG
Percentage of myasthenic patients	85%	8%
Muscle weakness distribution pattern	Ocular at onset, progressing to generalized proximal weakness in a craniocaudal direction	Generalized, often with weakness in bulbar and respiratory muscles
Tumour	Thymoma in 10% of patients	–
Responsiveness to AChE inhibitors	+++	–
HLA association	A1-B8-DR3 in early-onset MG	DR14-DQ5
IgG subclass	IgG1 and IgG3	IgG4
Target antigen	AChR	MuSK
MIR	α-subunit: amino acids 66–67	N-terminal Ig-like 1 domain
Additional epitopes within the main antigen	α-subunit β-subunit γ-subunit ε-subunit	Cysteine-rich domain Ig-like 2 domain
Epitope spreading outside of the main antigen	Antibodies against other proteins include: RyR1, RyR2, titin, actin, actinin, myosin, filamin, vinculin, rapsyn, HSP-70,	Additional antibodies against the AChRs or LRP4
Structural epitope	Yes	Yes
Pathogenic mechanisms:		
Complement mediated	+++	–
Antigenic modulation	+++	++
Ligand binding site competition	+ (with ACh)	+++ (with ColQ and LRP4)
Steric hindrance	–	Not known

the auto-antibody specificity towards the cytoplasmic domain. These antibodies are considered harmless, because they cannot access and bind to the AChR *in vivo*. There was also a slight shift in AChR auto-antibody subclass from less IgG2b (inflammatory in rats) to an increase in IgG1 (anti-inflammatory in rats), although this could not solely account for the marked decrease in myasthenic symptoms. This approach is considered safer as no potentially pathological significant epitopes are introduced.

Another epitope-specific approach was explored by Araga and colleagues. They used complementary peptides to generate an anti-idiotype antibody response. These anti-idiotype antibodies were able to neutralize two monoclonal pathogenic MIR AChR antibodies *in vitro* and prevented the onset of EAMG *in vivo* (65, 66). Similar results were also reported after immunization with a peptide that was developed to inhibit AChR reactive T cells by an anti-idiotype approach (67).

LRP4 MG	LEMS
5%	2%
Generalized, sometimes with bulbar muscle weakness	Proximal leg weakness at onset, progressing to generalized proximal weakness in caudocranial direction
–	Small cell lung cancer in 50–60% of patients
++	+
Not known	B8-DR3 (in early onset patients without tumour)
IgG1	Probably IgG1
LRP4	$Ca_v2.1$ (P/Q-type) VGCC
Not known	α1-subunit
Not known	β subunit
Additional antibodies against MuSK, AChR or VGCC	Antibodies against other proteins including synaptotagmin, m1 AChR, SOX proteins and ERC1
Not known	Probably both structural and linear epitopes
Not known	–
Not known	++
++ (with agrin)	–
Not known	Not known

Furthermore, specific removal of pathogenic AChR auto-antibodies has been attempted with affinity-based apheresis. This approach would modify and improve the current practice of total plasmapheresis, as it does not concomitantly remove other, potentially important, plasma components. Two expression systems (*Escherichia coli* and *Pichia pastoris*) have been used to produce the ectodomains of all human AChR subunits which were then immobilized on a column resin for immunoadsorption of the pathogenic antibodies from MG sera (68,69, 70). In a preliminary experiment using one EAMG rabbit, such selective apheresis prevented a further increase in AChR antibody titre (71).

Finally, another (non-epitope-related) therapeutic approach would be to force the immune system to shift to an IgG4 immune response. IgG4 is considered functionally monovalent and is unable to activate complement (72). *In vitro* it has been shown

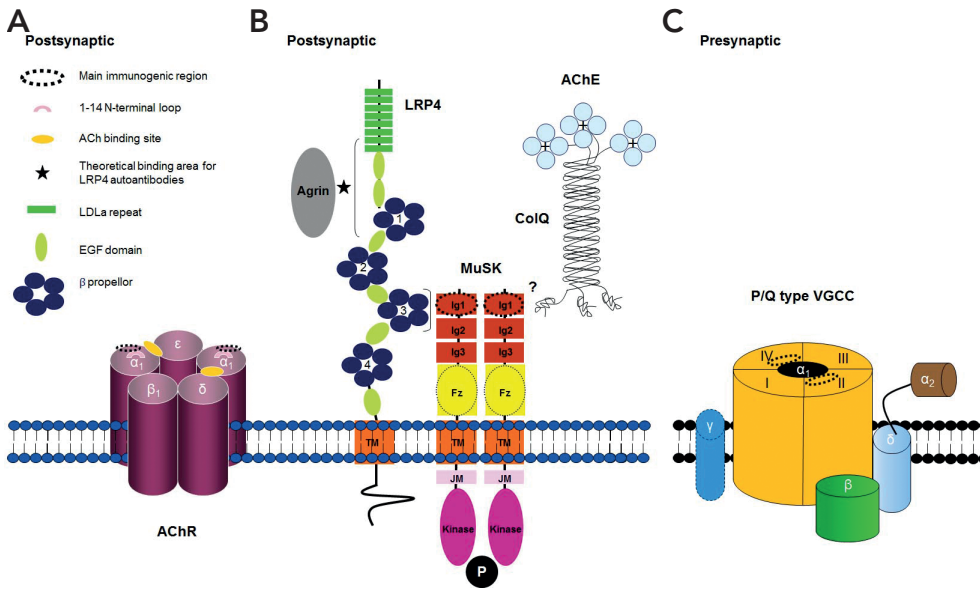

Figure 2. Overview of the antigenic targets in myasthenia gravis (MG) and Lambert–Eaton myasthenic syndrome (LEMS). The acetylcholine receptors (AChRs) are located in the postsynaptic membrane of the neuromuscular junction and each consists of five subunits. The main immunogenic region (MIR) for auto-antibodies in AChR MG is located on the outer side of the extracellular part of the α subunits of the AChR. Muscle-specific kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4) are also transmembrane proteins located on the postsynaptic muscle membrane. Once agrin binds to LRP4, the Ig-like 1 domain and part of the Ig-like 2 domain of MuSK interact with LRP4. This stimulates the dimerization of MuSK and the activation of its kinase domain, essential for AChR clustering and maintenance of the neuromuscular junction. MuSK also interacts with collagen Q (ColQ) [which anchors acetylcholinesterase (AChE) in the synaptic cleft], although the precise domains involved in this interaction are unknown. The MIR of MuSK is located in the first Ig-like domain. However, antibodies directed against other epitopes have also been described. Presynaptically, auto-antibodies against the Cav2.1 type (P-/Q-type) voltage-gated calcium channel (VGCC) cause LEMS. This channel protein complex consists of one pore-forming main subunit (α1), which has four repeating domains (I–IV), and several associated subunits. Only the α1 subunit and the α2 part of the α2δ subunit protrude into the extracellular space and are directly accessible for auto-antibodies *in vivo*. MIRs are contained in the extracellular parts of the α1 domains II and IV. Fz, Frizzled-like domain; TM, transmembrane domain; JM, juxtamembrane domain; LDLa, low-density lipoprotein-like domain class A; EGF, epidermal growth factor-like domain.

that Fab fragments from AChR antibodies can protect the AChR against antigenic modulation and internalization by patients' auto-antibodies (13). Interestingly, an experimental MG model in monkeys with cloned human IgG1 or IgG4 AChR antibodies revealed that the IgG4 antibodies could not induce myasthenia *in vivo*, in contrast to the IgG1 antibodies. Furthermore, administration of these IgG4 antibodies protected the animals from MG when they were subsequently challenged with the IgG1 antibodies (72).

MG associated with antibodies against MuSK (MuSK MG)

In 2001, auto-antibodies against MuSK, a postsynaptic neuromuscular junction transmembrane protein involved in AChR clustering, were discovered (73). MuSK MG is typically observed at an earlier age than when the AChR is involved, is more common in women and is characterized by the involvement of bulbar weakness and atrophy (74). It has been reported that 46% of patients experience respiratory crisis at some point in their lives (74). Moreover, MuSK MG is strongly associated with HLA-DR14-DQ5 (75, 76). In contrast to patients with AChR MG, in those with MuSK MG ocular weakness is less common in the chronic phase of disease, there is no association with thymoma and the response to treatment with AChE inhibitors is generally not effective. In fact, symptoms may worsen in some cases following this treatment (77, 78). In general, patients are treated with prednisone and other immunosuppressive drugs, similar to AChR MG. Recently, it was shown that rituximab, a B-cell-inhibiting monoclonal antibody, might be an effective drug (79).

Pathogenesis of MuSK MG

The involvement of IgG4 auto-antibodies in MuSK MG was suggested by the high prevalence of IgG4 MuSK antibodies in patients' serum and a correlation between IgG4 auto-antibody levels and disease severity (80, 81). Indeed, we have recently demonstrated that IgG4 antibodies alone from MuSK MG patients are able to induce myasthenia in mice (**Chapter 2**). In addition, we showed in this study (82), performed in NOD/SCID mice which lack a functional innate and adaptive immune system, that the IgG4 auto-antibodies against MuSK directly induce the disease. This is in contrast to AChR MG, which, as discussed above, is caused by IgG1 and IgG3 auto-antibodies that cause complement-mediated membrane destruction and cross-linking and internalization of the AChRs. How MuSK auto-antibodies induce pathology was largely unknown.

IgG4 antibodies are unable to activate complement and have a low affinity for Fc receptors on immune cells (72, 83). It is likely that the mechanism by which the auto-antibodies induce pathology directly depends on the epitope to which these antibodies bind. The extracellular domain of MuSK comprises three Ig-like domains and a Frizzled-like domain (an overview of the structure of MuSK and its main immunogenic regions is shown in Fig. 2). Epitope mapping revealed that most auto-antibodies recognize epitopes within the first two extracellular Ig-like domains (79, 84, **Chapter 4**). In one study, 30% of the patients had additional reactivity against the cysteine-rich domain (in the Frizzled-like domain) (85). The cysteine-rich domain has been implicated as a Wnt receptor and signalling through this pathway was shown to be essential for AChR clustering (86,87,88). The pathology seen in patients with antibodies against the cysteine-rich domain might therefore be due to interference with this pathway. Most patients however have auto-antibodies against the Ig-like

1 domain of MuSK. The Ig-like 1 domain has two essential functions. First, the external face of this domain mediates association between MuSK and LRP4, which, when bound to neuronal agrin, strengthens the interaction between MuSK and LRP4 and activates downstream signalling leading to AChR clustering. Secondly, the opposite medial side of the Ig-like 1 domain mediates MuSK dimerization (89, 90). We hypothesize that auto-antibodies against MuSK can induce myasthenia through three potential mechanisms: (i) antigenic modulation and internalization of surface MuSK, (ii) inhibition of MuSK dimerization and/or (iii) interference with MuSK binding part- ners (**Chapter 3**). Any of these mechanisms would cause defects in the maintenance of postsynaptic AChR clustering, thereby impairing neuromuscular transmission and thus causing clinical myasthenia. Indeed, loss of surface MuSK and AChRs was observed both *in vitro* and *in vivo* in passive transfer experiments (91).

The effects of MuSK antibodies on the interaction of MuSK with its binding partners have partly been addressed. Several proteins [e.g. collagen Q (ColQ), biglycan and LRP4] are known to interact with the extracellular domain of MuSK (92,93,94,95). ColQ is able to bind to MuSK when expressed by non-permeabilized COS cells, and it has been suggested that it may be involved in MuSK and AChE localization (93). However, the direct interaction between MuSK and ColQ could not be reproduced in another study (92). Nevertheless, the findings of a recent study indicated that MuSK auto-antibodies can prevent the interaction between MuSK and ColQ [96]. Passive transfer of patient IgG reduced neuromuscular junction ColQ and AChR levels. The authors of this study suggested that myasthenia is induced by a loss of ColQ–MuSK interaction and subsequent loss of AChR clustering (96). However, the same authors have recently shown that MuSK auto-antibodies induce myasthenia in *ColQ* deficient mice, thus suggesting that loss of MuSK-ColQ interaction is not the key pathomechanism in MuSK MG (97). Furthermore, they excluded effects of MuSK auto-antibodies on MuSK–LRP4 interaction in a plate-binding assay. However, agrin, which has been shown to be essential for appropriate association between MuSK and LRP4 (89), was not included in the assay. A possible effect of MuSK auto-antibodies may therefore have been overlooked.

Muscle-specific kinase mutant and knockout mouse models have shown that MuSK is essential for neuromuscular junction formation and maintenance (98, 99). In AChR MG patients and mouse models, the loss of AChR clusters is compensated by upregulation of presynaptic ACh release, via retrograde signalling at individual neuromuscular junctions (100). MuSK MG passive transfer (82,101) and active immunization experiments (102) in mice as well as patient muscle biopsy studies (103,104) have shown that this compensatory upregulation is missing in MuSK MG neuromuscular junctions. This might suggest that the retrograde signalling is regulated by the N-terminal Ig-like domains of MuSK or the interactions that occur at this site. Recently, Yumoto et al. (105) established that LRP4 is a bi-directional signalling molecule. LRP4 is able to bind outgrowing motor axons and induces

presynaptic differentiation independent of MuSK. In parallel, LRP4 is involved in transducing the neuronal agrin signal to stabilize postsynaptic AChR clustering. Thus, interference with patient MuSK auto-antibodies of MuSK–LRP4 interaction might explain the lack of presynaptic compensatory response to the decreased level of AChRs.

Epitope spreading in MuSK MG

The presence of multiple epitopes in MuSK MG suggests that epitope spreading occurs in this disease (**chapter 4**). Furthermore, it has been reported that some patients have auto-antibodies against multiple neuromuscular junction antigens including MuSK (106,107). Passive transfer MuSK MG mouse studies show that there is variation between patients' auto-antibodies in terms of disease potency (82, 108). This might also correlate with the exact epitopes for these auto-antibodies.

Therapeutic strategies from epitope mapping in MuSK MG

Although MuSK MG is an IgG4-mediated disease, many parallels can be drawn with AChR MG and LEMS. Both forms of auto-antibodies require structural epitopes, and their MIRs are located on the most protruding portion of their extracellular domains. Immune evasion therapies as described earlier might therefore be applicable for MuSK MG as well. The role of the IgG4 antibodies in this case is of particular interest. In one case study, it was reported that a patient underwent a class switch from IgG4 MuSK auto-antibodies to IgG1 MuSK auto-antibodies and went into stable remission (81). The loss of monovalent MuSK auto-antibodies might account for this improvement. However, in an active immunization EAMG rabbit model, both monovalent and divalent MuSK antibodies were able to interfere with proper MuSK functioning (109). It remains to be determined whether this is also true for human MuSK auto-antibodies. Drug-induced class switching for MuSK auto-antibodies might thus form a potential future therapy in other MuSK MG patients.

MG associated with antibodies against LRP4 (LRP4 MG)

Recently, LRP4 auto-antibodies were discovered in another subgroup of 'seronegative' MG patients (106,107,110). Antibodies of this type occur in an extremely variable proportion (3–92%) of 'seronegative' MG patients, which might depend on their ethnic origin or the selection criteria for inclusion. Four percent of the 574 samples tested were negative for AChR and MuSK antibodies but were positive for LRP4 antibodies. LRP4 antibodies were also found in 1% of this population where patients also had antibodies against either MuSK or VGCC. Most patients had a MuSK MG-like phenotype, experiencing generalized muscle weakness often combined with bulbar weakness (106,110). Moreover, no association with thymoma was found (106). As in AChR MG, patients with LRP4 MG in general respond well to AChE inhibitors.

Pathogenesis of LRP4 MG

Lipoprotein receptor-related protein 4 is a transmembrane protein of which the extracellular domain consists of eight LDLa domains, two EGF domains and four β-propellors which are each separated by one EGF-like repeat (an overview of the LRP4 protein structure is shown in Fig. 2). Epitope mapping has thus far not been performed for LRP4 MG. In one study, LRP4 auto-antibodies were classified as IgG1 subclass, which would suggest complement involvement (106); however, this has not yet been confirmed. Application of LRP4 antibodies in a solid-phase binding assay resulted in a loss of agrin–LRP4 interaction and in some cases reduced AChR clustering in a cellular system (106,110). Agrin is crucial for the LRP4–MuSK signalling cascade leading to AChR clustering (90). Therefore, interference in the interaction between agrin and LRP4 could result in myasthenia. Crystal structure analysis revealed that LRP4 and agrin can interact even if only the first β-propeller of LRP4 is present (111). However, in a solid-phase binding assay, the last two LDLa domains of LRP4 were shown to strengthen this interaction significantly (90). It is likely that the LRP4 auto-antibodies bind in this region to affect the agrin–LRP4 interaction (Fig. 2). It will be interesting to further pinpoint the binding epitope of the LRP4 antibodies and assess whether these auto-antibodies can also interfere with MuSK–LRP4 interaction and/or cause cross-linking and internalization of LRP4. Two patients with *LRP4* mutations and a progressive muscle weakness have been described. In these patients the mutations were located in the third beta-propeller domain and inhibited an effective binding with MuSK. (112) Moreover, compensatory upregulation of presynaptic ACh release, which may involve LRP4-mediated signalling, might also be affected by LRP4 antibodies.

Lambert-Eaton Myasthenic Syndrome (LEMS) with antibodies against P/Qtype VGCC

About 90% of patients with LEMS have auto-antibodies against presynaptic Cav2.1 (P-/Q-type) VGCCs (113,114). In about 50–60% of these patients, small cell lung cancer (SCLC) is detected (114,115,116). LEMS can occur at all ages and affects both men and women. A bimodal distribution is observed in patients with non-tumour-associated LEMS, as in those with MG, with a peak at about 35 years of age, predominantly in female patients. A second peak is observed above 60 years of age, with an equal sex distribution (117). In addition, above the age of 50 years, a larger peak of mostly male smokers with SCLC is seen (118,119). LEMS usually presents with proximal leg weakness and loss of tendon reflexes (115). Weakness progresses to other muscle groups with increasing disease severity; however, this occurs in a caudocranial direction in contrast to AChR MG (120). Furthermore, 80–90% of LEMS patients experience relatively mild symptoms of autonomic dysfunction which include dry mouth and eyes, erectile dysfunction, constipation and blurred vision (115,116,121).

Pathogenesis of LEMS

Antibodies against Cav2.1 VGCCs are presumed to be pathogenic in LEMS because the disease can be passively transferred into mice (113,114,122,123). Active immunization with parts of the α1 subunit also led to a LEMS phenotype in mice (124). Moreover, transfer of antibodies from an affected mother to child resulted in transient neonatal weakness (125).

The Cav2.1 VGCC is present in both SCLC cells and the motor nerve terminal (126). Freeze-fracture electron microscopy of mouse neuromuscular junction treated with purified IgG from LEMS patients shows a depletion and aggregation of active zone particles, which are presumed to represent VGCCs (127). The Cav2.1 VGCC is necessary for Ca^{2+} influx at these active zones, which enables release of ACh from the nerve terminal. In another passive transfer study, both LEMS IgG and divalent antibody fragments of LEMS patients caused a reduction in quantal content (i.e. the number of synaptic vesicles containing ACh exocytosed per nerve impulse) (128). Monovalent (Fab) fragments, however, did not affect neuromuscular transmission. The same effect of divalent but not monovalent antibody fragments was shown *in vitro* for Ca^{2+} flux in SCLC cells (128). The active zone particles are arranged in double parallel rows about 16–21 nm apart, enabling cross-linking. This suggests an important role for surface depletion of VGCCs through cross-linking and internalization as in AChR MG. Because IgG4 is considered functionally monovalent, the involvement of divalent antigen binding suggests that IgG1 and IgG3 subclass auto-antibodies might play a more important role than the IgG4 subclass antibodies.

In contrast to AChR MG, the effects of LEMS IgG seem to be complement independent, because passive transfer experiments in C5-deficient mice and after C3 depletion by cobra venom factor induced a LEMS phenotype (122, 129). Conductance of individual VGCCs remained intact after exposure to serum from LEMS patients, indicating that a competitive or direct blocking effect of antibodies is unlikely (130).

In summary, VGCC auto-antibodies induce LEMS by antigenic modulation and surface depletion of the ion channels. Complement-mediated membrane destruction, steric hindrance and/or competition with the Ca^{2+} binding site do not seem to play important roles in the pathogenesis of LEMS.

Epitope mapping in LEMS

The VGCC is a heteromultimeric protein complex on the presynaptic motor nerve terminal membrane and consists of an α1 subunit and α2δ, β and possibly γ accessory subunits (Fig. 2). The α1 subunit is the ion conducting pore, whereas the other subunits modulate gating and serve as a membrane anchor (131). The α1 and a part of the α2δ subunit extend into the synaptic cleft. The α1 subunit contains four domains (I–IV), each having six transmembrane segments (S1–S6). The search for binding epitopes has mostly focused on this subunit, especially the extracellular S5–S6 linker regions between domains which are exposed extracellularly. Antibodies against synthetic peptides corresponding to the S5–S6

linker region of domain II and IV have been detected in 43–75% of LEMS patients (132,133). The presence of antibodies that recognized domain IV seemed to be more common in patients without associated tumour compared with patients with SCLC (134). Conflicting results have been reported with regard to a binding epitope in the domain III S5–S6 linker region, which is the domain with the highest Ca^{2+} affinity (132,133,135). In an active immunization model, six of ten rats immunized with this synthetic peptide showed muscle weakness and defective neuromuscular transmission, suggesting that it is likely to be immunogenic (124).

Another target for auto-antibodies is the presynaptic protein synaptotagmin I (136), which is present in both SCLC cells and at presynaptic active zones where it is involved in the release of ACh. Immunizing rats with segments of synaptotagmin I induced electrophysiological abnormalities reminiscent of presynaptic dysfunction as seen in LEMS.

Epitope spreading in LEMS

Antibodies against the β subunit of the VGCC have also been described in some LEMS patients. Because this subunit is only present intracellularly, these antibodies are probably secondary to immune-mediated damage (137,138). The presynaptic active zone protein ERC1/ELKS1 has recently been reported as an antigen in a VGCC-positive LEMS patient, which is also only present intracellularly and unlikely to be of pathogenic relevance (139). Epitopes in other associated proteins might be more relevant for modulating the disease process. Antibodies against the M1-type muscarinic AChR, for example, can be found in most patients with LEMS (140). This receptor type is implicated in presynaptic compensation for impairment of Ca^{2+} entry that is necessary for ACh release. Epitope spreading to this receptor later in the disease course could impair this compensation mechanism. Upregulation of other VGCC subtypes capable of ACh release seems to be another compensatory mechanism in LEMS patients (141). Some patients harbour auto-antibodies against the Cav2.2 (N-type) and Cav1 (L- type) VGCC subtypes although the significance of this immune response is uncertain (142,143).

Epitope spreading also occurs in the antitumor immune response against SCLC. About 65% of LEMS patients with associated SCLC have antibodies against the intracellular nuclear SOX1 protein compared with 22–36% of patients with SCLC alone (144,145). Because these antibodies are rare in LEMS patients without associated tumour, they can be used as a serological marker for SCLC-associated LEMS. No survival effect or specific patient characteristics were reported for SOX antibodies; thus, a pathogenic role is unlikely (144,146). The significance of these antibodies in anti-tumour immune responses and development of autoimmunity remains unclear.

SCOPE OF THE THESIS

Studies from a broad spectrum of antibody-mediated autoimmune diseases, including the above described neuromuscular autoimmune diseases have emphasized that the pathomechanism, course of disease and treatment response of antibody-mediated autoimmune diseases can depend on:

1. Auto-antibody titre

2. Auto-antibody subclass

3. Epitope(s) bound by the auto-antibodies

4. Structural integrity of the MIR

Understanding what role these factors play in the development of autoimmune disease not only gives insight in the pathomechanism of disease, but also highlights which treatment strategy is most likely to be beneficial for the patient. Identifying the main binding epitopes, pathomechanism of disease and establishing the presence of auto-antibodies against a MIR can subsequently be useful for predicting the course of the disease and may provide insights for the development of new therapies. For MuSK MG these aspects had as yet not been investigated in detail.

The aim of this thesis is to investigate the pathomechanism of MuSK MG and to characterize the auto-antibodies causing the disease.

The first part of this thesis will focus on the research question:

How do MuSK auto-antibodies cause myasthenia gravis?

Chapter 2 investigates the pathogenicity of the IgG subclass antibodies from MuSK MG patient plasmapheresis material. IgG4 and IgG1-3 fractions were affinity purified and subsequently used in passive transfer studies in NOD/SCID mice.

Chapter 3 studies the exact mechanism by which the IgG4 MuSK auto-antibodies cause myasthenia *in vitro*.

The second part of the thesis investigates:

How MuSK auto-antibodies characteristics contribute to disease course?

Chapter 4 investigates the role of epitope spreading in disease severity and treatment responsiveness in MuSK MG patients.

Chapter 5 uses our experience in detecting MuSK auto-antibodies to study their role in patients with amyotrophic lateral sclerosis.

The last part of the thesis, **Chapter 6**, focusses on the relevance of our findings for other IgG4-mediated autoimmune diseases and provides future directions for (antigen-specific) treatment strategies.

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CHAPTER 2

Muscle-specific kinase myasthenia gravis IgG4 auto-antibodies cause severe neuromuscular junction dysfunction in mice

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Brain. 2012 Apr;135(Pt 4):1081-101.

ABSTRACT

Myasthenia gravis is a paralytic disorder with auto-antibodies against acetylcholine receptors at the neuromuscular junction. A proportion of patients instead has antibodies against muscle-specific kinase, a protein essential for acetylcholine receptor clustering. These are generally of the immunoglobulin-G4 subclass and correlate with disease severity, suggesting specific myasthenogenic activity. However, immunoglobulin-G4 subclass antibodies are generally considered to be 'benign' and direct proof for their pathogenicity in muscle-specific kinase myasthenia gravis (or other immunoglobulin-G4-associated disorders) is lacking. Furthermore, the exact electrophysiological synaptic defects caused at neuromuscular junctions by human anti-muscle-specific kinase auto-antibodies are hitherto unknown. We show that purified immunoglobulin-G4, but not immunoglobulin-G1-3, from patients with muscle-specific kinase myasthenia gravis binds to mouse neuromuscular junctions *in vitro*, and that injection into immunodeficient mice causes paralysis. Injected immunoglobulin-G4 caused reduced density and fragmented area of neuromuscular junction acetylcholine receptors. Detailed electrophysiological synaptic analyses revealed severe reduction of postsynaptic acetylcholine sensitivity, and exaggerated depression of presynaptic acetylcholine release during high-rate activity, together causing the (fatigable) muscle weakness. Intriguingly, compensatory transmitter release upregulation, which is the normal homeostatic response in acetylcholine receptor myasthenia gravis, was absent. This conveys extra vulnerability to neurotransmission at muscle-specific kinase myasthenia gravis neuromuscular junctions. Thus, we demonstrate that patient anti-muscle-specific kinase immunoglobulin-G4 is myasthenogenic, independent of additional immune system components, and have elucidated the underlying electrophysiological neuromuscular junction abnormalities.

INTRODUCTION

Myasthenia gravis is an autoimmune disease with antibodies against components of the neuromuscular junction causing fatigable muscle weakness. The most prevalent form of myasthenia gravis is caused by antibodies against the acetylcholine receptor (AChR), which induce damage of the postsynaptic membrane and defective neuromuscular transmission. A proportion of patients with myasthenia gravis (~5–10%) has antibodies against the postsynaptic transmembrane protein muscle-specific kinase (MuSK) (1). In complex with LRP4 and Tid1, this protein is involved in development and maintenance of AChR clustering under the influence of neural agrin (2,3,4,5). Although both forms of myasthenia gravis are caused by antibodies against neuromuscular junction components, there are several clinical, pharmacological and genetic arguments that separate them into two distinct disease entities (6,7,8,9). Notably, the immune response in MuSK myasthenia gravis is dominated by immunoglobulin (Ig)G4 auto-antibodies, instead of IgG1 or IgG3 in AChR myasthenia gravis (10,11,12,13), and anti-MuSK IgG4 but not IgG1–3 titres correlate with disease severity (14). The latter observation particularly suggests that pathogenic mechanisms differ between MuSK and AChR myasthenia gravis. IgG4 and IgG1–3 have different Fc (fragment, crystallizable) regions and will activate different immunological pathways (15). Human IgG1–3 is bivalent (enabling antigen cross-linking), activates complement and has strong pro-inflammatory properties. In contrast, IgG4 is functionally monovalent due to Fab (fragment, antigen binding) arm exchange (16), cannot activate complement and has low affinity for Fc receptors on immune cells, and has therefore been considered as benign and anti-inflammatory (17). In AChR myasthenia gravis, complement activation is indeed a major aspect of the antibody-mediated pathogenesis (18,19), while this seems not the case for MuSK myasthenia gravis (20,21).

After initial scepticism on the pathogenic role of MuSK myasthenia gravis IgG (20,21), a recent passive transfer study in mice has strongly indicated that MuSK myasthenia gravis IgG indeed contains the myasthenogenic factor (22). From this and further detailed experiments with MuSK myasthenia gravis whole-IgG, it has been postulated that the MuSK myasthenia gravis antibodies can dimerize, activate and internalize MuSK, which subsequently leads to reduction and dispersal of AChR clusters at the neuromuscular junction (23). These results, together with anti-MuSK IgG4 subclass being the predominant antibody in MuSK myasthenia gravis and the correlation of its titre with disease severity (14), suggest that anti-MuSK IgG4 antibodies are ultimately the myasthenogenic factor in MuSK myasthenia gravis serum. However, the human clinical studies provide only circumstantial evidence and there is no direct proof for this hypothesis. Furthermore, the exact electrophysiological defects at the neuromuscular junction induced by these antibodies are hitherto unknown.

Therefore, we performed passive transfer in mice with purified IgG4 and IgG1–3 fractions from plasma of patients with MuSK myasthenia gravis and characterized in detail their myasthenogenic effects with *in vivo* and *ex vivo* neuromuscular analyses.

To avoid immunity against injected human IgG and to exclude activation of immune pathways following antigenic binding of injected IgG we used NOD.CB17-Prkdc^{scid}/J (NOD/SCID) mice, which are immunodeficient due to lack of functional lymphoid cells and an incomplete complement system (24). The IgG4 but not the IgG1–3 fractions of patients with MuSK myasthenia gravis induced severe muscle weakness, which we demonstrate to be due to combined post- and presynaptic electrophysiological neuromuscular junction defects. Thus, we show that MuSK myasthenia gravis patient IgG4 auto-antibodies are directly myasthenogenic, independent of additional immune system components, and provide insight into their paralytic effect by elucidating the electrophysiological synaptic dysfunction these human auto-antibodies cause at single neuromuscular junction level.

MATERIALS AND METHODS

Patient material

Plasmapheresis fluid was obtained from therapeutic plasma exchange of four patients with MuSK myasthenia gravis, whom are clinically described in the supplementary material. Patients signed informed consent and the Medical Ethical Committee approved the study. Serum from two healthy individuals was obtained from Sanquin Blood Supply Foundation, Amsterdam, The Netherlands.

IgG4 and IgG1–3 purification

IgG4 and IgG1–3 were purified using a human IgG4 and a human total IgG-specific affinity resin (BAC BV, www.captureselect.com) on an AKTA explorer 900 (Pharmacia Biotech). The amount of plasma used per run was based on the IgG subtype concentrations, and the dynamic capacity of the column material, 6 and 15 mg/ml for the IgG4 and IgG total affinity resin, respectively. For both columns 25 ml affinity resin was used. The flow rate for all steps was set at 13 ml/min. Before addition of patient material, the column (XK 26) was equilibrated with 125 ml phosphate-buffered saline (PBS) pH 7.4. Thereafter, the plasma was diluted five times in citrate buffer and cleared from non-soluble material through a 0.22 µM filter (Millipore) and run through the anti-IgG4 column. The column was washed with 250 ml of PBS and bound material was eluted in 10 ml fractions with 375 ml 0.1 M glycine pH 3.0. These fractions were immediately neutralized with 1/10 volume 1 M Tris pH 8.0.

The non-bound fraction of the first purification step was again loaded on a regenerated 25 ml anti-IgG4 column to deplete all remaining IgG4. This IgG4 fraction was discarded and not used for the *in vitro* and *in vivo* experiments. A fraction of the IgG4 depleted plasma, depending on the total IgG content, was loaded on an anti-IgG total affinity resin using the same specifications as described above for the IgG4 affinity ligand.

Eluted fractions per purification containing high concentrations of IgG, based on OD280 measurements, were pooled and subsequently dialysed to PBS (molecular weight cut-off 3.5 kD), concentrated with a Vivaspin20 concentrator (Sartorius) and sterilized using a 0.22 µm filter (Millipore).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis

Samples were loaded on an 8% sodium dodecyl sulphate–polyacrylamide gel using sample buffer without a reducing agent. Separated proteins were either stained with Coomassie brilliant blue or transferred to PVDF membrane (Millipore). Transfer efficiency was checked by incubation in ponceau S buffer [0.1% (w/v) ponceau S; 0.5% (v/v) acetic acid] for 1 min and subsequent washes with milli-Q water. Blots were thereafter blocked in 4% Marvel skimmed milk powder in PBS (Marvel-PBS) for 1 h at room temperature. Blocked membranes were incubated for 1 h at room temperature with primary antibody diluted in Marvel-PBS. As primary antibody the following antibodies were used: mouse anti-IgG1 (1:5000) (Sanquin), mouse anti-IgG2 (1:5000), mouse anti-IgG3 (1:5000) or mouse anti-IgG4 (1:5000) (Nordic Immunological laboratories). Membranes were washed five times in 0.05% Tween-20 (v/v) in PBS (PBS–Tween) and subsequently incubated for 1 h at room temperature with IRDye 800CW conjugated goat anti-mouse secondary antibody (1:5000) diluted in Marvel-PBS. Excess of antibody was removed by four washes with PBS–Tween followed by two washes in PBS. Bound antibodies were detected on the Odyssey (LI-COR Biosciences GmbH).

Anti-MuSK titre determination

MuSK titres (nM) were determined with a commercial radioimmunoassay (RSR Ltd) in the patient plasmas and the sera of the passive transfer mice, obtained at the end of the passive transfer period.

Mouse passive transfer studies

To circumvent the potential problem of a mouse immune response against repetitively injected human IgG we used immunodeficient NOD.CB17-Prkdc^{scid}/J mice (24). Original breeders were purchased from Jackson Laboratory (Bar Harbor). Mice were bred and housed in sterile individually ventilated cages in the Leiden University Medical Centre animal facilities. Sterilized food and drinking water were provided *ad libitum*. We used 29 female NOD/SCID mice, aged 4–5 weeks at the start, for passive transfer of purified subclass IgG from patients with MuSK myasthenia gravis. Details are provided in supplementary Table 1. After establishing baseline values for the *in vivo* neuromuscular tests (see below) during 2 days, the mice were daily injected intraperitoneally at ~10 a.m. with a dose of the purified subclass IgG dissolved in a volume of 333 µl sterile PBS (for exact dosing information see relevant 'Results' sections and supplementary Table 1).

Before the injection, the body weight of each mouse was determined and neuromuscular performance was assessed. If mice became very weak (score >1, see below) or body weight loss occurred of >20% compared with the starting weight, or >15% in 1–2 consecutive days, passive transfer was terminated and mice were directly subjected to electromyographical testing, followed by CO_2 euthanasia and dissection of muscle nerve preparations for *ex vivo* neuromuscular junction

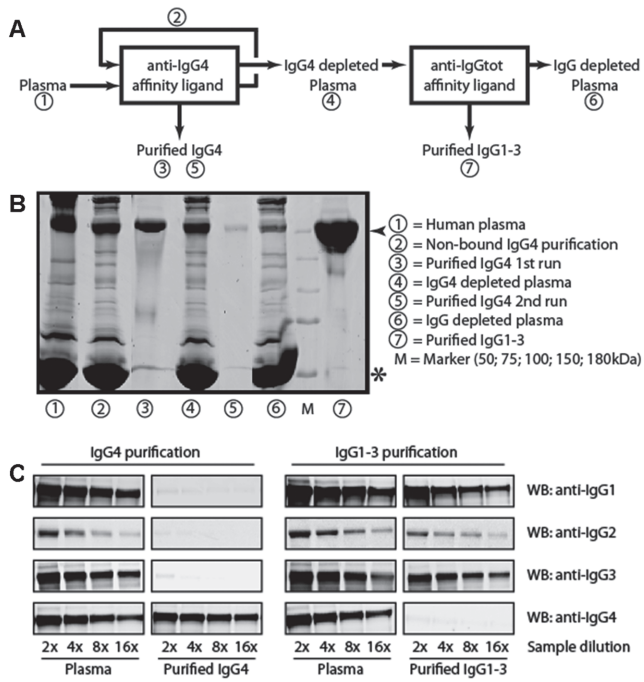


Figure 1. Purification of subclass IgG. (A) Schematic representation of the IgG subtype purification pipeline, in which plasmapheresis fluid is run over the IgG4 affinity resin two consecutive times to purify and deplete IgG4, followed by a run over the IgGtotal affinity resin to purify the remaining IgG1–3. (B) Representative results of the IgG4 and IgG1–3 purification of Patient 3 analysed on a Coomassie-stained gel to assess the purification of the IgG fractions (arrowhead indicates molecular weight of IgG; asterisk indicates molecular weight of human serum albumin). (C) Representative western blot of Patient 4 to assess the efficiency of depletion and purity of the IgG fractions. Only minimal reciprocal contamination of the different IgG subclass fractions was observed.

function tests and histology. All experiments were carried out according to Dutch law and Leiden University guidelines, including approval by the local Animal Experiments Committee.

In vivo assessment of neuromuscular function

Weakness in mice was visually scored (0 = no weakness, 1 = weakness upon activity, 2 = weakness at rest, 3 = severe weakness with breathing difficulty, 4 = death) (25). Forelimb and abdominal muscle strength was tested using a grip strength meter (type 303500, Technical and Scientific Equipment GmbH). The average peak force value of a trial of 10 consecutive pulls was calculated. The inverted mesh hanging test was used to assess fatigability of limb and abdominal muscles as described before (26). The test ended upon falling or completing the maximum hanging time, which was set at 120 s. Respiratory rate and tidal amplitude were assessed with non-invasive whole-body plethysmography in unrestrained animals (RM-80, Columbus Instruments). The signal was digitized using a Minidigi digitizer and Axoscope 10 (Axon Instruments/

Molecular Devices) and analysed with the event detection feature of Clampfit 9.2 (Axon Instruments/Molecular Devices).

Repetitive nerve stimulation electromyography

Mice were anaesthetized with a 1.5:1 (v/v) mixture of ketamine hydrochloride (Nimatek; 100 mg/ml, Eurovet) and medetomidine hydrochloride (Domitor; 1 mg/ml, Pfizer), at 1.25 µl/g mouse body weight, adjusted with Ringer solution to 200 µl volume and administered intraperitoneally. Mice were maintained at 37°C on a heating pad. A grounding needle electrode was inserted subcutaneously in the right thigh. Stimulation needle electrodes were inserted near the sciatic nerve in the left leg thigh. Subcutaneous recording needle electrodes were inserted near the calf muscles of the left leg. Grounding and recording electrodes were coupled via an AI402 pre-amplifier to a Cyberamp-380 signal conditioner (Axon Instruments/Molecular Devices). The nerve was stimulated supramaximally from a computer-controlled programmable electrical stimulator (AMPI). Trains of 10 stimuli were applied at increasing frequencies of 0.2, 1, 3, 5, 10, 20 and 40 Hz, with a 10–30 s pause between trains. Compound muscle action potentials (CMAPs) were digitized using a Digidata 1440 interface (Axon Instruments/Molecular Devices) and peak–peak amplitudes were determined in Clampfit 9.2 (Axon Instruments/Molecular Devices). After completing the recordings, mice were sacrificed by CO_2 inhalation without recovery from anaesthesia and muscles were dissected for the studies described below.

Ex vivo muscle contraction studies

Contraction force of left phrenic nerve-hemidiaphragms was recorded in Ringer's medium containing (in mM): NaCl 116, KCl 4.5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 23, glucose 11, pH 7.4) at room temperature (20–22°C) with a force transducer (type K30, Harvard Apparatus, Hugo Sachs Elektronik GmbH), connected to an amplifier TAM-A 705/1 (Hugo Sachs Elektronik). The signal was digitized using a Digidata 1440 digitizer (Axon Instruments/Molecular Devices), connected to a PC running Axoscope 10 (Axon Instruments). The phrenic nerve was stimulated supramaximally once every 5 min with 120 stimuli at 40 Hz. The safety factor of neuromuscular transmission was assessed by measuring contraction force in the presence of various concentrations (15–1000 nM) d-tubocurarine (Sigma-Aldrich). The amplitude of the recorded contractions was cursor-measured in Axoscope, at 2 s after the start.

Ex vivo neuromuscular junction electrophysiology

Intracellular recordings of miniature endplate potentials and endplate potentials at the neuromuscular junction were made in Ringer's solution at 26–28°C in right phrenic nerve-hemidiaphragm preparations. Just lateral of the main intramuscular phrenic nerve branch, muscle fibres were impaled with a glass microelectrode (10–20 MΩ, filled with 3 M KCl) connected to a Geneclamp 500B (Axon Instruments/Molecular devices) for amplifying and filtering (10 kHz low-pass). Although at the used low (×40)

light-microscopical magnification neuromuscular junctions are not directly visible (but the electrode-tip is), we know from fluorescence microcopical studies that neuromuscular junctions are strictly localized in that area. In controls, ~100% of the impalements yields synaptic signals with kinetical characteristics (i.e. a 0–100% rise time <2 ms) that assure correct placement of the electrode near the neuromuscular junction. The signal was digitized using a Digidata 1322A (Axon Instruments/Molecular Devices) and analysed using Clampfit 9.2 (Axon Instruments/Molecular Devices) and Mini Analysis 6.0.3 (Synaptosoft). Muscle action potentials were eliminated by using the skeletal muscle Na^+ channel blocker, μ-Conotoxin GIIIB (3 μM) (Scientific Marketing Associates). To record endplate potentials, the phrenic nerve was stimulated using a bipolar platinum electrode connected via an optical stimulus isolation unit to a computer-controlled programmable electrical stimulator (AMPI). Mean endplate potential and miniature endplate potential amplitudes at each neuromuscular junction were normalized to −75 mV, with the reversal potential for acetylcholine-induced current assumed 0 mV (27). To calculate the quantal content for each neuromuscular junction, the mean amplitude of the 20 endplate potentials recorded at low rate (0.3 Hz) stimulation were corrected for non-linear summation (28) and the normalized and corrected mean endplate potential amplitude was divided by the normalized mean miniature endplate potential amplitude (calculated from at least 20 miniature endplate potentials sampled). The quantal content is the number of acetylcholine quanta that is released upon a single nerve impulse. In each muscle, 40–60 muscle fibres were impaled to determine the percentage of neuromuscular junctions that were synaptically active or 'silent' (i.e. showing no miniature endplate potentials and no muscle action potential upon nerve stimulation). Thereafter, μ-Conotoxin GIIIB was applied and allowed to paralyze the preparation (usually within 15 min). Then, a measuring session of 20 endplate potentials evoked at 0.3 Hz nerve stimulation, spontaneous miniature endplate potentials during 2 min and a train of 35 endplate potentials at 40 Hz nerve stimulation was performed at 8–15 single neuromuscular junctions randomly sampled within the muscle.

Fluorescence microscopy of neuromuscular junctions

We assessed the binding capacity of purified patient IgG subclass fractions at the neuromuscular junction in muscle strips of dissected sets of small and thin cranial muscles from normal C57bl6/j mice, collectively referred to as levator auris longus, since most strips were from that particular muscle, but strips were also included of the closely underlying muscles (auricularis superior, abductor auris longus and interscutularis). These muscles are flat and thin and therefore exceptionally suitable for neuromuscular junction whole-mount imaging studies (29). Strips were fixed for 30 min at room temperature in 1% paraformaldehyde in PBS. After washing 30 min in PBS and incubating 30 min in 3% bovine serum albumin in PBS, muscles were incubated overnight at 4°C in 1:100 dilution of the purified IgGs from human MuSK myasthenia gravis Patients 1–3 and normal human control subjects. Samples were

washed for 1 h at room temperature with PBS, and subsequently incubated for 2.5 h at room temperature in a combination solution in PBS of 1:100 Alexa Fluor 546-conjugated goat anti-human IgG (Invitrogen) and 1 µg/ml Alexa Fluor 488 conjugated α-bungarotoxin (Invitrogen), followed by a PBS wash for 1 h. Muscle strips were mounted on microscope slides with Citifluor AF-1 antifadent and viewed under a Zeiss LSM 7 MP laser scanning microscope using a ×20 water immersion objective.

We also analysed morphology of neuromuscular junctions in diaphragm and levator auris longus muscles of the passively transferred mice. Diaphragm strips were fixed in 1% paraformaldehyde in PBS, washed in PBS and incubated for 3.5 h with 1µg/ml Alexa Fluor 488 conjugated α-bungarotoxin, followed by PBS wash (30 min), all at room temperature. AChR receptor staining at diaphragm neuromuscular junctions was quantified using ImageJ v1.44 (http://rsbweb.nih.gov/ij/). Ten randomly chosen neuromuscular junctions in the vertical midline of each of the lower-magnification pictures were selected and neuromuscular junction area was defined using the thresholding feature of the program. For each neuromuscular junction we determined stained area, mean pixel intensity and summed intensity (i.e. area multiplied by mean intensity). The mean ± SEM of these values was calculated for each picture. Differences were statistically tested with ANOVA and *post hoc* Tukey test. Levator auris longus neuromuscular junctions were incubated overnight at 4°C with rabbit anti-synaptic vesicle protein 2 (SV2) IgG antibody (produced from hybridoma cells purchased from The Developmental Studies Hybridoma Bank from the University of Iowa, USA), followed by PBS wash (30 min), Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) incubation for 2.5 h and again 30 min PBS wash (all at room temperature). Muscles were whole-mounted and viewed under the confocal laser scanning microscope.

Electron microscopy

Ultrastructural neuromuscular junction analysis was performed as described (30). Briefly, diaphragm muscles were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in PBS for 1 h at 4°C, post-fixed with 1% osmium tetroxide, dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections were viewed with a Philips CM 100 electron microscope. At least six endplate regions were photographed per muscle. Quantitative morphometry of the folding index (length of postsynaptic membrane/length of presynaptic membrane) was performed as described (31,32).

Statistical tests

Wherever appropriate, Student's *t*-test or ANOVA with Tukey's *post hoc* test were performed, as indicated. Differences with *P*-values < 0.05 were considered statistically significant.

RESULTS

Purification of IgG4 and IgG1–3

Using VHH-based affinity resins, we purified IgG1–3 and IgG4 from plasmapheresis fluid of four patients with MuSK myasthenia gravis (clinically described in the supplementary material) and from pooled serum of two healthy individuals, using IgG subclass-specific affinity chromatography (Fig. 1A). Only minimal contamination with other proteins remained in both the IgG4 and the IgG1–3 fractions in a Coomassie-stained gel (Fig. 1B). Western blots demonstrated almost complete absence of IgG4 in the IgG1–3 fraction, and of IgG1, 2 and 3 in the IgG4 fraction (Fig. 1C). The minimal enrichment of IgG4 compared with the IgG4/IgG1–3 ratio in the starting plasma sample was ~200 times, which may be an underestimation due to the possibility that the secondary IgG subclass-specific antibodies used for detection may have some degree of cross-reactivity (12).

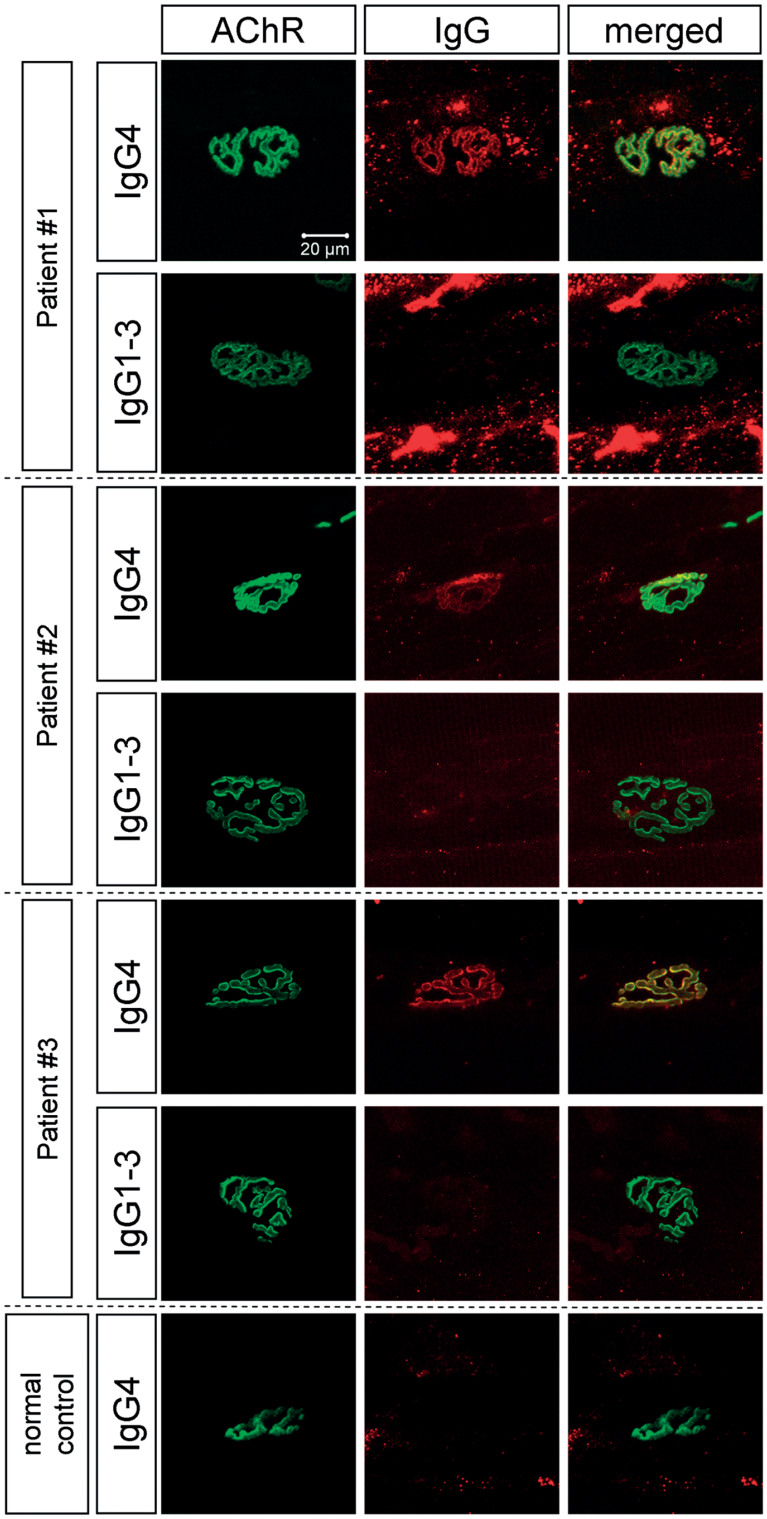
Selective binding of IgG4 from patients with MuSK myasthenia gravis to the neuromuscular junction

In levator auris longus muscles from untreated C57Bl6/J mice we assessed the neuromuscular junction binding potency of purified patient IgG4 and IgG1–3. Muscles were incubated with IgG and with fluorescently labelled α-bungarotoxin, which binds to AChRs and thus delineates the neuromuscular junction. Confocal laser scanning microscopy showed clearly co-localized IgG and AChR staining in preparations incubated with the purified IgG4 fractions from the three patients, while there was no IgG staining with patient IgG1–3 fractions or purified normal human IgG4 (Fig. 2). This shows that IgG4 of patients with MuSK myasthenia gravis selectively binds to the neuromuscular junction.

Passive transfer of purified IgG4 from patients with MuSK myasthenia gravis induces muscle weakness in mice

We injected young female NOD/SCID mice with different daily doses of MuSK myasthenia gravis IgG4 (14 mice) and IgG1–3 (8 mice). For details see supplementary Table 1. Daily injection of mice with 4 mg (from Patients 1 and 2), 0.5 mg or more (Patient 3) and 4 mg or more (Patient 4) purified MuSK myasthenia gravis IgG4 caused progressive body weight loss and overt muscle weakness (visual scoring grade 1–2), starting after ~1 week (Fig. 3A). We quantified the (fatigable) muscle weakness with

Figure 2. Demonstration of the selective binding of MuSK myasthenia gravis IgG4 to neuromuscular junctions. Maximum intensity projections of confocal laser scanning microscopical images of mouse levator auris longus neuromuscular junctions co-stained for AChRs with Alexa Fluor 488 α-bungarotoxin (green) and with purified MuSK myasthenia gravis patient IgG4, IgG1–3 or purified normal human IgG4 (all in red). The IgG4 from each of the three tested patients with MuSK myasthenia gravis stained the neuromuscular junction, while IgG staining at neuromuscular junctions was absent with the matched IgG1–3 fractions or normal human IgG4.


AChR
IgG
merged
Patient #1
IgG4
IgG1-3
20 µm
Patient #2
IgG4
IgG1-3
Patient #3
IgG4
IgG1-3
normal control
IgG4

grip strength and inverted mesh testing. After ~10 days of injection, the affected mice pulled <20–25% of the initial force (Fig. 3D) and fell off the inverted mesh in ~0–40 s, much faster than initially when they completed the allowed 120 s hanging time (Fig. 3G). Concomitant progressive reduction of tidal volume, detected in whole-body plethysmography, suggested increasing weakness of breathing muscles (Fig. 2J). In total, we observed 10 clinically weak mice after receiving MuSK myasthenia gravis IgG4 injection (supplementary Table 1). Eight control NOD/SCID mice that received 4 mg/day purified IgG1–3 of the same four patients with MuSK myasthenia gravis (two mice per patient IgG1–3 tested, supplementary Table 1) showed no such body weight loss or (fatigable) muscle weakness (Fig. 3), nor did control NOD/SCID mice that received either 4 mg/day normal human IgG4 or only the vehicle, PBS (Fig. 3).

At the end of the experiment, anti-MuSK antibodies in the serum of the mice treated with IgG4 reached levels 5–10 times higher than those in the patient plasmas, while no anti-MuSK reactivity could be detected in the IgG1–3 injected mice or the mouse injected with 4 mg/day healthy control IgG4 (supplementary Table 2).

Depending on the patient material, lower daily doses IgG4 (0.13–1.5 mg) caused no *in vivo* weakness but showed subclinical weakness (in muscle contraction tests and neuromuscular junction electrophysiological experiments, supplementary Fig. 2).

After ending passive transfer, *in vivo* neuromuscular junction function was assessed in calf muscles with repetitive nerve stimulation electromyography at various frequencies (0.2–40 Hz, 10 stimuli per frequency). All of the clinically weak MuSK myasthenia gravis IgG4-treated mice tested showed considerable reduction of CMAPs (Fig. 4 and supplementary Table 1), indicating progressive loss of successfully transmitting neuromuscular junctions during stimulation and explaining the observed fatigable muscle weakness. CMAP reduction depended on stimulation frequency, becoming apparent from 3 Hz stimulation and maximizing at frequencies of ≥10 Hz. CMAP reductions at 10 Hz stimulation ranged from ~20% (Mouse 2, passively transferred with Patient 1 IgG4, to almost 100% in Mouse 9, treated with Patient 2 IgG4 (supplementary Table 1). Figure 4C shows 10 consecutive CMAPs recorded at 10 Hz in Patient 2 IgG4-treated Mouse 5, decrementing by ~70%. No CMAP reduction was found in the control NOD/SCID mice, injected with either MuSK myasthenia gravis IgG1–3, normal human IgG4 or PBS. On average, 10 Hz stimulation resulted in 51.5 ± 10% CMAP amplitude decrement in the clinically weak MuSK myasthenia gravis mice tested ($n = 8$), whereas IgG1–3-treated control NOD/SCID mice ($n = 8$) showed 3.1 ± 0.7 increment ($P < 0.001$, Student's t-test, Fig. 4D).

MuSK myasthenia gravis IgG4 disturbs synaptic transmission at the neuromuscular junction

In *ex vivo* contraction experiments we tested muscle strength, fatigability and the safety factor of neuromuscular transmission in muscles of the passively transferred mice. Mean force delivered upon 40 Hz tetanic nerve stimulation by hemidiaphragms of patient IgG4-injected mice was 10.8 ± 0.9 g, while that of pooled controls was

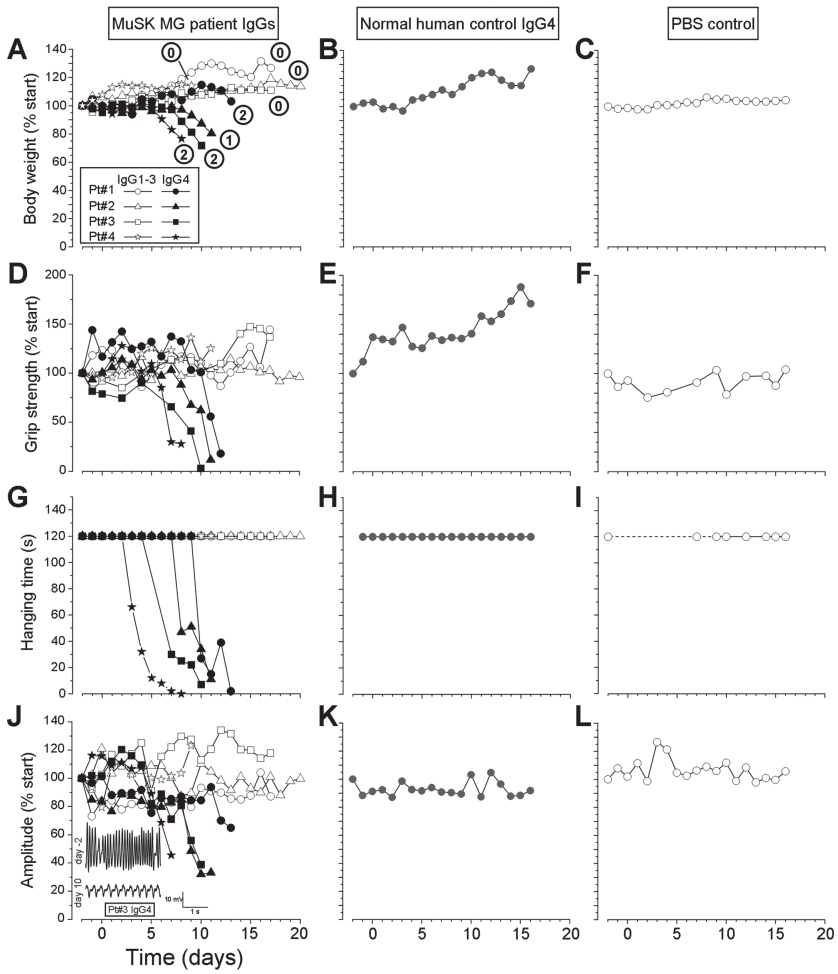


Figure 3. Passive transfer of MuSK myasthenia gravis IgG4 into NOD/SCID mice causes clinical myasthenia gravis. Exemplary *in vivo* neuromuscular tests of four individual young (3–5 weeks old) female NOD/SCID mice each injected with MuSK myasthenia gravis (MG) IgG4 from a different patient (4 mg/day, Patient 1, 2 and 4 or 2 mg/day, Patient 3) and four NOD/SCID mice, each injected with IgG1–3 from the same patients (all 4 mg/day), one NOD/SCID mouse injected with normal human IgG4 (4 mg/day, pooled from two healthy donors) and one NOD/SCID mouse injected with 333 µl PBS alone. For overview of all injected NOD/SCID mice of this study, see supplementary Table 1. Baseline was determined 2 days before start of injections (on Day 0). (A–C) Body-weight loss of the MuSK myasthenia gravis IgG4 mice and muscle weakness score of ≥1 at the end of the experiment (encircled values). All controls showed neither body-weight loss nor muscle weakness (all scored 0). (D–F) Grip-strength became severely diminished in MuSK myasthenia gravis IgG4 mice. (G–I) Inverted mesh hanging-time became much shorter for MuSK myasthenia gravis IgG4 mice. All control mice completed the maximum period (2 min). The PBS-injected mouse initially showed some premature falls (dashed line) due to excessive exploratory behaviour, but later completed the test, when MuSK myasthenia gravis IgG4-injected mice failed. (J–L) Respiration amplitude of MuSK myasthenia gravis IgG4-injected mice became reduced. Inset in J shows recorded signals before treatment and after 10 days injection with Patient 3 IgG4. Respiration amplitude of all control mice remained stable.

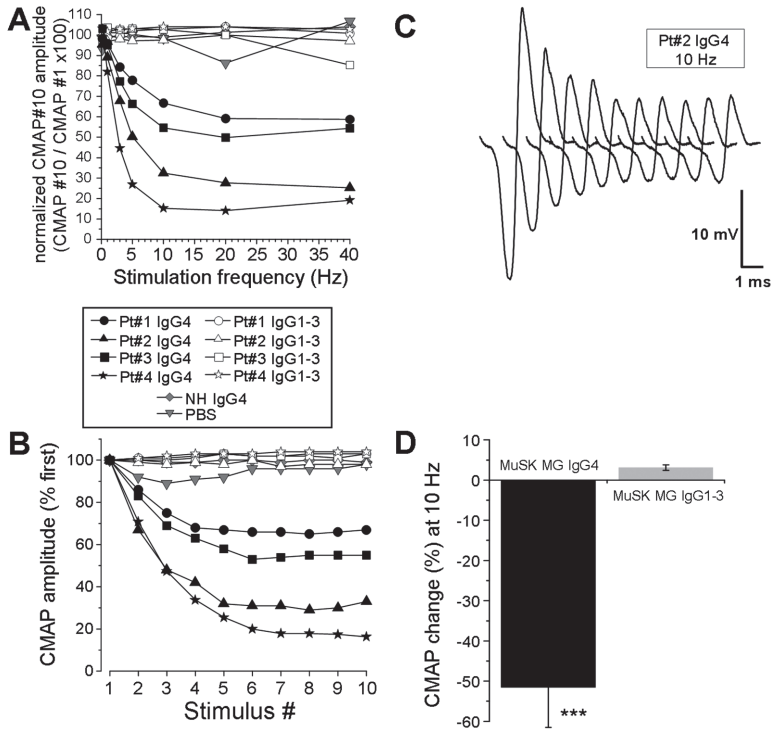


Figure 4. Passive transfer of MuSK myasthenia gravis IgG4 causes severe CMAP reduction. Repetitive nerve stimulation electromyography in anaesthetized mice revealed frequency-dependent reduction of CMAPs, compatible with a myasthenia gravis-like neuromuscular junction defect. (A) Stimulation frequency-dependency of CMAP decrements in the same exemplary mice as used for Fig. 3. (B) Development of CMAP amplitude during 10 stimuli at 10 Hz. (C) Example CMAPs in a mouse injected with Patient 2 IgG4. (D) Group comparison of CMAP amplitude change during 10 Hz nerve stimulation. ***$P < 0.001$, clinically weak MuSK myasthenia gravis IgG4 ($n = 8$ mice) versus MuSK myasthenia gravis IgG1–3 ($n = 8$ mice).

17.6 ± 0.5 g ($P < 0.001$, Student's t-test, n = 10 and 8 mice, respectively; for example contraction profiles see Fig. 5A). Tetanic fade (i.e. rundown of contraction force in spite of continuous nerve stimulation) was observed with most (8/10) muscles from the clinically weak mice injected with MuSK myasthenia gravis IgG4. Next, muscles were exposed to several concentrations of the reversible AChR antagonist d-tubocurarine and the inhibiting effect on tetanic contraction force was determined. Hemidiaphragms of the 10 clinically weak mice injected with MuSK myasthenia gravis patient IgG4 were more sensitive, with (intrapolated) 50% inhibiting d-tubocurarine concentrations of ~50 nM, while that of the eight MuSK myasthenia gravis IgG1–3, one normal human IgG4 and two PBS-injected controls was ~400 nM (Fig. 5B, $P < 0.001$, MuSK myasthenia gravis IgG4 versus IgG1–3, Student's t-test). This shows that MuSK myasthenia gravis IgG4 severely reduces the safety factor of neuromuscular transmission at the neuromuscular junction.

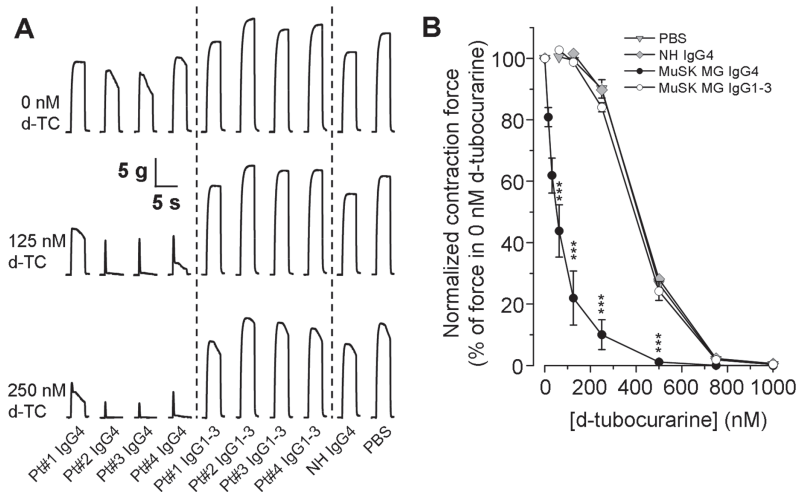


Figure 5. Passive transfer of MuSK myasthenia gravis IgG4 causes tetanic fade and reduced safety factor of neuromuscular transmission. Contraction experiments on left hemidiaphragm from passive transfer mice. (A) Example muscles from mice injected with Patient 1–4 IgG4, three of them showing tetanic fade upon 40 Hz nerve stimulation (upper row of traces). All MuSK myasthenia gravis IgG4 muscles delivered a lower absolute force as compared with the controls and were much more sensitive to reduction of force by the reversible AChR antagonist d-tubocurarine (d-TC). Examples of the contraction profiles in the presence of 125 and 250 nM are shown in the second and third row of traces. (B) Concentration–effect relationship of d-tubocurarine and contraction force, showing a large leftward shift of the curves of the muscles of 10 tested clinically weak mice injected with MuSK myasthenia gravis IgG4 (from an estimated EC_{50} of ~400 nM in controls to ~50 nM), demonstrating greatly reduced safety factor of neuromuscular transmission at neuromuscular junctions. ***$P < 0.001$, clinically weak MuSK myasthenia gravis IgG4 ($n = 10$ mice) versus MuSK myasthenia gravis IgG1–3 ($n = 8$ mice).

To elucidate the exact transmission defects at the neuromuscular junction underlying the fatigable muscle weakness caused by MuSK myasthenia gravis IgG4, we performed detailed ex vivo electrophysiological studies. Micro-electrode recordings of synaptic signals at neuromuscular junctions of diaphragm muscles from the NOD/SCID mice injected with IgG4 revealed small mean miniature endplate potential amplitudes (on average 0.67 ± 0.09 mV, i.e. ~50% reduction), as compared with 1.31 ± 0.03 mV observed in IgG1–3 injected NOD/SCID controls ($P < 0.001$, Student's t-test, $n = 10$ and 8 mice, respectively; Fig. 6A–D). Miniature endplate potentials occurred considerably less frequently (−55%) at neuromuscular junctions from all weak MuSK myasthenia gravis patient IgG4-treated mice, being on average 0.29 ± 0.02 s, compared with 0.65 ± 0.03/s in controls ($P < 0.001$; Fig. 6E). In addition, there was more amplitude variation amongst miniature endplate potentials recorded at individual neuromuscular junctions in muscles from weak mice injected with IgG4 from Patients 1, 2 and 4, group mean variance coefficients 0.39 ± 0.02 compared with 0.29 ± 0.01 in IgG1–3 NOD/SCID controls ($P < 0.001$; Fig. 6F). Furthermore, there was a tendency of slowed miniature endplate potential kinetics,

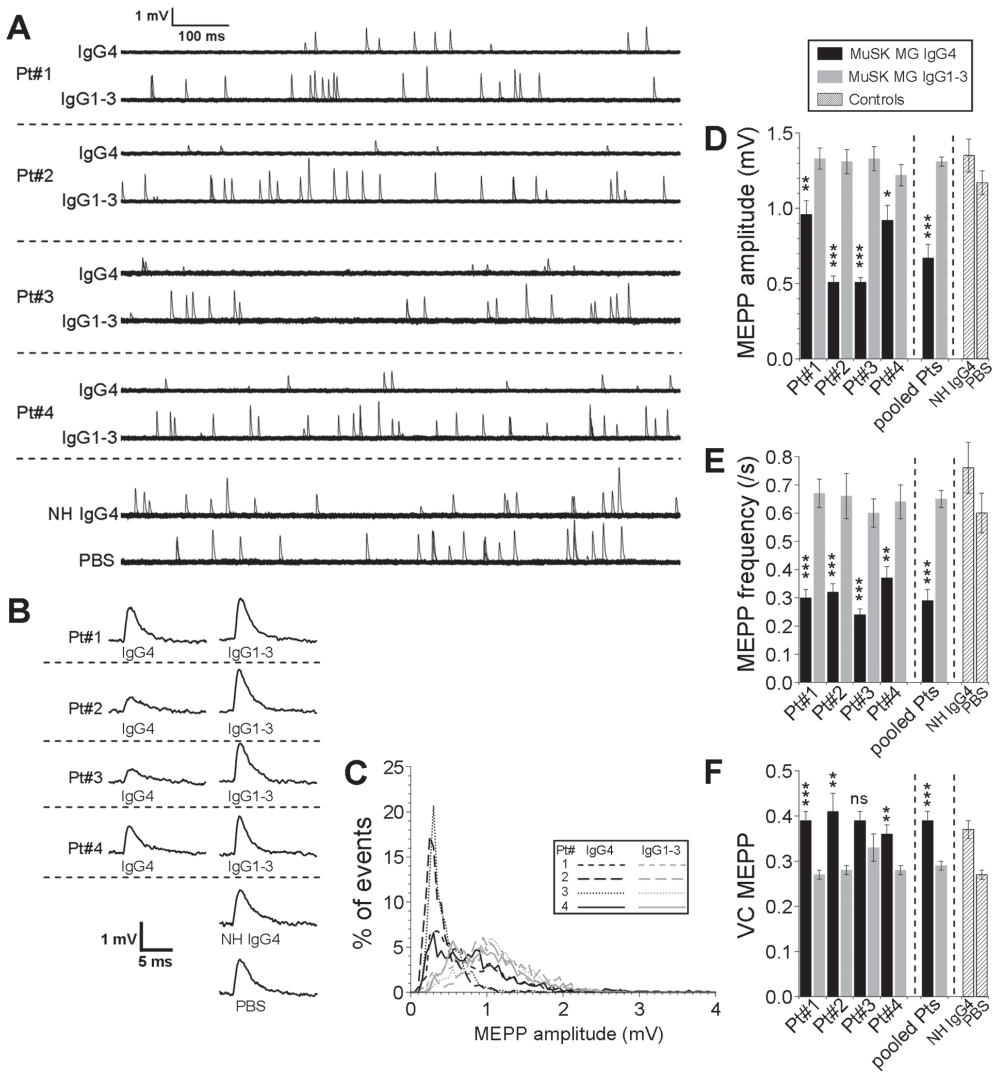


Figure 6. MuSK myasthenia gravis IgG4 causes reduction of the postsynaptic electrophysiological sensitivity for acetylcholine at neuromuscular junctions. *Ex vivo* intracellular electrophysiological microelectrode measurements of miniature endplate potentials at neuromuscular junctions of right hemidiaphragm muscles from passive transfer mice. (A) Examples of 1 s recording traces, 30 traces superimposed. (B) Representative miniature endplate potentials, 15 ms traces. (C) Miniature endplate potential (MEPP) amplitude distributions (based on 896–2316 miniature endplate potentials per condition), showing shift of the miniature endplate potential amplitudes towards smaller values in the neuromuscular junctions from mice treated with MuSK myasthenia gravis IgG4. MuSK myasthenia gravis IgG3 curves were similar to PBS and normal human IgG4 controls (data not shown). Reduced average miniature endplate potential amplitudes (D) and uniquantal spontaneous acetylcholine release, measured as miniature endplate potential frequency (E), at neuromuscular junctions from mouse muscles injected with MuSK myasthenia gravis IgG4. (F) Increased variance coefficient (VC) of miniature endplate potential amplitude at neuromuscular junctions of muscles from mice injected with IgG4 from patients with MuSK myasthenia gravis. Individual patient data in D–F based on 2–4 mice per patient IgG subclass with 8–15 neuromuscular junctions per hemidiaphragm from each

mouse tested; bars represent mean ± SEM of $n = 25–43$ neuromuscular junctions. Pooled patients' bars represent mean ± SEM of $n = 10$ mice treated with MuSK myasthenia gravis IgG4 treated and $n = 8$ mice treated with MuSK myasthenia gravis IgG1–3. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test, ns = not significant, IgG4 group versus IgG1–3 group. Normal human (NH) IgG4 control bar represents mean ± SEM of 10 neuromuscular junctions from one injected mouse. PBS control bar represents mean ± SEM from 22 neuromuscular junctions from two mice.

10–90% rise times being on average 0.82 ± 0.08 ms with MuSK myasthenia gravis IgG4 and 0.61 ± 0.03 ms with IgG1–3 ($P < 0.05$; supplementary Fig. 1A). In diaphragm from one mouse (Mouse 11, supplementary Table 1) treated with Patient 3 IgG4, 15% of neuromuscular junctions (6 of 40 sampled) showed no miniature endplate potentials at all, and nerve stimulation did not evoke a postsynaptic response. In the other tested muscles, no or only an occasional (<2%) silent neuromuscular junction was encountered, similar to previous observations using our methods in normal, untreated muscles (33). We also recorded endplate potentials at neuromuscular junctions, which result from nerve impulse-induced acetylcholine release from the presynaptic motor nerve terminal. Endplate potentials evoked at 0.3 Hz were considerably smaller at neuromuscular junctions from clinically weak MuSK myasthenia gravis IgG4-treated mice.

The mean amplitude was 16.23 ± 1.25 mV, while the IgG1–3 injected control NOD/SCID group value was 29.23 ± 0.83 mV ($P < 0.001$, Fig. 7A and B) and PBS and normal human IgG4 control values were in the same range (Fig. 7A and B). As with miniature endplate potentials, there was more amplitude variation within individual neuromuscular junctions of patient IgG4-treated mice, showing a mean endplate potential variance coefficient of 0.13 ± 0.01, while the IgG1–3 value was 0.06 ± 0.005 ($P < 0.001$; Fig. 7C), with PBS and normal human IgG4 control values in the same range. Furthermore, there was a tendency of slower endplate potential kinetics, with half-widths and 100–0% decay times being on average 12% increased with MuSK myasthenia gravis IgG4, compared with IgG1–3 ($P < 0.05$; supplementary Fig. 1C and D). From the mean endplate potential (0.3 Hz) and miniature endplate potential amplitudes at each neuromuscular junction we calculated the quantal content (i.e. the number of acetylcholine quanta released per nerve impulse). Surprisingly, in view of the well-known phenomenon of homeostatic upregulation of quantal content when postsynaptic sensitivity for acetylcholine is reduced in myasthenia (34,35,36,37,38), quantal contents at all patient IgG4-treated neuromuscular junctions were similar to all controls, i.e. ~35 quanta per nerve impulse ($P = 0.5$; Fig. 7D). We also stimulated the phrenic nerve at 40 Hz, the approximate physiological firing frequency of rodent motor neurons (39), as used in the tetanic contraction experiments described above. At control neuromuscular junctions, this led to rundown of endplate potential amplitudes to a plateau phase reached after the 10th endplate potential of ~75–80% of the first endplate potential. At neuromuscular junctions of muscles from mice treated with Patients 2, 3 and 4 IgG4, endplate potential run-down was much more

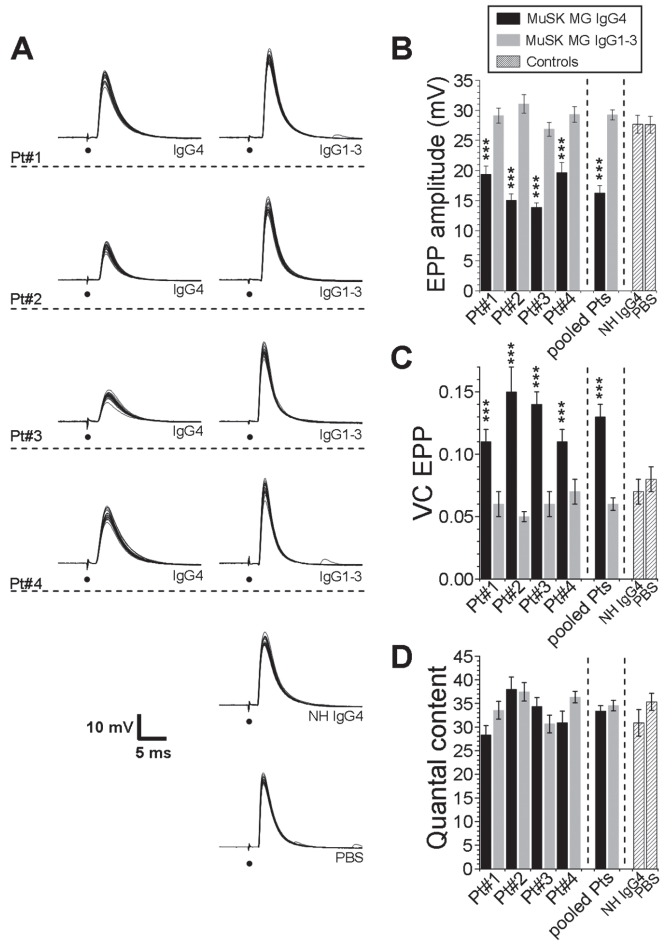


Figure 7. Neuromuscular junctions from mice injected with MuSK myasthenia gravis IgG4 have small endplate potentials and lack compensatory increased acetylcholine release. *Ex vivo* intracellular electrophysiological microelectrode measurements of 0.3 Hz nerve stimulation-evoked endplate potentials at neuromuscular junctions of right hemidiaphragm muscles from passive transfer mice. (A) Exemplary endplate potentials (20 subsequently recorded endplate potentials, superimposed). Black dots indicate the moment of nerve stimulation, causing a stimulation artefact. (B) Mean endplate potential (EPP) values amplitude. (C) Increased variance coefficient (VC) of endplate potential amplitude at neuromuscular junctions of muscles from mice injected with IgG4 from patients with MuSK myasthenia gravis. (D) In spite of reduction of miniature endplate potential amplitude (see Fig. 6), which normally leads to homeostatic increase of acetylcholine release at neuromuscular junctions, neuromuscular junctions of muscles from mice injected with MuSK myasthenia gravis IgG4 do not have increased quantal contents, all being ~35 quanta released per nerve impulse. Individual patient data in B–D based on 2–4 mice per patient IgG subclass with 8–15 neuromuscular junctions per hemidiaphragm from each mouse tested; bars represent mean ± SEM of $n = 25–43$ neuromuscular junctions. Pooled patients bars represent mean ± SEM of $n = 10$ mice treated with MuSK myasthenia gravis IgG4 treated and $n = 8$ mice treated with MuSK myasthenia gravis IgG1–3. ***$P < 0.001$, Student's *t*-test, IgG4 group versus IgG1–3 group. Normal human (NH) IgG4 control bar represents mean ± SEM of 10 neuromuscular junctions from one mouse injected. PBS control bar represents mean ± SEM from 22 neuromuscular junctions from two mice.

pronounced, to a plateau value of 57, 53 and 59%, respectively ($P < 0.001$; Fig. 8A–C). For Patient 1 IgG4, this was not the case. As with 0.3 Hz evoked endplate potentials, there was more amplitude variation amongst subsequent endplate potentials during the plateau phase at individual neuromuscular junctions. While the mean variance coefficient of all controls was 0.08 ± 0.004, the patient IgG4 value was 0.20 ± 0.01 ($P < 0.001$; Fig. 8D). The electrophysiological measurements show that MuSK myasthenia gravis IgG4, but not IgG1–3, causes combined pre- and postsynaptic electrophysiological defects that eventually lead to defective transmission and completely explain the (fatigable) muscle weakness.

MuSK myasthenia gravis IgG1–3 plus additional human complement does not induce muscle weakness

NOD/SCID mice lack an active haemolytic complement system (24), meaning that a potential complement-mediated effect of injected MuSK myasthenia gravis patient IgG1–3 might have been missed. To control this possibility we injected 0.5 ml normal human serum as complement source into NOD/SCID mice pretreated for 10–18 days with 4 mg/day MuSK myasthenia gravis patient IgG1–3 (each of the four patient IgG1–3 tested in one mouse). This treatment paradigm has been shown to cause complement-dependent neuromuscular junction damage in a mouse model for another autoimmune neuromuscular disease, Miller Fisher syndrome (using anti-GQ1b ganglioside antibodies) (33). However, no muscle weakness occurred in the following 3 h, and no CMAP reduction was observed with electromyography. Dissected diaphragms had normal d-tubocurarine sensitivity in contraction experiments and electrophysiological neuromuscular junction parameters were within range of IgG1–3 alone, normal human IgG4- and PBS-injected NOD/SCID control mice (data not shown). This shows there was no substantial *in vivo* binding of MuSK myasthenia gravis IgG1–3 to neuromuscular junctions, confirming the *in vitro* immunofluorescence studies (Fig. 2). Together this excludes complement-mediated effects at the neuromuscular junction of the used MuSK myasthenia gravis IgG1–3.

Disturbed pre- and postsynaptic neuromuscular junction geometry in MuSK-myasthenia gravis IgG4-treated mice

After finishing the functional studies, diaphragms from passive transfer mice were stained for AChRs to enable neuromuscular junction imaging. Confocal laser scanning microscopy of neuromuscular junctions of mice treated with MuSK myasthenia gravis IgG4 showed severe morphological abnormalities (Fig. 9A–I), including very small (~25% of control area, $P < 0.01$, ANOVA, Fig. 9D) and less intensely stained AChR clusters (summed pixel intensity/neuromuscular junction <20% of controls, $P < 0.01$, ANOVA, Fig. 9F). Many neuromuscular junctions had an irregular, dispersed and punctuate staining pattern (Fig. 9G). A proportion of neuromuscular junctions showed remarkable striping, with multiple elongated clusters running in parallel along the longitudinal muscle fibre axis (Fig. 9G). Some neuromuscular junctions showed

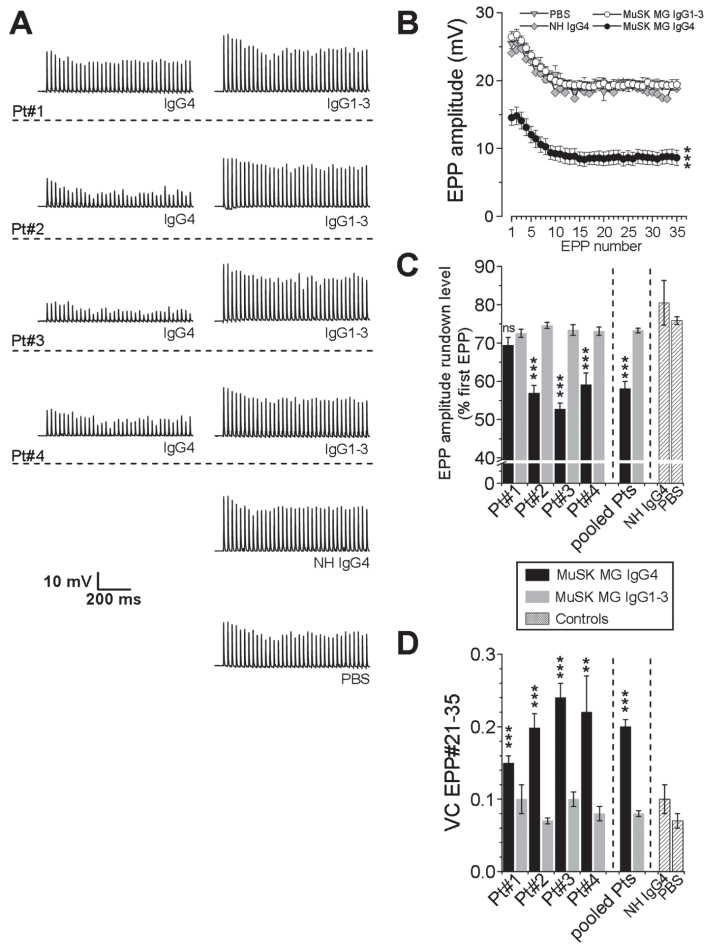


Figure 8. Passive transfer with MuSK myasthenia gravis IgG4 causes extra depression of acetylcholine release during high-rate use of neuromuscular junctions. *Ex vivo* intracellular electrophysiological microelectrode measurements of 40 Hz nerve stimulation-evoked endplate potentials at neuromuscular junctions of right hemidiaphragm muscles from passive transfer mice. (A) Examples of endplate potential trains recorded; 1 s duration traces. Stimulation artefacts have been partially removed for clarity. (B) Average endplate potential (EPP) amplitudes during 35 pulses of 40 Hz nerve stimulation. Pooled data from 10 clinically weak MuSK myasthenia gravis IgG4-injected mice, eight MuSK myasthenia gravis IgG1–3 injected mice, one normal human IgG4-injected mouse and two PBS-injected mice. (C) Mean run-down level is exaggerated at neuromuscular junctions from muscles from mice injected with MuSK myasthenia gravis Patients 2, 3 and 4 IgG4. (D) Neuromuscular junctions from MuSK myasthenia gravis IgG4-injected mice showed more amplitude variation during the plateau phase of the endplate potential trains. Individual patient data in B–D based on 2–4 mice per patient IgG subclass with 8–15 neuromuscular junctions per hemidiaphragm from each mouse tested; bars represent mean ± SEM of $n = 25–43$ neuromuscular junctions. Pooled patients bars represent mean ± SEM of $n = 10$ mice treated with MuSK myasthenia gravis IgG4 treated and $n = 8$ mice treated with MuSK myasthenia gravis IgG1–3. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test, IgG4 group versus IgG1–3 group. Normal human (NH) IgG4 control bar represents mean ± SEM of 10 neuromuscular junctions from one mouse injected. PBS control bar represents mean ± SEM from 22 neuromuscular junctions from two mice.

a vague remnant of the normal 'pretzel-like' structure, readily observed at bright intensity in NOD/SCID controls (MuSK IgG1–3, normal human IgG4 and PBS, Fig. 9H and I). In separate imaging experiments on levator auris longus muscles from passive transfer mice we double-stained neuromuscular junctions for AChRs and the presynaptic marker SV2. As with diaphragm from MuSK myasthenia gravis IgG4-treated mice, many AChR clusters in levator auris longus neuromuscular junctions were fragmented, more faintly and punctately stained, again sometimes with vague remnants of a pretzel-like structure. Again, striping was observed at a proportion of the neuromuscular junctions (Fig. 10A). Patient IgG1–3 or normal human IgG4 injected NOD/SCID controls showed more continuous, brightly stained pretzel-like structures (Fig. 10A). SV2 staining in these controls clearly co-localized with AChRs, and was rather continuous.

In MuSK myasthenia gravis IgG4-treated neuromuscular junctions this was less clear, SV2 staining being somewhat more punctuate and co-localized with the vaguely stained remnant of the AChR pretzel-like structure than with the dispersed AChR puncta (Fig. 10A).

Electron microscopic investigation of diaphragm neuromuscular junctions from passively transferred mice confirmed the postsynaptic membrane defects. Many neuromuscular junctions from MuSK myasthenia gravis IgG4-treated mice showed less extensive postsynaptic foldings, the postsynaptic folding index (length of the postsynaptic membrane normalized to the length of the presynaptic membrane) being reduced by ~25%, compared with the neuromuscular junctions from IgG1–3-treated mice ($P < 0.001$, Student's t-test, Fig. 10B and C).

These morphological studies clearly show that MuSK myasthenia gravis IgG4 induces fragmentation and reduction of the postsynaptic AChR area and that presynaptic geometry changes too, albeit less dramatically. It should be realized that the extent of the remaining postsynaptic area may have been overestimated considerably, due to a complete disappearance of a proportion of neuromuscular junctions, especially at diaphragm, as suggested by the electrophysiological measurements and our visual assessments of the AChR immunofluorescence preparations. Overall, the morphological neuromuscular junction defects induced by MuSK myasthenia gravis IgG4 showed much similarity with those observed in active and (whole-IgG) passive MuSK myasthenia gravis mouse models by others (22,23,40,41).

DISCUSSION

IgG4 is traditionally considered as an anti-inflammatory IgG, as opposed to IgG1 and IgG3 subclasses, which activate immune cells and complement. Therefore, the role of elevated and antigen-specific IgG4 in a number of autoimmune diseases has thus far been enigmatic (17,10,15,41). We show here that IgG4 from patients with MuSK myasthenia gravis binds to mouse neuromuscular junctions and causes severe muscle weakness, without requiring other immune system components. Detailed synaptic function analyses showed severely reduced electrophysiological postsynaptic

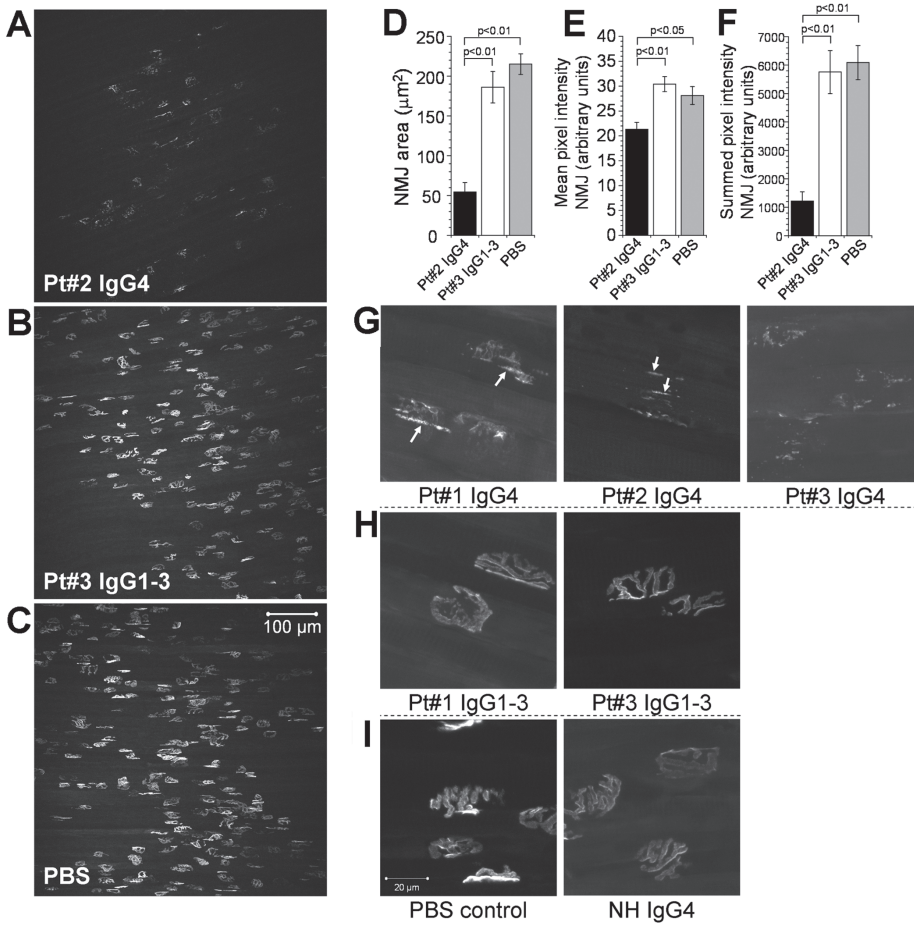


Figure 9. Disturbed postsynaptic neuromuscular junction (NMJ) morphology in MuSK myasthenia gravis IgG4 passive transfer mice. Example confocal laser scanning maximum intensity *z*-stack projections of AChR-stained neuromuscular junctions from mice treated with (A) Patient 2 MuSK myasthenia gravis IgG4, (B) Patient 3 IgG1–3, or (C) PBS. Muscles were processed together in one identical experimental run, allowing direct comparison of area and intensity of staining, which were greatly reduced at MuSK myasthenia gravis IgG4-treated neuromuscular junctions (D–F), mean ± SEM of $n = 10$ random neuromuscular junctions; *P*-value at least < 0.05, ANOVA. (G) Typical stripes (white arrows) were often present at neuromuscular junctions with disintegrated AChR area. (H) Normal AChR staining at example control neuromuscular junctions treated with MuSK myasthenia gravis IgG1–3 treated neuromuscular junctions or (I) normal human (NH) IgG4 or PBS. Scale bar = 100 µm (A, B, C); = 20 µm (G, H, I).

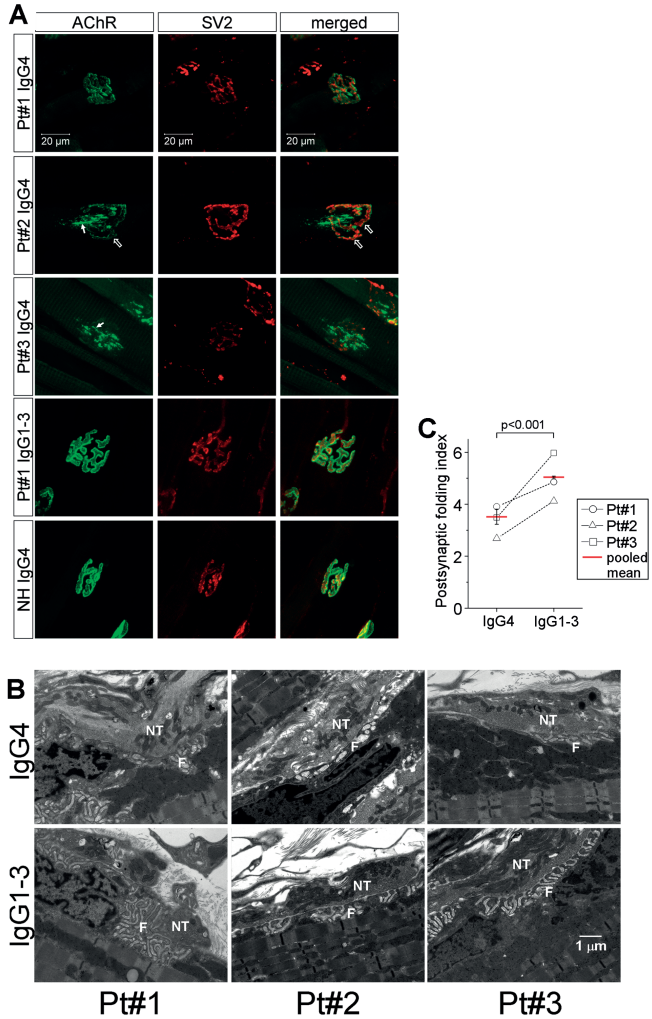


Figure 10. Disturbed presynaptic neuromuscular junction morphology and postsynaptic ultrastructure in MuSK myasthenia gravis IgG4 passive transfer mice. (A) Levator auris longus neuromuscular junctions co-stained for AChRs (green) and presynaptic SV2 (red). Disrupted AChR geometry was present, albeit less outspoken than in diaphragm, especially with Patient 1 IgG4. Striping (white arrows) and vague remnants of normal structure (white hollow arrow) were sometimes encountered. SV2 staining was somewhat more punctuate and dispersed, as compared with control neuromuscular junctions from MuSK myasthenia gravis IgG1–3 or normal human IgG4-treated mice, showing more clear and continuous staining, co-localizing with the clear AChR staining. (B) Ultrastructure of diaphragm neuromuscular junctions showed less extensive postsynaptic foldings. NT = nerve terminal area, F = folding area. Scale bar = 1 µm. (C) Electron microscopy morphometry demonstrates lower folding index (i.e. simplified postsynaptic membranes) at neuromuscular junctions of MuSK myasthenia gravis IgG4-injected mice ($P < 0.001$, pooled mean value of $n = 34$ MuSK myasthenia gravis IgG4 profiles versus $n = 68$ MuSK myasthenia gravis IgG1–3 profiles, Student's t-test).

acetylcholine sensitivity at neuromuscular junctions and an extra rundown of presynaptic transmitter release during intense synaptic activity jointly underlie the weakness. Of particular interest, MuSK myasthenia gravis mouse model neuromuscular junctions lacked compensatory upregulation of acetylcholine release, which is the normal homeostatic presynaptic response to reduced postsynaptic acetylcholine sensitivity. This renders them more vulnerable to transmission block. We are the first to pinpoint human anti-MuSK IgG4 as specific myasthenogenic and to reveal the exact pre- and postsynaptic functional defects it causes at the neuromuscular junction.

Our study clearly demonstrates that MuSK myasthenia gravis IgG4 targets the neuromuscular junction. *In vitro* staining of levator auris longus neuromuscular junctions of normal mice showed binding of IgG4, but not IgG1–3, which entirely co-localized with AChRs. Any unexpected neuromuscular junction binding of IgG4 antibodies per se was excluded in control incubations with purified normal human IgG4. The (fatigable) muscle weakness and ~20–100% electromyographical CMAP decrement observed in clinically weak MuSK myasthenia gravis IgG4-injected mice indicated neuromuscular junction dysfunction. Similar electromyographical observations were made previously in MuSK myasthenia gravis whole-IgG passive transfer mice (22). Fatigable muscle weakness hallmarks (MuSK) myasthenia gravis (42) and, provided testing clinically weak muscles, CMAP reduction is found in most patients with MuSK myasthenia gravis (43). Myasthenia was absent in NOD/SCID control mice receiving either MuSK myasthenia gravis IgG1–3, normal human IgG4 or PBS alone. This shows that MuSK myasthenia gravis IgG4 specifically caused muscle weakness and, together with the correlation of anti-MuSK IgG4 titre with disease severity (14), strongly suggests it is the crucial pathogenic factor in MuSK myasthenia gravis. Earlier studies suggested that anti-MuSK auto-antibodies might only be bystanding disease markers, in view of absence of AChR reduction and IgG deposits at biopsied MuSK myasthenia gravis neuromuscular junctions (20,21). However, subsequent animal studies including this one, strongly suggest anti-MuSK antibody to be the cause. First, post-natal deletion of the MuSK gene in mouse muscle causes severe weakness due to AChR loss at neuromuscular junctions, demonstrating that post-developmental removal of MuSK leads to myasthenia (44). Second, immunization of rabbits (45) or mice (40,41,46) with (rat) MuSK extracellular domain fragments yields paralytic animals with myasthenic features, i.e. CMAP decrement and reduced AChR density at neuromuscular junctions. Third, injection of high doses (45 mg/day) of MuSK myasthenia gravis total-IgG into mice causes myasthenia, also with CMAP reduction and AChR loss (22,23). Local injection of MuSK myasthenia gravis whole-plasma induced subclinical myasthenia in foot muscle (47). Fourth, the weak mice in our present study pinpoint anti-MuSK IgG4 as the crucial myasthenogenic factor. One reason for not observing AChR loss at biopsied human MuSK myasthenia gravis neuromuscular junctions, at least in one study (21), may have been that extremity muscle was used, which is normally not clinically weak in MuSK myasthenia gravis (42).

NOD/SCID mice are immunodeficient and defective in complement ((24)). The induced weakness in them shows that MuSK myasthenia gravis IgG4 can cause myasthenia by itself, without additional immune system components. Complement-independency was already suggested by anti-MuSK antibodies being mainly IgG4 (10,11,12), an IgG subclass unable to activate complement (48), and the observation that complement was not or only scarcely present at biopsied MuSK myasthenia gravis neuromuscular junctions (20,21). Although many MuSK myasthenia gravis sera activate complement in a cellular assay, likely due to some anti-MuSK IgG1 presence (49), injection of human complement in NOD/SCID mice pretreated with either of the four MuSK myasthenia gravis patient IgG1–3 s did not cause weakness here. Collectively, this supports the idea that MuSK myasthenia gravis differs from AChR myasthenia gravis, with AChR antibodies being IgG1 and IgG3 (13), and readily detectable complement at biopsy neuromuscular junctions (19,18). Although MuSK myasthenia gravis IgG1–3 did not cause weak mice, we cannot exclude some contribution in the few patients with MuSK myasthenia gravis with additional anti-MuSK IgG1–3.

We observed some variability in potency amongst MuSK myasthenia gravis IgG4s from different patients (supplementary Table 1), and dose-dependency of effects as exemplified by IgG4 from Patient 2 with low daily doses (0.13 and 1 mg) causing only subclinical myasthenia (detected in muscle contraction experiments using d-tubocurarine), while 4 mg/day induced overt clinical weakness. Others induced weakness in mice with high doses of 45 mg total MuSK myasthenia gravis IgG per day (22). Because IgG4 constitutes between 5% and 14% of total IgG (at least in the MuSK myasthenia gravis sera that were used in our current study; R. Klooster, unpublished data), we estimate this total IgG must have roughly contained 2–6 mg IgG4. This is in the range of the daily doses of purified IgG4 that produced weak mice in the present study and suggests that the effects in the study of (22) were due to the IgG4 component of the injected total IgG.

Electrophysiological study of neuromuscular junctions of MuSK myasthenia gravis IgG4-injected mice revealed clear postsynaptic abnormalities, explaining the muscle weakness. Considerable reductions (~50%) in miniature endplate potential amplitude, indicated greatly reduced AChR density, a hallmark of myasthenic neuromuscular junctions in patients with AChR myasthenia gravis and animal models (34,50,36,37).The faint and fragmented AChR staining observed with confocal fluorescence microscopy, similar to observations in active and passive MuSK myasthenia gravis mouse models by others (22,23,40,41), is compatible with this postsynaptic electrophysiological defect. In further agreement, electron microscopic investigation revealed simplified postsynaptic membrane ultrastructure, as shown in a MuSK myasthenia gravis muscle biopsy (20). Due to the fragmented AChR area, acetylcholine quanta released from different presynaptic sites will act on different local postsynaptic AChR densities. This may explain the higher (miniature) endplate potential amplitude variations at individual neuromuscular junctions. Another factor may be the distribution of the acetylcholine degrading acetylcholinesterase, which

by interaction with perlecan and collagenQ, is determined by MuSK (51). Immune attack on MuSK might create less uniform acetylcholinesterase density in the synaptic cleft and thus local variation of acetylcholine hydrolysis, causing increased (miniature) endplate potential amplitude variation. In addition, the somewhat slower (miniature) endplate potential kinetics we observed may indicate some overall reduction of acetylcholinesterase, because (miniature) endplate potential broadening is a hallmark of acetylcholinesterase inhibition (52,53). Notably, many MuSK patients with myasthenia gravis do not benefit from acetylcholinesterase inhibiting drugs, standard and beneficially used in AChR myasthenia gravis, and may even display symptoms of overdosing when receiving only moderate doses (6,54,55). Collectively, this suggests that AChR reduction at neuromuscular junctions of patients with MuSK myasthenia gravis may be paralleled by (partial) acetylcholinesterase loss. In agreement, very recent active immunization and MuSK myasthenia gravis whole-IgG passive transfer mouse studies showed reduction of acetylcholinesterase protein expression at neuromuscular junctions (56) and acetylcholinesterase messenger RNA at some muscle types (41). Alternatively, changes in (miniature) endplate potential kinetics may result from the disturbed AChR geometry and density by itself, forcing the acetylcholine molecules released to diffuse further to encounter AChR molecules.

One mouse treated with MuSK myasthenia gravis Patient 3 IgG4 had 15% 'silent' neuromuscular junctions, i.e. with no synaptic electrophysiological signals. This agrees with some muscle fibres having barely or no detectable AChRs in confocal microscopy, as also shown by others in MuSK myasthenia gravis total-IgG passive transfer mice (23). Significant numbers of silent neuromuscular junctions were not encountered with IgG4 from the other patients, nor in (weak) muscles from mice injected with lower doses Patient 3 IgG4 (data not shown). This demonstrates that this phenomenon is dose- and patient-dependent, and probably represents the most extreme form of neuromuscular junction disruption by anti-MuSK IgG4. Silenced neuromuscular junctions in the diaphragm of the mouse treated with Patient 3 IgG4 likely also contributed to the lower absolute tetanic contraction force measured *ex vivo*.

The amplitudes of 0.3 Hz nerve stimulation-evoked endplate potentials were severely reduced (on average by ~50%) at neuromuscular junctions of muscles from clinically weak MuSK myasthenia gravis IgG4 injected mice. At many neuromuscular junctions they were smaller than 12 mV, about the minimal endplate potential required to trigger a muscle fibre action potential in rodents (57). At 40 Hz, a physiological rate for rodent neuromuscular junctions (39), endplate potentials at many more neuromuscular junctions became <12 mV due to exaggerated amplitude depression (on average by 42% at MuSK myasthenia gravis IgG4-treated neuromuscular junctions, as compared with only 27% depression in IgG1–3 controls). Thus, subthreshold endplate potentials (either continuous or evolving at high-rate nerve firing) at many neuromuscular junctions explains both the (fatigable) weakness in vivo as well as the low absolute contraction force and tetanic fade of diaphragm

muscles in contraction experiments. Endplate potentials of control mice, without *in vivo* or *ex vivo* weakness, were much larger (>25 mV), demonstrating the large safety factor at healthy neuromuscular junctions (58). The greatly increased d-tubocurarine sensitivity of contraction of MuSK myasthenia gravis IgG4-treated mice diaphragms indicates a severely reduced safety factor at those neuromuscular junctions that still had suprathreshold endplate potentials.

Ex vivo myasthenic features of one mouse injected with Patient 1 IgG4 were the least outspoken, in spite of equal dosing (4 mg/day) as the Patient 2 and 4 IgG4 mice, and even twice that of the Patient 3 IgG4 mouse, which was the most affected. This shows potency variation amongst different MuSK myasthenia gravis IgG4s, as shown for total-IgGs by others (22). However, *in vivo* weakness of this one Patient 1 IgG4 mouse was overt and there was rapid weight loss, suggesting that muscles other than the *ex vivo* investigated diaphragm were more affected. Furthermore, we show clear dose-dependency of MuSK myasthenia gravis IgG4, exemplified by Patient 2 IgG4, which in low-dose (1 mg/day) induced temporary weakness *in vivo* (data not shown) and subclinical myasthenia in *ex vivo* analyses (supplementary Fig. 2).

Besides postsynaptic functional defects, MuSK myasthenia gravis IgG4 also induced presynaptic changes: 55% reduction of spontaneous uniquantal acetylcholine release (miniature endplate potential frequency) and greatly exaggerated depression of acetylcholine release at 40 Hz (resulting in extra endplate potential rundown). The miniature endplate potential amplitude distribution curves (Fig. 6C) excluded that the low miniature endplate potential frequency simply resulted from miniature endplate potentials becoming too small to detect. Rather, low miniature endplate potential frequency may indicate small presynaptic terminal size (59). Indeed, we observed somewhat fainter, more punctuate presynaptic SV2 staining. In agreement, neuromuscular junctions from mice injected with MuSK myasthenia gravis total-IgG showed impaired pre- and postsynaptic apposition, indicating reduced functional presynaptic area (i.e. with opposite AChR presence) (22). In theory, anti-MuSK IgG4 may act directly on the presynaptic motor nerve terminal. However, neuronal MuSK expression is unlikely (4), although it cannot be completely ruled out (60). It is more conceivable that autoimmune attack of postsynaptic MuSK, either directly or indirectly, disturbs functional and structural synaptic homeostasis pathways at neuromuscular junctions. Neuromuscular junctions from patients with AChR myasthenia gravis and rodent models display 50–200% upregulation of acetylcholine release, counteracting postsynaptic AChR loss and involving yet unidentified retrograde signalling factors (36,37,61), and similar synapse homeostasis in response to various challenges is observed in many species (62,63,64,65,66). At neuromuscular junctions of MuSK myasthenia gravis IgG4-injected myasthenic mice we observed failure of this important homeostatic response, aggravating weakness. This suggests a role for MuSK in the underlying pathways that sense AChR loss or release retrograde messaging molecules. Interestingly, some MuSK-signalling pathway members interact with or take part in the postsynaptic dystrophin glycoprotein complex (e.g. agrin and

rapsyn) (67,68,69,70), and deletions from this complex affect neuromuscular junction structure, function and synaptic homeostasis (68,71). Alternatively, lack of appropriate homeostasis at MuSK IgG4-treated neuromuscular junctions may be due to secondary presynaptic damage, merely following the severe postsynaptic disruption, preventing the nerve terminal to respond to retrograde signals. Disturbance of pre- and postsynaptic apposition (22) may be relevant here because if the total nerve terminal would in fact release extra acetylcholine, a partial lack of opposing AChR area would obscure this. The observed increase in endplate potential rundown may be seen as an indication that such 'hidden' quantal content increase indeed exists, causing a more rapid exhaustion of transmitter quanta, which is a feature of myasthenic motor nerve terminals once homeostatic transmitter upregulation is achieved (37). In any case, lack of upregulated functional acetylcholine release at MuSK myasthenia gravis neuromuscular junctions renders transmission more vulnerable to AChR loss, as compared with AChR myasthenia gravis neuromuscular junctions with adequate upregulation.

In conclusion, we provide strong evidence of anti-MuSK IgG4 being the crucial pathogenic factor in MuSK myasthenia gravis, causing combined pre- and postsynaptic functional neuromuscular junction defects with absence of an adequate synaptic homeostatic response, all contributing to muscle weakness. Microelectrode studies of neuromuscular junctions in MuSK myasthenia gravis muscle biopsies agree with the present mouse study: reduced miniature endplate potential amplitude without compensatory increased acetylcholine release (20,72), paralleled by low miniature endplate potential frequency and extra endplate potential rundown (72). This adds clinical relevance to our MuSK myasthenia gravis IgG4 mouse model. The role of IgG4 in MuSK myasthenia gravis as well as in other IgG4-associated autoimmune diseases has hitherto been uncertain (17,15,12,49). So far, only IgG4 (directed against an epidermal protein) in a variant of the autoimmune blistering disease pemphigus has been shown to cause blisters upon intradermal injection of mice (73,74). However, the precise pathological effects were not clarified. We are the first to demonstrate pathogenic action of an IgG4 auto-antibody on the neuromuscular system. The results may provide rationale for selective IgG4 depletion from MuSK myasthenia gravis patient plasma as therapy, rather than the currently practiced total plasmapheresis (75).

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SUPPLEMENTARY MATERIAL

Supplementary description of patients

Patient #1. This female patient presented at the age of 26 with intermittent diplopia and ptosis. A few months later, she experienced shortness of breath, dysarthria and mild proximal arm weakness during her work as a ballet teacher. Ten months after onset of symptoms, dysphagia occurred, she lost more than 10% of her body weight, and had to quit working. Antibodies to the AChR were negative. During the next years, she suffered from recurrent episodes with unexplained fatigue, dyspnea, dysphagia and anxiety for which she received long-term psychiatric treatment. Ten years later, she was referred for analysis of an exacerbation of bulbar weakness and dyspnea. She also complained about a dropping head and worsening of symptoms in the course of the day. Examination showed facial weakness, a feeble voice, fatigable neck and proximal arm weakness, but no ptosis or diplopia. Repetitive nerve stimulation electromyography revealed abnormal decrement of CMAP amplitude in two facial and the hypothenar muscles. Stimulated single fiber showed abnormal jitter in the orbicularis oculi muscle. Now, anti-MuSK antibodies could be demonstrated in the absence of anti-AChR antibodies. Forced vital capacity was 87% of predicted. Pyridostigmine was initiated, causing only muscle twitching and cramps. She underwent five courses of plasma exchange. Plasma was frozen for the studies described here. Because no improvement occurred, she was then treated with high dose steroids. Azathioprine caused liver enzyme reaction and was replacement by mycophenolate mofetil with gradual improvement of symptoms. One year later, steroids could be withdrawn and postintervention status was classified as minimal manifestations.

Patient #2. At age 30, being 14 weeks pregnant, this female experienced intermittent diplopia and mild ptosis. Pyridostigmine improved ocular symptoms, although muscle twitching occurred when dosing increased to 60 mg tds. Anti-AChR antibodies were negative. Severe bulbar symptoms started one week after delivery of a healthy boy, i.e. severe ptosis, ophthalmoparesis, facial weakness with a vertical smile and dysarthria. She also developed mild neck weakness but could still raise her arms >3 min. Repetitive nerve stimulation electromyography of hypothenar and trapezius muscles was normal. Stimulated single fiber electromyography of both orbicularis oculi muscles showed markedly increased jitter and blockings. She refused immunosuppressive therapy, despite recurrent exacerbations with dysphagia and severe shortness of breath. Her weight fell from 56 to 43 kg before she accepted high dose prednisone two years after onset. Now symptoms clearly improved and only occasional diplopia, mild nasal speech and slight difficulty in swallowing solid food remained. Symptoms relapsed when she reduced prednisone on her own initiative. In following years, more continuous bulbar weakness and atrophy of the tongue and facial muscles developed. Seven years after onset, she received intravenous IgG

treatment, without effect. By now, serious concerns existed about her weight loss, complicated by paranoid delusions regarding food and body perception. One month later, plasmapheresis was performed, leading to minimal improvement. Tube feeding was initiated, but she refused antipsychotic treatment. Unfortunately, prednisone was stopped and she was readmitted a few months later with severe respiratory insufficiency requiring ventilation, her weight now being only 36 kg. A second plasma exchange, followed by steroids and intravenous IgG, enabled extubation 3 weeks later. Antibodies to MuSK were demonstrated. Plasma from the second treatment was frozen for the present studies.

Patient #3. This male first experienced tiredness and intermittent diplopia at age 54. One month later, stair climbing became difficult. Being a frequent swimmer, he felt unable to keep his head above the water when doing breaststroke for a few minutes. In the evening his eyelids dropped, left more than right. A neurologist also noticed marked facial weakness. Diagnosis of MG was made and oral pyridostigmine treatment initiated. This slightly improved diplopia, but none of the other symptoms, and only in the first week. Antibodies to AChR were negative. One month later, he had progressive shortness of breath and wheezed during inspiration, especially at night. Repetitive nerve stimulation electromyography showed abnormal CMAP decrement in trapezius muscle. Stimulated single fiber electromyography in orbicularis oculi muscle showed increased jitter. Vital capacity was 68-74% of predicted. Anti-MuSK antibodies were positive. High dose prednisone and azathioprine was initiated, initially leading to some improvement of diplopia and dyspnea. However, six months after onset his speech became less intelligible, diplopia persisted during the day and his legs became weaker, requiring holding onto two banisters while stair climbing. In contrast, he experienced no arm weakness. He had lost 7% body weight in one month. Neurological examination showed marked ptosis, ophthalmoparesis, dysarthria and weakness of facial muscles and both neck flexors and extensors. Noticeable was the focal weakness of both hip extensors (MRC 3 range) and the ankle dorsiflexors (MRC 4 range), whereas he could still do 20 squads with some difficulty. Plasma exchange was performed, markedly improving symptoms. Plasma was frozen for the present studies. The quantitative MG score decreased in 10 days from 14 to 4, out of 39. One month later he felt asymptomatic, had regained weight and walked normally. Neurological examination showed only mild ptosis and minimal weakness of ankle dorsiflexors and hip extensors.

Patient #4. This female patient presented with unilateral ptosis, diplopia, dysphagia and mild proximal limb muscle weakness at age 6. Dysphagia led to 17% body weight loss in one month. Repetitive nerve stimulation of thenar muscles showed a decrement of 34%. Anti-AChR antibodies were negative. Oral pyridostigmine was ineffective. After two respiratory crises (vital capacity 55% of predicted), requiring intubation and assisted ventilation, a thymectomy was performed at age 7. She was treated with

prednisone and azathioprine, but had repeated respiratory crises in the following 3 years. She also needed continuous non-invasive ventilation at night between age 7 and 23. At age 10 she was treated for the first time with plasma exchange during a respiratory crisis leading to rapid clinical improvement. At age 13, weekly plasma exchange treatment was initiated. For this purpose an arteriovenous shunt was created in the left forearm. Mycophenolate mofetil was started, prednisone and azathioprine were withdrawn. At age 15, anti-MuSK antibodies were demonstrated. Weekly plasma exchange therapy was continued for 7 years. During these years she had no recurrence of respiratory crisis and she was able to finish high school and an international management study. Mycophenolate mofetil and pyridostigmine were withdrawn at age 19 and 20 respectively. The plasma exchange interval was increased to 2 weeks at age 21 and to three weeks at age 23 without a clinical deterioration. At this age plasma was frozen and used for the present studies. Examination showed mild facial weakness, rhinolalia, tongue atrophy, and a mild proximal muscle weakness.

Table S1. Overview of all passive transfer mice tested in this study. Indicated is the daily dose of i.p. injected purified subclass IgG in mg, whether or not mice developed visible *in vivo* weakness, body weight change during the last three days of passive transfer and change of compound muscle action potential (CMAP) amplitude recorded at 10 Hz nerve stimulation during subcutaneous needle electromyography at the calf muscles in anesthetized mice. NHS=normal human serum; n.d. = not done; n/a= not applicable.

	mouse#	patient#	daily dose (mg)	*in vivo* weakness	body weight change in last 3 days (%)	CMAP change at 10 Hz nerve stimulation (%)
MuSK MG IgG4	1	1	4	yes	-10	-33
	2	1	4	yes	-10	-21
	3	2	0.13	no	+4	+5
	4	2	1	no	+2	-2
	5	2	4	yes	-17	-67
	6	2	4	yes	-18	-26
	7	3	0.13	no	+1	+6
	8	3	0.5	yes	-15	n.d.
	9	3	1	yes	-25	-98
	10	3	1	yes	-20	-45
	11	3	2	yes	-26	n.d.
	12	4	1.5	no	+4	n.d.
	13	4	4	yes	-5	-38
	14	4	6	yes	-22	-84
MuSK MG IgG1-3	15	1	4	no	+3	+3
	16	1	4	no	+5	+3
	17	2	4	no	+5	-1
	18	2	4	no	+2	+5
	19	3	4	no	0	+3
	20	3	4	no	+4	+2
	21	4	4	no	+2	+4
	22	4	4	no	-1	+6
MuSK MG IgG1-3	23	1	4	no	+6	+5
+ NHS at end	24	2	4	no	-2	+4
	25	3	4	no	+3	+3
	26	4	4	no	+6	+2
Normal human IgG4	27	n/a	4	no	+7	-1
PBS	28	n/a	n/a	no	+1	-2
	29	n/a	n/a	no	+4	+4

Table S2. Comparison of the anti-MuSK antibody titres in patient plasma and serum of passive transfer mice. MuSK titres (nM) were determined with radioimmunoassay in the patient plasmas and the sera of the mice (obtained at the end of the experiment), injected with the different IgG fractions purified from the patient plasmas. Anti-MuSK reactivity in the mice was only observed after injection with the MuSK MG IgG4 fractions and not the IgG1-3 fractions. No anti-MuSK antibodies were detected in the serum of the mouse injected with normal human IgG4.

		Anti-MuSK titre (nM)	
Subject	**Patient plasma**	**IgG4-injected mouse serum**	**IgG1-3 injected mouse serum**
Patient #1	6.3	66.4	0.0
Patient #2	39.8	297.5	0.0
Patient #3	28.0	146.4	0.0
Patient #4	n.d.	n.d.	n.d.
Normal human control	0.0	0.0	n.d.

n.d. = not done

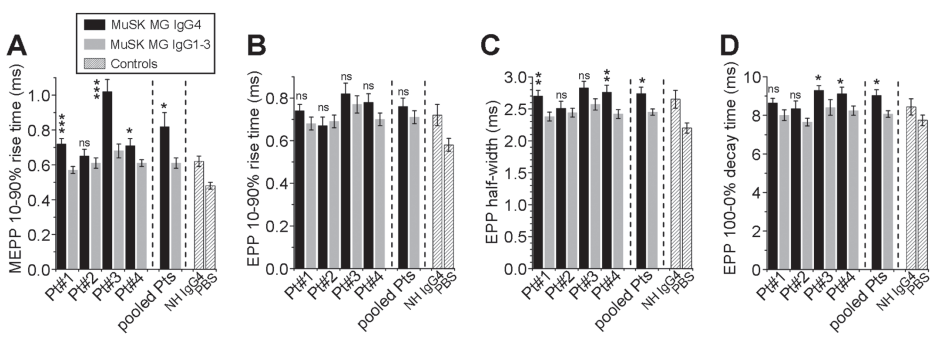

Figure S1. Tendency of slower kinetics of electrophysiological synaptic signals recorded at NMJs from mice injected with MuSK MG IgG4. *Ex vivo* intracellular electrophysiological micro-electrode measurements of MEPPs at NMJs of right hemidiaphragm muscles from passive transfer mice. (A) Rise times of MEPPs showed tendency of increase, especially at NMJs from the diaphragm muscle from the mouse treated with MuSK patient #3 IgG4. (B) Rise times of EPPs were similar in IgG4 and IgG1-3 treated NMJs. (C) EPP width at half the peak height tended to be larger at IgG4-treated NMJs, as well as the time needed to decay from 100 to 0% of the peak amplitude (D). These tendencies for slower MEPP and EPP kinetics at NMJs from MuSK MG IgG4 treated NMJ may point towards reduced presence of acetylcholinesterase in the synaptic cleft (see Discussion). Individual patient data based on 2-4 mice per patient IgG subclass with 8-15 NMJs per hemidiaphragm from each mouse tested; bars represent mean ± S.E.M. of n=25-43 NMJs. Pooled patients bars represent mean ± S.E.M. of n=10 mice treated with MuSK MG IgG4 treated and n=8 mice treated with MuSK MG IgG1-3. *p<0.05, **p<0.01, ***p<0.001, Student's t-test, n.s.=not significant, IgG4 group vs. IgG1-3 group. Normal human (NH) IgG4 control bar represents mean ± S.E.M. of 10 NMJs from one mouse injected. PBS control bar represents mean ± S.E.M. from 22 NMJs from two mice.

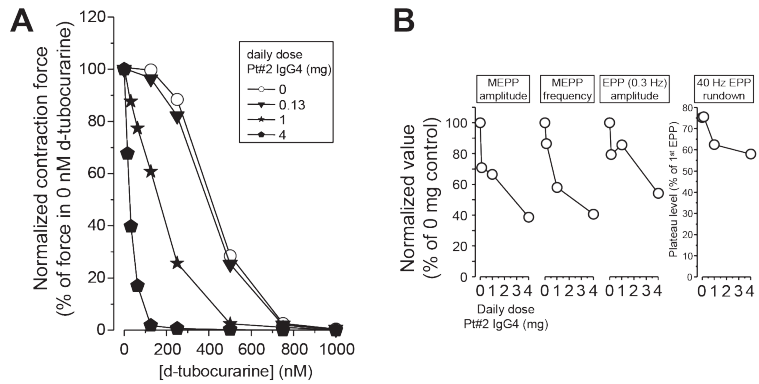

Figure S2. Dose-dependency of the (subclinical) MG induced by passive transfer of MuSK MG patient #2 IgG4. Three mice received either 0.13, 1 or 4 mg/day MuSK MG IgG4 for 38, 32 and 12 days, respectively. Only the mouse injected with 4 mg/day showed overt muscle weakness. (A) In *ex vivo* contraction measurements of their diaphragms there was a dose-dependent increase in the sensitivity of the contraction to d-tubocurarine, indicated by the leftward shift of the concentration-effect curves compared to the control curve which was composed from the pooled data of the MuSK MG IgG1-3, normal human IgG4 and PBS controls of Figure 5B. (B) Similarly, the changes of electrophysiological parameters MEPP amplitude, MEPP frequency, EPP amplitude and EPP rundown induced by the MuSK MG patient #2 IgG4 were dose-dependent.

CHAPTER 3

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

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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

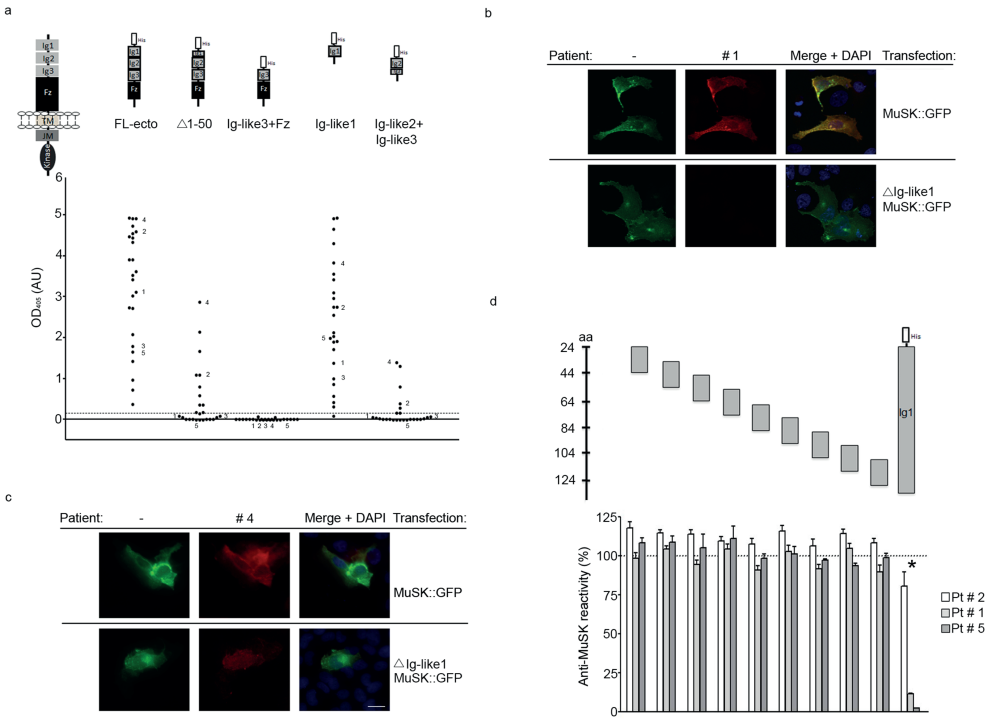

Figure 1. MuSK MG IgG4 antibodies bind predominantly to the first Ig-like domain in MuSK. (a) An ELISA shows that antibodies from all 25 patients bind to the extracellular region of MuSK. The predominant binding sites reside in the first Ig-like domain, as antibodies bind to this domain nearly as well 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

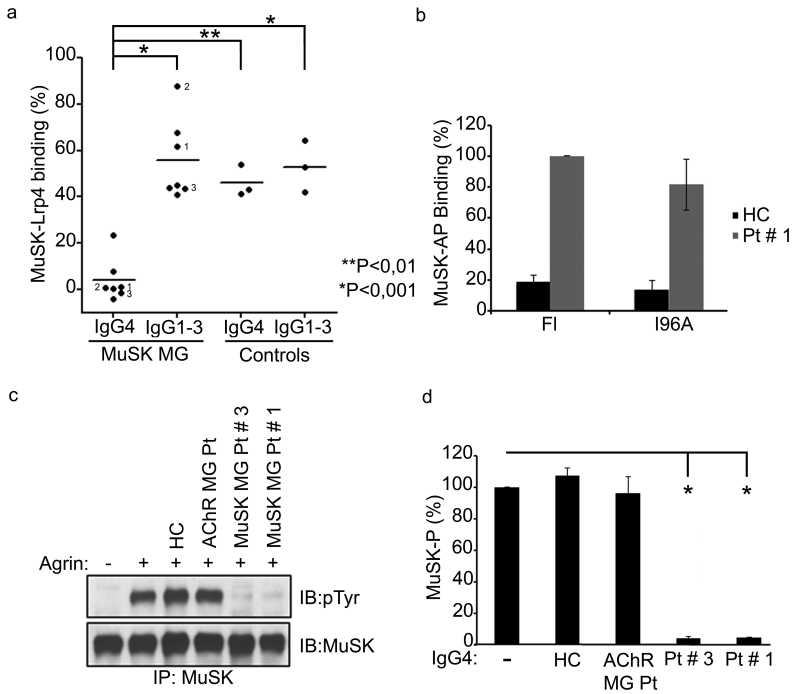

Figure 2. MuSK MG IgG4 antibodies block binding between LRP4 and MuSK and inhibit Agrin-stimulated MuSK phosphorylation in muscle. (a) IgG4 antibodies from MuSK MG patients but not IgG1-3 from the same patients significantly inhibit Agrin-dependent binding between MuSK and LRP4. IgG1-3 antibodies from 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 auto-antibodies 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.

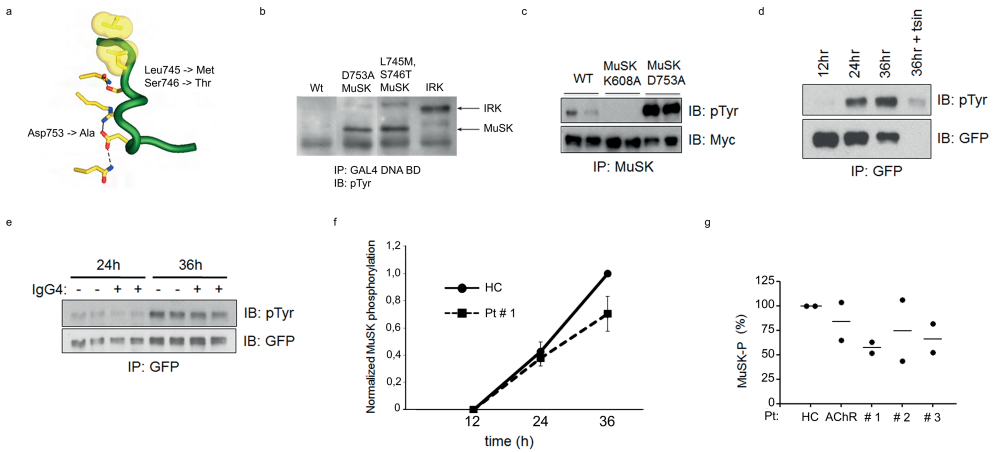

Figure 3. IgG4 antibodies from MuSK MG patients fail to reduce MuSK phosphorylation in 3T3 cells transfected with MuSK but not LRP4. (a) The diagram of the MuSK activation loop shows the substitutions in rodent MuSK that increase MuSK kinase activity. (b) MuSK tyrosine phosphorylation in yeast is enhanced 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

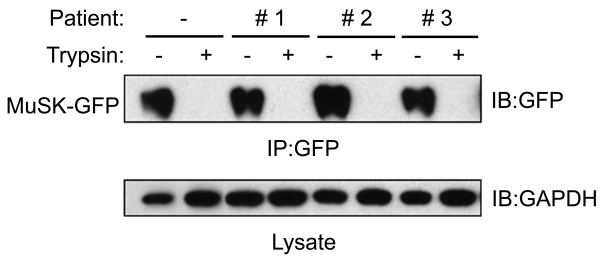

Figure 4. IgG4 antibodies from MuSK MG patients do not reduce MuSK cell surface expression. 3T3 cells, which were transiently transfected with MuSK-GFP, were treated with MuSK MG IgG4 antibodies for 24 h (Figure 3). Cells were harvested, or treated with trypsin prior to harvesting. MuSK-GFP was immunoprecipitated 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.

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SUPPLEMENTARY DATA

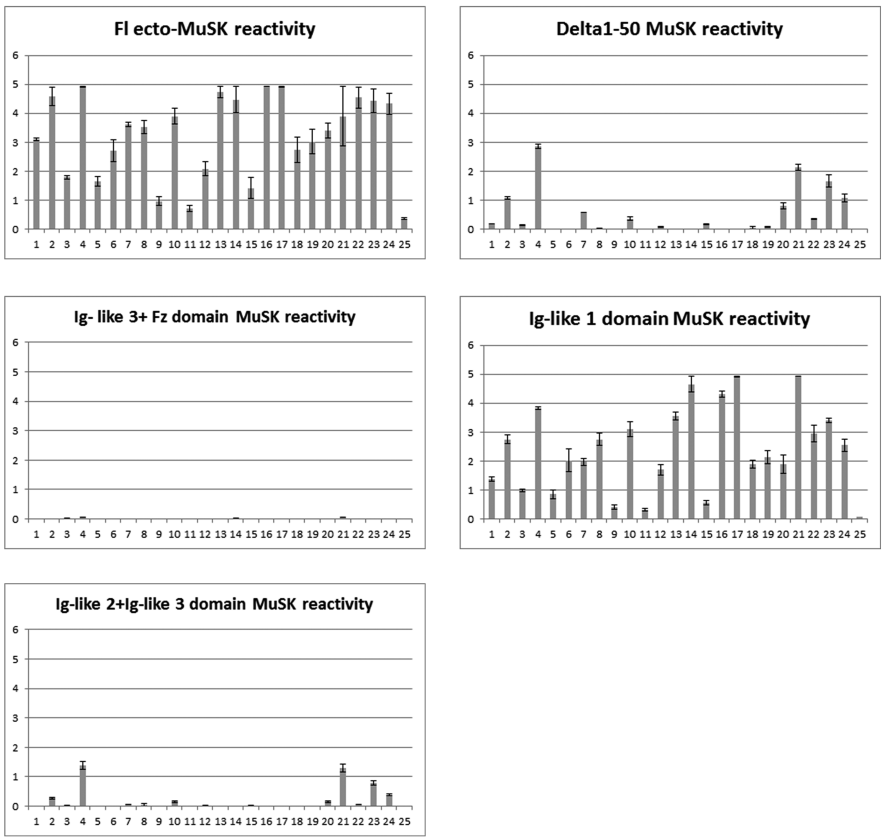

Figure S1 Overview of individual patient antibody reactivity to the full length extracellular domain or truncated versions of recombinant MuSK proteins. The graph represents average reactivity based on three independent experiments with error bars depicting the SEM values.

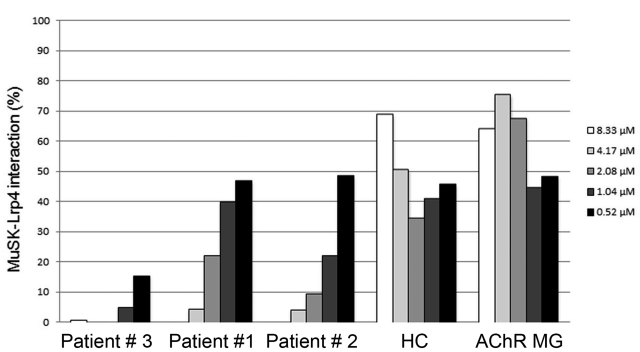

Figure S2 MuSK MG IgG4 antibodies inhibit MuSK-LRP4 interaction in a dose-dependent manner.

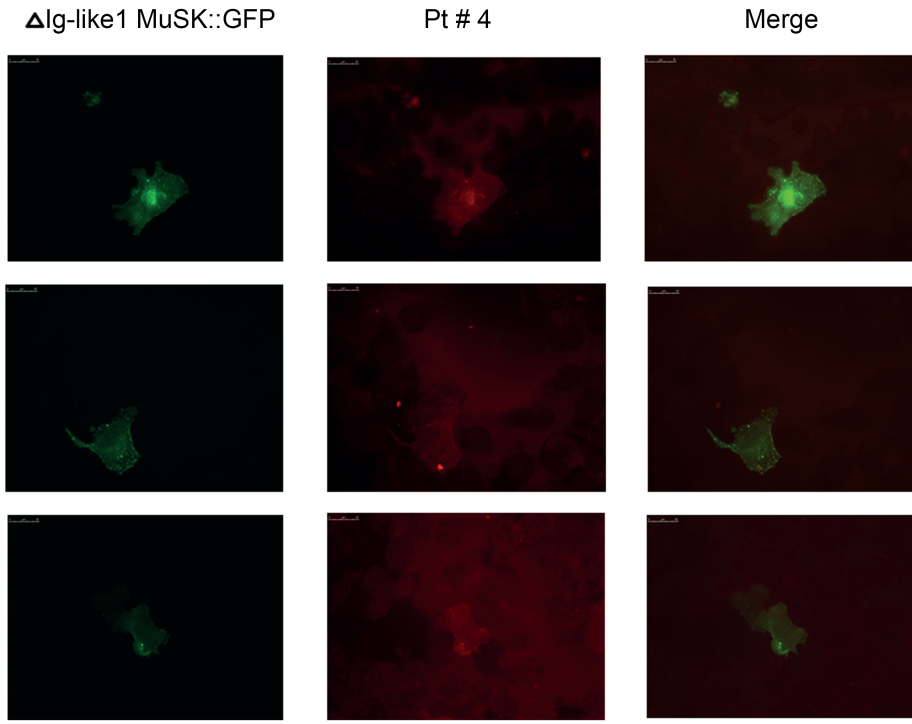

Figure S3 Examples of MuSK MG pt # 4 antibody staining of cells expressing ΔIg-like 1 MuSK::GFP.

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

CHAPTER 4

Longitudinal epitope mapping in MuSK myasthenia gravis: implications for disease severity

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Journal of Neuroimmunology 2016 Feb 15;291:82-8.

ABSTRACT

Muscle weakness in MuSK myasthenia gravis (MG) is caused predominantly by IgG4 antibodies which block MuSK signalling and destabilize neuromuscular junctions. We determined whether the binding pattern of MuSK IgG4 antibodies change throughout the disease course ("epitope spreading"), and affect disease severity or treatment responsiveness.

We mapped the MuSK epitopes of 255 longitudinal serum samples of 53 unique MuSK MG patients from three independent cohorts with ELISA.

Antibodies against the MuSK Iglike-1 domain determine disease severity. Epitope spreading outside this domain did not contribute to disease severity nor to pyridostigmine responsiveness. This provides a rationale for epitope specific treatment strategies.

INTRODUCTION

MuSK myasthenia gravis (MG) is caused by antibodies to the receptor tyrosine kinase MuSK at the neuromuscular junction (Klooster et al. 2012;McConville et al. 2004;Niks et al. 2008). Unique to the disease are the prevalent IgG4 MuSK antibodies that prevent MuSK-Lrp4 interactions in a complement-independent manner and lead to functional inhibition of the AChR clustering pathway (Huijbers et al. 2013;Koneczny et al. 2013;Mori et al. 2012). The extracellular domain of MuSK consists of three N-terminal Ig-like domains and a Frizzled-like domain (MuSK-Fz-like). Most patients carry antibodies to the Ig-like domain 1 (MuSK-Ig1), which contains residue I96 essential for MuSK-Lrp4 interaction (Zhang et al. 2011). Antibodies to MuSK-Ig1 are likely to inhibit either by physically obstructing MuSK-Lrp4 binding, or by changing the conformation of MuSK rendering it unable to interact with Lrp4 and other interacting proteins. Antibodies to the Ig-like 2 domain (MuSK-Ig2) and MuSK-Fz-like have also been described, but their role in the disease process is unclear (Huijbers et al. 2013;McConville, Farrugia, Beeson, Kishore, Metcalfe, Newsom-Davis, & Vincent 2004;Ohta et al. 2007). Moreover, intermolecular epitope spreading has been reported involving antibodies against MuSK and Lrp4, AChR or agrin (Gasperi et al. 2014;Higuchi et al. 2011;Zhang et al. 2012). Intramolecular and intermolecular epitope spreading has previously been described in bullous pemphigus where it correlated with disease severity (Di et al. 2011). Whether this is the case for MuSK MG is not known. Responsiveness to treatment with acetylcholine esterase inhibitor (AChEi) varies in MuSK MG. In AChR MG this treatment results in improvement of the symptoms by preventing breakdown of ACh. Thirty-fifty percent of MuSK MG patients treated with AChEi experience cholinergic side effects, ranging from cramps to worsening of symptoms (Evoli and Padua 2013). The AChE-ColQ complex is stabilized in the neuromuscular junction by interactions with MuSK and could be blocked by MuSK antibodies (Kawakami et al. 2012). Therefore, we hypothesized that increased AChEi sensitivity might be correlated with a specific epitope pattern of MuSK antibodies (Cartaud et al. 2004;Otsuka et al. 2015).

To investigate epitope spreading and the association with disease severity, reactivity patterns and treatment responsiveness in MuSK MG, we mapped and independently confirmed the epitopes for a large set of (longitudinal) serum samples from 53 patients.

PATIENTS AND METHODS

Patient material

Patients were retrospectively selected based on clinical weakness typical for MuSK MG and a positive MuSK RIA assay (RSR Ltd., Cardiff, UK) and the availability of longitudinal serum samples. The patients were followed at the Leiden University Medical Centre (LUMC), the University Medical Centre Groningen, the Hospital Santa Creu i Sant Pau in Barcelona or the Università Cattolica del Sacro Cuore in Rome.

The control group consisted of six healthy individuals, eight patients with Lambert-Eaton myasthenic syndrome (LEMS), and nine patients with seronegative MG. All patients and controls gave written informed consent and the study was approved by the LUMC medical ethical committee.

Severity of symptoms was evaluated retrospectively by experienced neurologists (JV, JK, EN, and II) using the disease severity score (DSS) (Niks et al. 2008). Neurologists were blinded for MuSK antibody titres and used information from patients' charts to evaluate the severity of symptoms on the date of each serum sample.

Cloning of target genes and recombinant protein purification

The coding region of nine MuSK protein fragments were amplified from full length human *MuSK* cDNA using primers containing *Nde*I and *Xho*I restriction sites (Supplementary table 1). The *MuSK* containing inserts were *Nde*I and *Xho*I digested and cloned into the pET28a vector (EMD Biosciences, Novagen Brand, Madison, WI). All vectors were sequence verified and were used to produce partially overlapping recombinant MuSK protein fragments (Supplementary table 2).
Protein production was performed as described previously (Huijbers et al. 2013).

Epitope mapping MuSK ELISA

Insoluble protein fragments were diluted in 1M urea and soluble protein fragments were diluted in PBS to a concentration of 3 µg/ml. 96-wells Maxisorp plates (Thermo Scientific, Nunc, Roskilde, Denmark) were coated with 100 µl diluted protein per well, and incubated overnight at 4 °C. After overnight incubation, the plate was processed as described previously (Huijbers et al. 2013).

Each ELISA experiment also included two negative control serum samples and one coating control per six plates to control for inter-plate and inter-experimental differences. As internal positive control, each plate contained a duplicate reactivity test for the full-length extracellular MuSK protein with a standard MuSK MG patient serum. All samples were tested in duplicate.

Statistical analysis

Each duplicate was averaged and corrected for the average PBS background signal. Each optical density value was next corrected for the internal positive control value. The 23 negative controls were used to determine the average background level. Signal detected in patients above this average background level plus three times the standard deviation were considered positive.

For statistical analysis the data was analysed using IBM SPSS statistics version 20 (SPSS Inc., Chicago, IL, U.S.A.). To assess the association between DSS and reactivity levels to MuSK-Ig1, taking into account the correlation between repeated measurements within patients, we fitted a linear mixed model with a fixed effect for the MuSK-Ig1 reactivity and random slopes and intercepts per patient. To

address whether there was additional effect of reactivity against other domains on disease severity we entered them separately into the model together with the MuSK-Ig1 variable.

RESULTS

Patient characteristics

To study epitope spreading in MuSK MG, 233 longitudinal serum samples of 20 Dutch and 11 Spanish patients were studied for their immunoreactivity against partially overlapping domains of human MuSK. Moreover, 22 samples of Italian patients were included to confirm our findings and study AChEi sensitivity in a separate cohort. Table 1 gives an overview of the demographic features of the patients included in this study. Mean age at onset was 42 years (49.2 in the Dutch population, 40.4 in the Spanish population, and 34.5 in the Italian cohort). The average age at onset in females was 8.89 years earlier compared to males although this difference was not significant (p=0.335). Average follow-up for the Dutch patients was 6.1 years (1.02 to 19.17). Mean follow up among men was 6.52 yrs (1.02-19.17) and for women 5.67 yrs (1.52-11.05) with substantial variation between patients (Table 1, Fig. 1A).

Table 1. Overview of demographic and clinical features of the patients included in this study.

	Dutch (n = 20)	**Spanish (n = 11)**	**Italian (n=22)**
Age at onset (range)	18.7 – 80.7	14 – 65	13-61
Sex F:M	10:10	8:3	16:6
Average follow-up in years (range)	5.8 (1.02-19.2)	5.07 (0-7.9)	-
Average number of included samples (range)	10.5 (3-21)	2.9 (1-4)	1
Mestinon at any point during disease	7	5	18
Azathioprine at any point during disease	14	2	12
Prednisone at any point during disease	17	8	19
Rituximab at any point during disease	0	6	2
IVIG at any point during disease	4	3	2
Plasmapheresis at any point during disease	6	0	10
Thymectomy	5	2	4
Co-morbidity	Diabetes mellitus type II: 3 Psoriasis: 1	-	Thyroiditis: 1 CIDP: 1

Epitope spreading is uncommon in MuSK myasthenia gravis

We defined epitope spreading as: 'the occurrence of reactivity to other epitopes in any of the serum samples of a patient compared to the reactivity pattern in the first available serum sample of this patient'. All Dutch and Spanish patients (n=31) showed reactivity to MuSK-Ig1 at the time of diagnosis. Sixteen patients showed additional

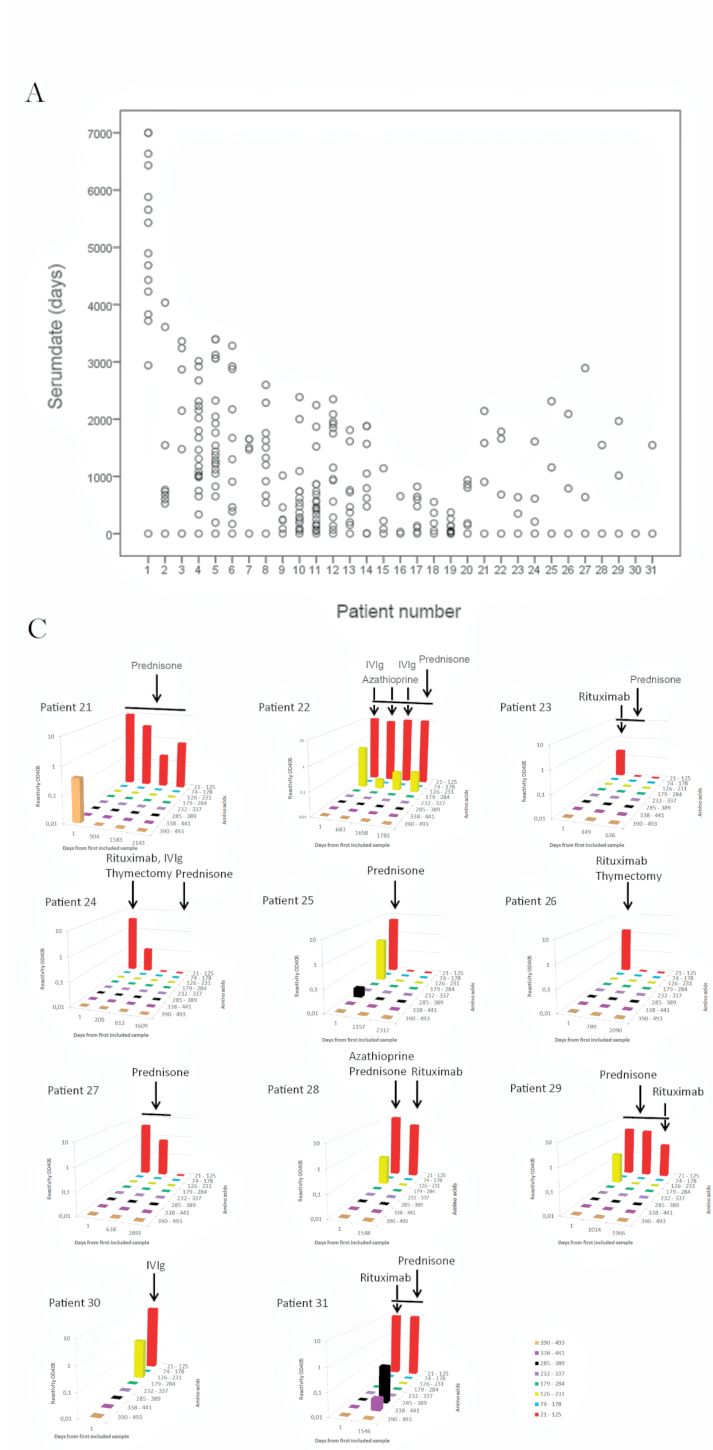
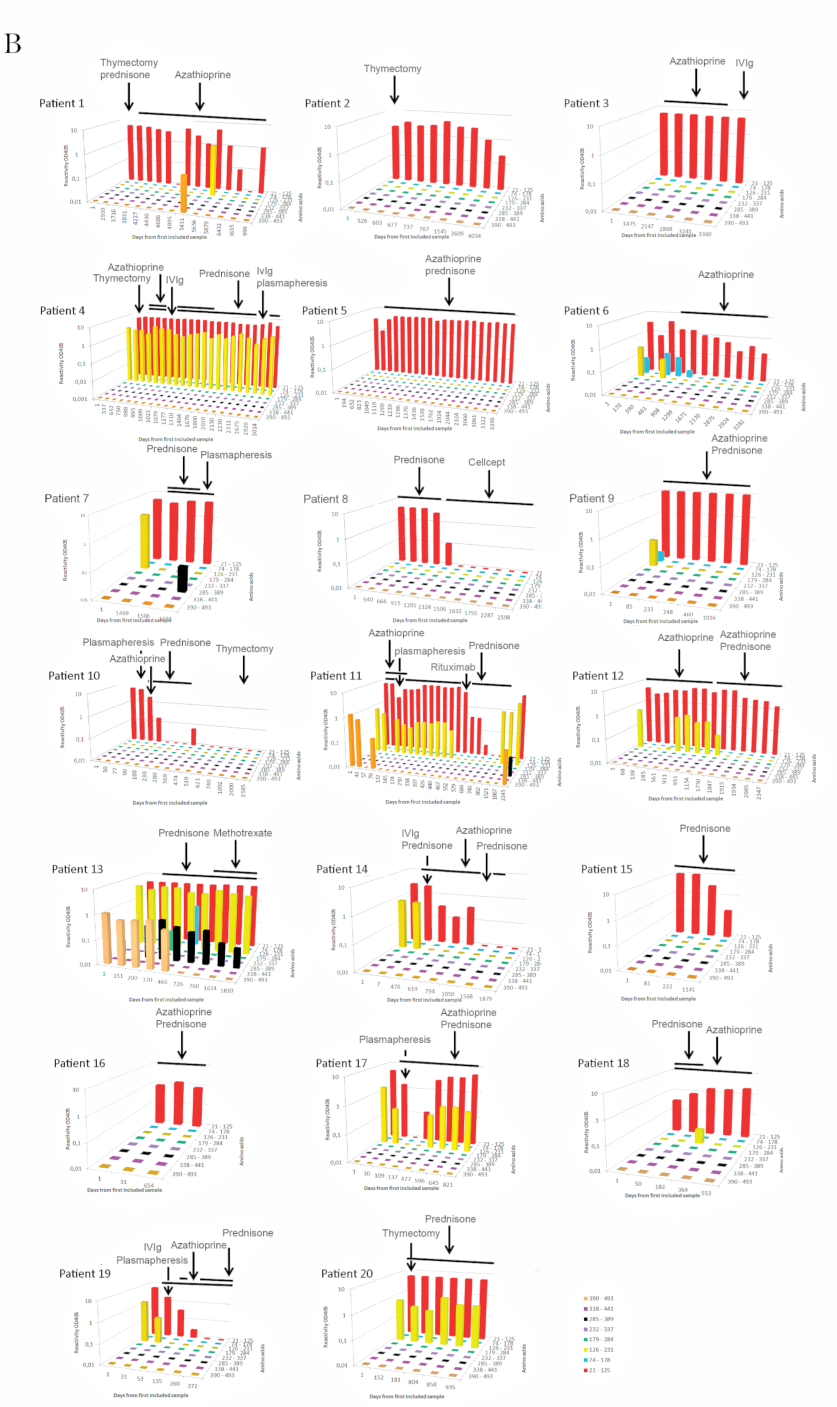


Figure 1. Overview of follow up and longitudinal epitope mapping patterns for 31 MuSK MG patients. Panel A shows the distribution of samples over the time of follow up for each patient. Reactivity patterns to the different MuSK proteins over time per patient reveal that epitope spreading is relatively uncommon (B Dutch patients, C Spanish patients). All patients show reactivity to ▸



the N-terminal MuSK protein domain. Reactivity to the MuSK-Ig2 is observed at any point during disease in 18 patients. The lines above each graph indicate the relevant treatment. Thymectomy had been performed in 6 patients previously to the discovery of MuSK antibodies in 2001.

reactivity to the MuSK-Ig2 and four patients had antibodies to the MuSK-Fz-like domain in the first available serum sample. In subsequent sera, epitope spreading was observed in 6 out of 31 patients accounting for 19% of MuSK MG patients tested (patients 1, 7, 11, 13, 18 and 31). When epitope spreading occurred, the majority of them developed reactivity against the MuSK-Fz-like domain (Fig. 1B, C). Three of these patients (7, 11, 13) already had reactivity against MuSK-Ig2 at the first time of examination, of which two (11 and 13) also had autoantibodies against the MuSK-Fz-like domain.

Of the patients who did not develop epitope spreading, 11 of 25 (44%) had only reactivity against MuSK-Ig1 (amino acids 21-125) whereas 48% also had reactivity against MuSK-Ig2 in their first available sample. Only two patients (8%) had reactivity against either the MuSK-Ig3 or the MuSK-Fz-like domain in addition to MuSK-Ig1 reactivity. None of the patients had reactivity against the intracellular domain at any point during their illness (data not shown).

Fig. 1 also illustrates the timing of the various treatments in the individual patients. As the treatment paradigms differed strongly between the patients it was not possible to statistically assess the effect of the treatments on reactivity against the different domains of MuSK. However, on the individual level the effects of treatment on antibody titres can be observed. Moreover, in five Italian patients, who went into remission, no reactivity against the MuSK-Ig1 domain could be detected, suggesting that these titres reflect their clinical status.

MuSK MG disease severity correlates with immunoreactivity against MuSK-Ig1 longitudinally

Since epitopes have been considered crucial determinants of the effectiveness and pathogenicity of an auto-immune response, we assessed whether reactivity against any domain of MuSK corresponded with the course of the disease and severity of the symptoms. A linear mixed effect model confirmed that reactivity against the N-terminal part of MuSK significantly correlates with DSS (combined cohorts: mean β-coefficient 0.159, $p<0.000002$, Dutch cohort: β-coefficient 0.175, $p<0.0001$, Spanish cohort: β-coefficient 0.107, $p<0.036$). This analysis took into account the individual correlation of each patient. This observation was subsequently confirmed in a third cohort of Italian patients (β-coefficient 0.167, $p< 0.026$). The average correlation between DSS and reactivity against MuSK-Ig1 for all patients is shown in Fig. 2A and for the individual cohorts in supplementary Fig.1. When including gender, age or treatment regimen in the mixed effect model this did not affect the association. Reactivity to other domains of MuSK did not contribute to disease severity, after correcting for the level of MuSK-Ig1 reactivity. Table 2 gives an overview of the significance of the correlation between the DSS and additional reactivity against the different domains of MuSK. In conclusion, antibodies against epitopes outside the N-terminal Ig-like 1 domain, and thus epitope spreading, do not seem to contribute to disease severity in our cohorts.

MuSK-Ig1 reactivity positively predicts disease severity between patients

Next, we investigated whether titres measured by our MuSK-Ig1 ELISA correlated more strongly to DSS than the values obtained by using the standard diagnostic RIA assay for MuSK MG, which is based on reactivity to the complete extracellular domain of MuSK. To address this we took the first samples of all our MuSK MG patients (Dutch and Spanish cohort) and established their DSS and their ELISA reactivity against MuSK-Ig1. When using a linear regression analysis the β-coefficient was 0.2882 (p=0.0013) (Fig.2B). When performing this test for the first available RIA value and corresponding DSS score for each patient (Fig.2C), the MuSK RIA values did not correlate with disease severity between patients (slope=0.322, p=0.083).

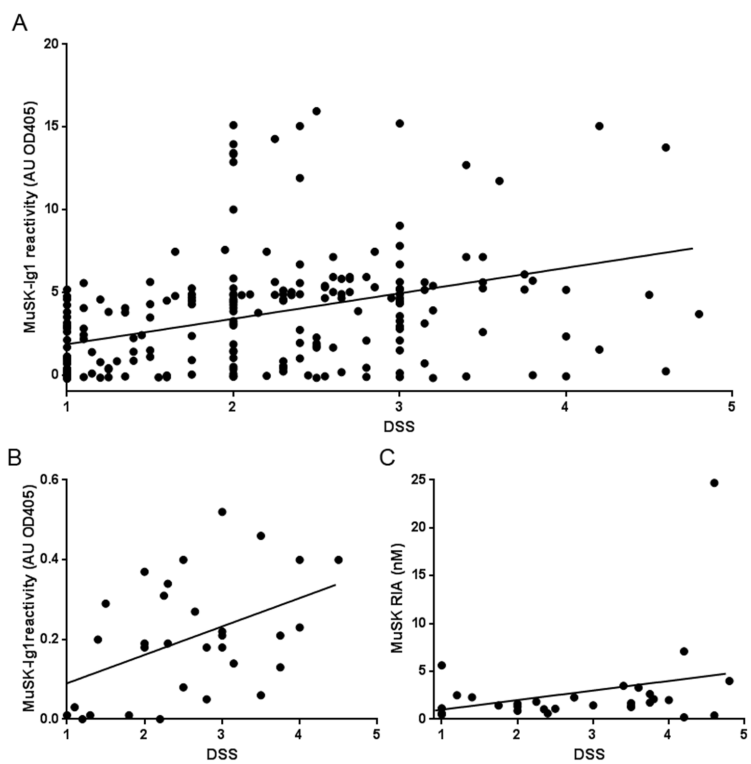


Figure 2. Overview of the average correlation between DSS and reactivity against the N-terminal domain for 53 MuSK MG patients (A). The multiple measures of each patient are represented by the dots. The correlation between the DSS and MuSK-Ig1 reactivity (B) or MuSK RIA results (C) from the first time point of each individual patient.

Reactivity patterns to MuSK differ between male and female patients

The data also allowed for the comparison of reactivity patterns with other demographic features of the three cohorts. Patients were stratified based on the maximum reactivity pattern. Thus when a patient at any point had reactivity against all MuSK Ig-like

Table 2. Overview of the significance level of each of the analysed MuSK proteins correlating to disease severity when reactivity to the MuSK-Ig1 is included as a covariate.

Protein fragment	p-value	β-coefficient	95% Confidence Interval
N-terminal Ig-like 1 MuSK 21-125	0.000002	0.157	0.108-0.204
MuSK 74-178	0.258	-0.77	-2.10-0.57
MuSK 126-231	0.065	0.40	-0.31-0.82
MuSK 179-284	0.326	-1.37	-4.27-1.54
MuSK 232-337	0.110	-2.00	-4.45-0.45
MuSK 285-389	0.848	-0.13	-1.51-1.24
MuSK 338-441	0.061	-2.51	-5.13-0.119
MuSK 390-493	0.278	0.53	-2.29-3.31

domains and the MuSK-Fz-like domain, even if this was only detected in a single sample, the patient was categorised in the Ig1+Ig2+Ig3/Fz group. Surprisingly, this distribution was significantly different between males and females (Fig. 3). Females more often had a restricted immune response against MuSK-Ig1 only, whereas all, but three, males had a broader immune response with at least antibodies to one additional protein fragment (Pearson Chi-Square p=0.039). This difference was not caused by variation in age at onset or duration of follow up.

Epitope patterns do not predict AChEi responsiveness

To investigate whether the presence of antibodies against other domains of MuSK correlate with treatment effects of AChEi we studied 14 Dutch and 18 Italian patients.

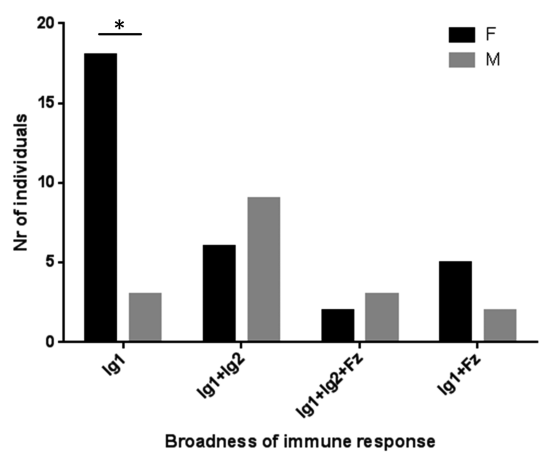


Figure 3. Reactivity patterns differ between male and females. Female MuSK MG patients have significantly more reactivity restricted to the Ig-like domain 1 compared to men.

When judging the antibody response to the maximal number of protein fragments, epitope patterns did not correlate with treatment responsiveness (Pearson Chi-square p=0.232). Also when patients were separated in two groups having either only MuSK-Ig1 domain antibodies or having a broader response to MuSK, this immune response did not correlate with a beneficial effect of AChEi (p=0.06). The well-known side effects of AChEi also did not correlate with the broadness of the anti-MuSK response (Pearson Chi-square test: p= 0.580). Moreover, as epitope patterns differ between males and females we investigated whether sex correlates with treatment responsiveness. Both sexes were distributed equally among the groups of responders and patients with side effects (Pearson Chi-square test: benefit p=0.948, side effects p=0.283). This data suggests that AChEi effectiveness and hypersensitivity are not predicted by sex nor the epitope specificity of the immune response against MuSK.

DISCUSSION

We here show that disease severity positively correlates with immunoreactivity against the N-terminal Ig-like domain of MuSK. This domain is crucial for interaction with Lrp4, in mediating the dimerization of the Agrin/Lrp4/MuSK heterotrimer complexes, MuSK activation/phosphorylation, and ultimately for AChR clustering and NMJ maintenance. This supports the observation that the main mode of action of MuSK IgG4 antibodies is interference with Lrp4 MuSK signalling (Huijbers et al. 2013, Otsuka et al. 2015). The importance of MuSK-Ig1 as the MIR of MuSK is supported by the observation that epitope spreading is uncommon and reactivity to other domains does not seem to contribute to a more severe disease outcome. This is different from other studies suggesting that epitope spreading is a beneficial process distracting the immune response away from the pathogenic epitope (Vincent et al. 1998)Others suggest that epitope spreading occurs early in the disease and significantly worsens the clinical outcome (Di et al 2011). We cannot exclude that the epitope spreading occured at an earlier or later disease stage. However, the epitope specificity appeared rather confined and stable during the disease course in the majority of MuSK MG patients over a period of minimally 5 years. Taken together these observations provide a rationale for using MIR (i.e. MuSK-Ig1) specific interventions for the treatment of MuSK MG.

In MuSK MG there appears to be a limited role for IgG1/IgG3 mediated structural damage of the neuromuscular synapse as seen in AChR antibody mediated MG (Engel and Arahata 1987). In AChR MG IgG1 or IgG3 mediated damage to the synapse is thought to expose the complete AChR leading to the generation of secondary reactivity against intracellular epitopes (Di et al. 2010;Di et al. 2011). In our cohorts of MuSK MG patients we found no reactivity against intracellular MuSK domains (data not shown). This corroborates on the observation that MuSK antibodies do not cause extensive physical breakdown of the NMJ or local inflammatory response, but rather disturb AChR clustering by preventing the AChR clustering signalling cascade (Ghazanfari et al. 2014;Klooster and Plomp et al. 2012;Mori et al.2012). It might also

suggest that the autoimmune response in MuSK is not the causative antigen that is presented in total to elicit the initial immune response, but that another antigen primes the immune system and induces crossreactive antibodies. If antigenic mimicry has a role in the initiation of MuSK MG, the MuSK-Ig1domain is the obvious candidate to search for crossreactive epitopes.

In autoimmune disease absolute antibody titres often do not predict disease severity, while within serum samples from a single MuSK MG patient a correlation between the antibody titre and disease severity can be observed (Niks et al. 2008). Indeed, within patients immunoreactivity against the MuSK extracellular domain often corresponds with clinical status (Bartoccioni et al. 2006). A decrease in antibody titre coincided with remission of the clinical features. In our cohort disease severity correlated well with the ELISA testing for MuSK-Ig1 reactivity both within and among patients. This suggests that the titre of antibodies against MuSK-Ig1 is a good predictor of disease severity.

The functional effects of ColQ and biglycan binding to MuSK are unknown (Amenta et al. 2012;Cartaud, Strochlic, Guerra, Blanchard, Lambergeon, Krejci, Cartaud, & Legay 2004). Both biglycan and ColQ interact with the MuSK-Ig1and the MuSK-Fz-like domain (Amenta et al. 2012, Otsuka et al. 2015). It would be interesting to explore whether the loss of these protein interactions by MuSK patient antibodies is relevant to the disease. One study has shown a dose-dependent loss of MuSK-ColQ interaction when exposed to MuSK antibodies derived from an active immunization model in rabbits (Kawakami et al. 2011). In line with this some have hypothesized that the AChEi hypersensitivity observed in many MuSK MG patients might be the result of loss of this interaction. In our study we could not confirm the occurrence of epitope dependent AChEi effectivity or hypersensitivity.

The MuSK-Fz-like domain functions as a Wnt receptor (Wu et al. 2010). Antibodies against this domain are seen in 22.6% of our MuSK MG patients. This is in concordance with a Japanese cohort were 30% of patients were shown to have antibodies against the MuSK-Fz-like domain (Takamori et al. 2013). Our study did not find a positive correlation between MuSK-Fz-like domain reactivity and disease severity. Perhaps the levels of MuSK-Fz-like domain antibodies were too low, or do not interfere with Wnt signalling. Although geographic effects in MuSK autoimmunity and higher involvement in Asians (Suzuki et al. 2011) suggest a genetic and/or environmental contribution to the development of MuSK autoimmunity, the epitope data available from three different European cohorts and a Japanese cohort does not support population differences in the immunoreactivity pattern.

One of the more striking observations in our study is the difference between men and women in their immune reactivity against MuSK. An antibody response restricted to the MuSK-Ig1 domain was almost exclusively found in women. Sera from men recognize a larger number of MuSK epitopes than sera from female MG patients. Although we do not have an explanation for this finding, it seems robust, as it was present in all three cohorts of patients that were studied. The three male patients,

with a restricted MuSK antibody profile, all had a relatively low titer. Also, epitope spreading in these patients might have been missed due to the lack of follow-up sera in these patients. Differences in age at onset or follow up time did not explain the differences in the reactivity pattern.

In conclusion, epitope spreading occurs in a minority of the MuSK MG patients. The correlation between MuSK Ig1-like domain reactivity with disease severity indicates that blocking of the Lrp4 MuSK interaction is a key factor in developing myasthenic weakness in MuSK MG.

ACKNOWLEDGEMENTS

We would like to thanks Ingrid Hegeman for providing us with excellent support in serum maintenance. This study was supported by funding of the Prinses Beatrix Spierfonds and the Association Française contre les Myopathies.

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SUPPLEMENTARY DATA

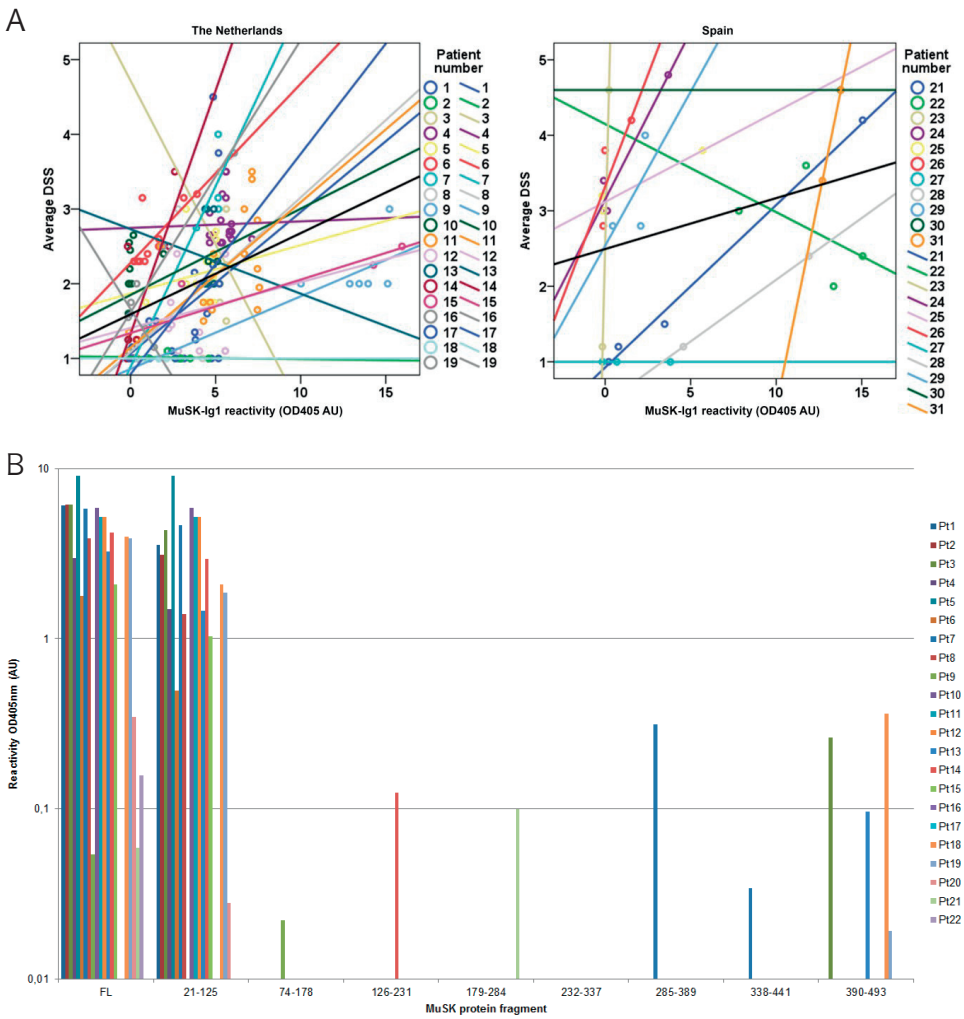

Figure S1. Overview of the association between DSS and MuSK-Ig1 reactivity for each patient and separated on origin (A) and of the epitope patterns of 22 Italian MuSK MG patients (B).

Table S1. Primer pairs for amplifying the different MuSK proteins DNA fragments.

Primer name	Sequence	
MuSK 21-125	Fw	5′ –GCA CAT ATG ACT GAG AAA CTT CCA AAA GCTC– 3′
	Rev	5′ –AG ATG AAA CCT AAA ATA ACT CGC TAG CTC GAG GC– 3′
MuSK 74-178	Fw	5′ –GCA CAT ATG CGG TAC AGC ATC CGG GAG A– 3′
	Rev	5′ –TCT GGG CGA TTG AGG ATT CAT TAG CTC GAG GCA– 3′
MuSK 126-231	Fw	5′ –GCA CAT ATG CCT CCC ATA AAT GTG AAA ATA ATA– 3′
	Rev	5′ –TT GGC TTT GTG ACC CTG TAG CTC GAG GCA– 3′
MuSK 179-284	Fw	5′ –GCA CAT ATG AAC GTA CAA AAG GAA GAT GCA G– 3′
	Rev	5′ –GGA CTC TAC ACA TGC GCG GCT TAG CTC GAG GCA– 3′
MuSK 232-337	Fw	5′ –GCA CAT ATG CAC TGT ACA GCA ACA GGC ATT– 3′
	Rev	5′ –CA AAA GAT GCT CTT GTT TTT CGC TAG CTC GAG GCA– 3′
MuSK 285-389	Fw	5′ –GCA CAT ATG ACC AAT AAG CAT GGG GAG AAG– 3′
	Rev	5′ –GT CCT GGA GTA GTG CCT ACT TAG CTC GAG GCA– 3′
MuSK 338-441	Fw	5′ –GCA CAT ATG AAC ACC TCC TAT GCG GAC C– 3′
	Rev	5′ –GC AAG CTT CCC AGC ATG CAT TAG CTC GAG GCA– 3′
MuSK 390-493	Fw	5′ –GCA CAT ATG CCT ATT CCC ATT TGC AGA GAG– 3′
	Rev	5′ –TCT GTC TCA CCT ACA TAC TCC TAG CTC GAG GCA– 3′
MuSK 441-773	Fw	5′ –GCA CAT ATG TCA GCA GCA GTA ACC CTC A – 3′
	Rev	5′ –CGC ATG TGT GAG AGG GCA CTC GAG GCA– 3′

Table S2. Overview of amino acid sequences of the recombinant MuSK proteins used in the ELISA assays

Recombinant protein	Amino acid sequence
MuSK 21-125	TEKLPKAPVITTPLETVDALVEEVATFMCAVESYPQPEISWTRNKILIKLFDTRYSIRENGQ LLTILSVEDSDDGIYCCTANNGVGGAVESCGALQVKMKPKITR
MuSK 74-178	RYSIRENGQLLTILSVEDSDDGIYCCTANNGVGGAVESCGALQVKMKPKITRPPINVKIIE GLKAVLPCTTMGNPKPSVSWIKGDSPLRENSRIAVLESGSLRIH
MuSK 126-231	PPINVKIIEGLKAVLPCTTMGNPKPSVSWIKGDSPLRENSRIAVLESGSLRIHNVQKEDAG QYRCVAKNSLGTAYSKVVKLEVEVFARILRAPESHNVTFGSFVTL
MuSK 179-284	NVQKEDAGQYRCVAKNSLGTAYSKVVKLEVEVFARILRAPESHNVTFGSFVTLHCTATGI PVPTITWIENGNAVSSGSIQESVKDRVIDSRLQLFITKPGLYTCIA
MuSK 232-337	HCTATGIPVPTITWIENGNAVSSGSIQESVKDRVIDSRLQLFITKPGLYTCIATNKHGEKFS TAKAAATISIAEWSKPQKDNKGYCAQYRGEVCNAVLAKDALVFL
MuSK 285-389	TNKHGEKFSTAKAAATISIAEWSKPQKDNKGYCAQYRGEVCNAVLAKDALVFLNTSYA DPEEAQELLVHTAWNELKVVSPVCRPAAEALLCNHIFQECSPGVVPT
MuSK 338-441	NTSYADPEEAQELLVHTAWNELKVVSPVCRPAAEALLCNHIFQECSPGVVPTPIPICREY CLAVKELFCAKEWLVMEEKTHRGLYRSEMHLLSVPECSKLPSMH
MuSK 390-493	PIPICREYCLAVKELFCAKEWLVMEEKTHRGLYRSEMHLLSVPECSKLPSMHWDPTACA RLPHLDYNKENLKTFPPMTSSKPSVDIPNLPSSSSSSFSVSPTYS
MuSK 441-773	SAAVTLTTLPSELLLDRLHPNPMYQRMPLLLNPKLLSLEYPRNNIEYVRDIGEGAFGRVF QARAPGLLPYEPFTMVAVKMLKEEASADMQADFQREAALMAEFDNPNIVKLLGVCAV GKPMCLLFEYMAYGDLNEFLRSMSPHTVCSLSHSDLSMRAQVSSPGPPPLSCAEQLCI ARQVAAGMAYLSERKFVHRDLATRNCLVGENMVVKIADFGLSRNIYSADYYKANENDA IPIRWMPPESIFL

CHAPTER 5

Myasthenia gravis with muscle specific kinase antibodies mimicking amyotrophic lateral sclerosis.

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Neuromuscular Disorders, 2016 June; 26(6):350-3.

ABSTRACT

Muscle-specific kinase (MuSK) myasthenia gravis (MG) is hallmarked by the predominant involvement of bulbar muscles and muscle atrophy. This might mimic amyotrophic lateral sclerosis (ALS) presenting with bulbar weakness. We encountered four cases of MuSK MG patients with an initial misdiagnosis of ALS.

We analysed the clinical data of the four misdiagnosed MuSK MG patients, and investigated the presence of MuSK autoantibodies in a group of 256 Dutch bulbar-onset ALS patients using a recombinant MuSK ELISA and a standard MuSK radio immunoprecipitation assay.

Clues for changing the diagnosis were slow progression, clinical improvement, development of diplopia and absence of signs of upper motor neuron involvement. No cases of MuSK MG were identified among a group of 256 bulbar ALS patients diagnosed according to the revised El Escorial criteria.

A misdiagnosis of ALS in patients with MuSK MG is rare. We recommend to carefully consider the diagnosis of MuSK MG in patients presenting with bulbar weakness without clear signs of upper motor dysfunction.

INTRODUCTION

In myasthenia gravis (MG) with muscle-specific kinase (MuSK) antibodies, bulbar muscles are severely affected throughout the course of the disease (1). Exacerbations often consist of worsening of bulbar symptoms, while ocular muscles are generally spared (2). In addition, MuSK MG patients may have manifestations of muscle fiber hyperexcitability, including fasciculations (3). MuSK MG could be confused with amyotrophic lateral sclerosis (ALS) with bulbar-onset, as previously reported in patients presenting with progressive dyarthria, dropped head or dysphagia (4;5). Obviously, confusing MuSK MG, a treatable disorder, with a fatal disease like bulbar onset ALS, should be avoided. The authors recommended testing for MuSK antibodies as part of the diagnostic work-up of patients with predominant bulbar ALS (3).

Four MuSK MG patients that were referred to one of our neuromuscular centres were initially diagnosed with ALS. This prompted us to investigate the occurrence of MuSK antibodies in a large cohort of ALS patients.

CASE REPORTS

A summary of the four cases is given in table 1.

Case 1

A 69 year old man had problems with swallowing since one year. He lost weight from 75 to 69 kg and developed a dysarthria. Complaints tended to get worse during the day, but did not fluctuate, indicating slow progression of symptoms over time. After walking for half an hour he was unable to hold his head upright. He was known with alternating strabismus since childhood and denied double vision. On physical examination he had severe nasal dysarthria, and could not lift his soft palate. He could not put his tongue in the cheeks nor perform fast alternating movements with his tongue. The left eye was in abducted position, but he did not complain about diplopia. Flexion of the neck was weak, while the limb muscles were strong. Tendon reflexes were brisk, with flexor plantar responses.

Electromyogram showed spontaneous muscle fiber activity in the trapezius and gastrocnemius muscles, and fasciculations in trapezius and deltoid muscles. In several muscles, signs of reinnervation were found. Small polyphasic motor unit action potentials were present in the paraspinal muscles at C7 level.

A diagnosis of progressive bulbar spinal atrophy was made in the referring hospital, based on a pure motor syndrome without fluctuations or diplopia or ptosis. Patient did not fulfill the revised El Escorial criteria, the dysarthria had the aspect of nasal speech, and upper motor neuron signs were lacking. Subsequent doubt, because there was no further progression, led to testing for MuSK antibodies and a muscle biopsy, showing slight variation in fibres size, that were classified as non-specific. Serum MuSK antibodies were found one and a half year after onset of the dysphagia. Treatment with cholinesterase inhibitors worsened his symptoms.

Table 1. Overview of MuSK MG patients with a previous diagnosis of ALS

Patient	Sex	Age at onset	Presenting symptom	Diagnostic delay (months)
1	Male	69	Bulbar weakness	18
2	Female	61	Extremities and bulbar weakness	22
3	Male	56	Bulbar weakness	168
4	Female	64	Dyspnea	4

Immunosuppressive treatment with plasmapheresis, prednisone and azathioprine led to a complete remission.

Case 2

A previously healthy 61 year-old woman presented with progressive weakness in arms and legs since two years. The complaints had started in the right arm, with an inability to raise her arm. In the following two years she developed a comparable weakness in the left arm. She also complained of weakness in both legs with difficulties walking stairs, dysarthria, dysphagia, and difficulty keeping her head erect. There were no fluctuations during the day and no diplopia.

Physical examination showed dysarthria with a nasal speech. Neck extensors, deltoid, triceps, and biceps were weak with wasting of the deltoid muscle bilaterally. She had hip flexor weakness and could not rise from a chair without using the arms. No fasciculations were noted. Knee tendon jerks were brisk bilaterally, with bilateral ankle clonus. Plantar responses were flexor. Laboratory investigations, including CK, thyroid, MRI of head and neck were normal.

Electromyogram showed spontaneous muscle fiber activity and polyphasic potentials in the right flexor carpi radialis muscle, the right first dorsal interosseus muscle and left biceps muscle. In the legs there were signs of denervation and re-innervation bilaterally in gastrocnemius muscle. In the quadriceps and anterior tibial muscle there were only signs of re-innervation. In the paravertebral muscles there were signs of denervation at all investigated levels. Fasciculations were seen in almost all investigated muscles. The patient refused examination of the bulbar muscles.

Bulbar ALS was considered because of weakness and atrophy in the brainstem, cervical and lumbosacral region with denervation in muscles from 3 regions. Riluzole was started. However, the patient did not fulfill the revised El Escorial criteria as signs of upper motor neuron dysfunction were lacking She needed a speech enhancer,

Diagnostic clue	Therapy	Post-intervention status
No progression after initial severe symptoms	Prednisolone, azathioprine, nocturnal ventilator support	Pharmacologic remission
Spontaneous improvement after 12 months	Prednisolone, azathioprine, mycophenylate mofetil, rituximab	Pharmacologic remission
Slow progression and diplopia 4 years after onset of nasal speech and swallowing difficulties	Plasmapheresis, pyridosti gmine, azathioprine	Improved. Stable, moderate bulbar weakness with severe atrophy
Asymmetric ptosis	Plasmapheresis, prednisolone, mycophenolate mofetil, nocturnal ventilator support	Pharmacologic remission

there were increasing swallowing difficulties and progressive axial weakness. Twenty-two months after the initial presentation however, speech and strength spontaneously improved. She was able to speak without mechanical assistance and could lift her arms above her head for the first time in 3 years. ALS was considered unlikely, and the patient was reanalyzed. At that time EMG showed a decrement of 23% on repetitive stimulation in the left m. nasalis, 23% in the right m. trapezius, but no decrement in the right m abductor digiti minimi. Single fiber EMG of the left m. frontalis was normal, with only 2 out of 20 fibers with a jitter above the normal range and no blocking. Serum MuSK antibodies were present. She was treated with prednisolone, azathioprine, mycophenolate mofetil, and rituximab and made a clinical complete remission without any treatment for the last 14 months.

Case 3

A 56-year-old man complained about difficulties moving his lips or tongue, nasal speech and regular choking. He had had a few moments of diplopia, which quickly resolved. In addition to the clinical symptoms above, physical examination showed an increased masseter, nasopalpebral, knee jerk and Achilles tendon reflexes, and absent abdominal skin reflexes. Both plantar reflexes were reported as equivocal extensor. The palmomental and snout reflexes were negative. No fasciculations were seen. Extensive additional tests were normal and included blood, urine, CSF, X-thorax, ECG, and EEG. EMG of the mm. orbicularis oculi and orbicularis oris dexter showed signs of spontaneous muscle fiber activity, while the trapezius, sternocleidomastoid, deltoid, and pectoralis major muscles were normal. A muscle biopsy of the sternocleidomastoid muscle was reported normal. A neostigmine test was negative, but fasciculations were reported to be present in both the right and left mm. pectoralis major and serratus anterior. Bulbar ALS was diagnosed based on weakness, atrophy and spontaneous muscle fiber activity on EMG in the brainstem region, in spite of the fact that signs of upper motor neuron involvement were lacking.

Thus, the patient did not fulfil the revised El Escorial criteria. In the following years the disease stabilized. Myasthenia gravis was considered, but rejected because of the atrophy. Several years later he was seen again and complained about a "thick" tongue, nasal speech, and regular choking, He needed to drink to be able to swallow the food more easily. He lost weight from 67 to 62,5 kg. He experienced difficulties in moving the tongue and complained of weak lip closure. The symptoms showed a slow progression. When asked, he admitted that there might be some minor fluctuations. He had no complaints about weakness of arm or leg muscles.

Physical examination showed pronounced atrophy of the facial musculature and tongue. Frowning, whistling or smiling was not possible. There was severe dysarthria with a nasal voice. The pharynx did not move, also not after sensory stimulation. Eye movements were completely normal. There were no pseudobulbar or increased reflexes, plantar reflexes were normal. Serum MuSK antibodies were positive. He was treated with plasmapheresis without a clear response. Immunosuppressive treatment with prednisone and azathioprine for several months induced a mild speech improvement, but no major change was seen, most likely due to the severe atrophy.

Case 4

A 64 year old female, without relevant medical history, suffered from unexplained dyspnea by the end of 2002. After a couple of weeks, she was referred to a cardiologist, who found no abnormalities. In the next four weeks, some nasal dysarthria and hoarseness developed with a diurnal variation. Furthermore slight swallowing problems were reported. There were no abnormalities at clinical examination, but a progressive pseudobulbar palsy was surmised. Electromyography showed no abnormalities, for reasons of completeness 3 Hz repetitive nerve stimulation was performed in the upper extremities without finding any decrement. Clinical symptoms worsened, especially her dyspnea, and the diagnosis of ALS was made, although there were no signs of upper motor neuron involvement, and the patient did not fulfil the revised El Escorial criteria. Another EMG-investigation again showed no neurogenic abnormalities. Eventually, she was referred to another center for chronic ventilary support and she ended up in the respiratory care unit with severe dyspnea and moderate bulbar symptoms as described. There the diagnosis myasthenia was suggested by an asymmetric ptosis. No acetylcholine receptor antibodies were found, EMG with repetitive nerve stimulation at the ICU was normal again. She was treated with plasma-exchange and high-dose steroids and recovered completely within 2 weeks. Thereafter the clinical course was fluctuating with tapering and increasing of the medication. She remained on nocturnal ventilator support for four years. Anti-MuSK-antibodies were found in 2005. She is now free of symptoms on a decreasing dose of mycophenolate mofetil since 2008.

METHODS

Sera of 256 patients diagnosed with ALS, and fulfilling the revised El Escorial Criteria for possible, probable laboratory-supported, probable or definite ALS were included. Patients were identified from 1 January 2006 to 1 September 2011 in a large, prospective population based case control study on ALS in the Netherlands (6). All ALS patients had a bulbar onset. Twenty patients with MuSK MG and oculobulbar weakness were used as positive controls. Twelve healthy controls or patients with other autoimmune diseases were included as controls. Information on the previous diagnosis in 18 other Dutch MuSK MG patients was collected from the patient files. The studies were approved by the Medical Research Ethics Committee of the University Medical Centers of Utrecht and Leiden.

Sera were tested twice for MuSK antibodies using an ELISA based on recombinant MuSK (7). These experiments were blinded and performed in duplicate. A sample was considered positive when it had reactivity to MuSK above average background reactivity plus 3 times standard deviation. Samples that fulfilled these requirements were tested in duplicate in a standard diagnostic radio-immunoprecipitation assay (RIA) for MuSK reactivity (RSR, Cardiff, UK).

RESULTS

Average MuSK reactivity in ELISA is presented in Figure 1a. Twenty-four (9%) ALS sera gave signals just above background at least once. All these samples were measured a third time and 9 tested above background at least twice in three experiments. In a MuSK RIA 24 double positive samples were negative (Fig. 1b). In conclusion, none of the 256 tested ALS sera contained MuSK antibodies. In 18 confirmed MuSK MG patients an earlier diagnosis of seronegative MG (12x), Lambert-Eaton myasthenic syndrome (1x), chronic progressive external opthalmoplegia (1x), facioscapulohumeral muscular dystrophy (1x), or acquired inflammatory myopathy (1x) was made. However, motor neuron disease was not considered in any of these patients before the diagnosis of MuSK MG.

DISCUSSION

The clinical presentation of MuSK MG can be confused with that of bulbar ALS, or progressive spinal muscular atrophy, causing a diagnostic delay or inappropriately withholding an effective treatment. The diagnostic delay in our four patients was between one and 3 years, while an earlier publication described one patient that was diagnosed four years after onset of symptoms (3). All patients presented with prominent bulbar weakness without diplopia. One patient experienced temporary diplopia at the onset of the disease. In MuSK MG, 5% of patients never have ocular muscle involvement. This is in contrast to non–MuSK MG, where ocular muscles are almost always involved at some point (2). Two patients admitted to have fluctuations at the onset of symptoms, but around the time of diagnosis, weakness appeared

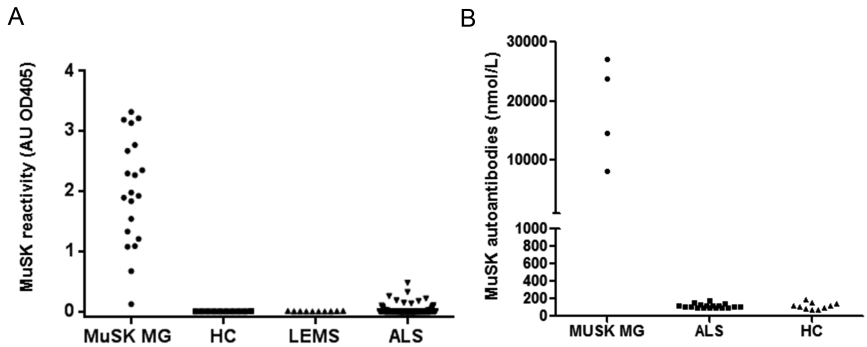

Figure 1: MuSK reactivity levels in sera from ALS patients, MuSK MG patients and controls measured by ELISA (a) and RIA (b).

rather stable. In addition, fasciculations were recorded in three patients, which often are seen as a sign of motor neuron disease. Preferential involvement of bulbar muscles in MuSK MG has been well documented and is probably due to the lower expression level of the MuSK protein in these muscles (2;8). Also, one third of patients with ALS start with bulbar weakness (9). It should be stressed that none of our four patients had clear signs of upper motor neuron dysfunction, and the dysarthria was characterized by a nasal voice, although clinical distinction between flaccid, spastic or mixed dysarthria can be quite difficult (10). Thus, none of these patients fulfilled the revised El Escorial criteria for ALS. We tested 256 sera from bulbar ALS patients diagnosed according to revised El Escorial criteria in a tertiary center and none were positive for MuSK antibodies, suggesting that a false-negative diagnosis of MuSK MG is not common among patients with bulbar ALS diagnosed if the revised El Escorial criteria are properly used. In conclusion, we would not recommend to routinely check all ALS patients for MuSK antibodies. However, prominent bulbar weakness, a prolonged disease course or minor fluctuations, and the absence of signs of upper motor neuron involvement should alert the physician.

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CHAPTER 6

General discussion

Adapted from:
The expanding field of IgG4-mediated neurologic autoimmune disease

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European Journal of Neurology. 2015; 22(8):1151-61.

GENERAL DISCUSSION

The relevance of this thesis transcends MuSK MG as recently several new IgG4-mediated autoimmune diseases have been described. Currently, thirteen different antigens residing in the central nervous system (CNS) or peripheral nervous system (PNS), but also in connective tissue of the skin or kidneys have been shown to be associated with immunoglobulin G4 (IgG4). The identification of new antigenic targets in autoimmune diseases led to the designation of the subclass of the causative antibodies. The antibody subclass is pivotal for the effector function of the antibodies and the pathophysiological mechanism responsible for the disease. Moreover, the development of antibody specific treatments is facilitated by this knowledge. Table 1 gives an overview of the main characteristics of the IgG4-mediated autoimmune diseases that are presently known.

Myasthenia gravis with MuSK antibodies

Myasthenia gravis with antibodies against muscle specific kinase (MuSK) is hallmarked by fluctuating weakness with prominent involvement of bulbar and axial muscles. A large proportion of patients require respiratory support at some point during the disease (1,2). MuSK MG has the highest prevalence below the age of 40 years and mainly affects young women (1,3). The incidence of MuSK MG shows distinct geographical variation with a lower prevalence at higher latitudes estimated at 0.3 patients per million per year, and with a prevalence of 2.9.(4) In MuSK MG there is an association with the HLA DR14-DQ5 haplotype.(5,6,7)

After the discovery of MuSK antibodies in 2001 it soon became evident that they were predominately of the IgG4 subclass, and that their titres correlated with disease severity (8,9,10). Purified IgG4 from MuSK MG patients, but not IgG1-3 from the same patients or control IgG4 was able to bind neuromuscular junctions in whole mount mouse muscle and in addition upon passive transfer it induced a myasthenic phenotype in immune-compromised mice (**Chapter 2,** 11,12,13). These experiments unequivocally proved the pathogenicity of MuSK IgG4 antibodies (14). Active immunization of mice, rats, and rabbits with MuSK causes a myasthenic phenotype (15,16,17,18,19). Moreover, monovalent antibodies generated by papain digestion inhibited ACh receptor (AChR) clustering and MuSK phosphorylation *in vitro* (20). As IgG4 is considered to be functional monospecific this supports the notion that the MuSK antibodies cause disease by functional interference rather than by crosslinking and internalizing MuSK. Whether patient MuSK antibodies are in fact functionally monospecific and bivalent has yet to be established. Sequencing the CH3 and hinge region of the IgG4 gene in four MuSK MG patients showed the presence of residues essential for half-antibody exchange, suggesting that these patients' antibodies are able to undergo half-antibody exchange (Unpublished data).

Neuromuscular junction maintenance is maintained by the agrin-LRP4-MuSK signalling cascade which facilitates AChR clustering (21). The extracellular domain of

MuSK consists of three Ig-like domains and a Frizzled-like domain. The I96 residue in the first Ig-like domain of MuSK is essential for the interaction with LRP4 (22). Since the main immunogenic region of MuSK was located in this domain, we and others investigated whether IgG4 MuSK antibodies were able to inhibit agrin-dependent LRP4-MuSK binding. Indeed, IgG4 from MuSK MG patients inhibits this interaction, and the downstream signalling cascade, and is therefore considered a key effector mechanism of the MuSK autoantibodies (**Chapter 3,** 23,24). Furthermore, epitope mapping studies confirmed that antibodies against the first Ig-like domain correlate significantly with disease severity, whereas this was not the case for antibodies binding to other parts of the MuSK protein (**Chapter 4)**. Thus supporting the notion that IgG4 MuSK antibodies cause myasthenia by abolishing AChR clustering through inhibition of LRP4-MuSK signalling.

MuSK also interacts with the neuromuscular junction protein ColQ and the extracellular matrix protein biglycan. Passive transfer studies with total IgG from MuSK MG patients also showed interference with the MuSK-ColQ interaction (25). If and how this contributes to the disease is currently unknown. However, it might explain the adverse effects in many of the MuSK MG patients to acetylcholinesterase inhibitors, because acetylcholinesterase is anchored in the synaptic cleft by ColQ. In fact, animals treated with MuSK MG patient antibodies and pyridostigmine show increased AChR loss and exacerbation of muscle weakness (26).This hypersensitivity is not related to the epitope pattern of the auto-antibodies (**Chapter 4)**.

Chronic inflammatory demyelinating polyneuropathy with antibodies against (para)nodal proteins

Chronic inflammatory demyelinating polyneuropathy (CIDP) is the most frequent chronic inflammatory neuropathy. Diagnosis is based on clinical features, supported by electromyographical findings, but comprises of a heterogeneous group of clinical phenotypes (27,28). The presence of IgG and complement deposition in nerve biopsies (29), the results of passive transfer studies in rats (30), and the favourable response to human intravenous immunoglobulin or plasmapheresis (31) all support an autoimmune pathogenesis. In three out of 46 patients with CIDP, characterized by a rapidly progressive clinical course with predominantly motor symptoms, antibodies to contactin1 (CNTN1) or the contactin1-associated protein-1 (CASPR1) were found (32,33). In another series of 53 CIDP patients two were positive for antibodies to neurofascin 155 (27). These two patients also had a severe phenotype, poor response to IVIg, and a disabling tremor.

Neurofascin 155, CASPR, and CNTN1 patient antibodies bind the paranode of Ranvier and to a variety of regions in the CNS (34,27). An ELISA testing for IgG4 subclass specific antibodies against neurofascin 155 and CNTN1 confirmed that the majority of these antibodies were of the IgG4 subclass (35,27). Low levels of antigen specific IgG2 antibodies were also detected. To optimize nerve conduction and prevent current leakages, neurofascin 155 , CNTN1, and CASPR establish the axon-

glial junction separating nodal voltage-gated sodium channels from juxtaparanodal voltage-gated Kv1 channels (36). Deletion of *neurofascin155*, *CNTN1* or *CASPR* in mice causes severe neuropathy, decreased nerve conduction velocity, and other symptoms that mimic the CIDP phenotype (37,38,39). The exact mode of action of the IgG4 antibodies has not been investigated. However, the addition of patient total IgG in cultures of myelinated dorsal root ganglion neurons revealed that the antibodies prevent cell-cell interactions (35). In three patients with CNTN1 antibodies electrophysiological evidence of demyelination was found (32). Future experiments should further elucidate the cause and consequence of the IgG4 immune response against these proteins.

Parasomnia with antibodies against Iglon5

A recent report described Iglon5 antibodies in eight patients with abnormal sleep movements, behavior and obstructive sleep apnoea, as confirmed by polysomnography (40). The median age at disease onset was 59 years and six patients had chronic progression. In four patients the sleep disorder was the initial and most prominent feature. Other associated symptoms with disease were gait instability, dysarthria, dysphagia, ataxia, and chorea. Two patients had a rapid progression and died within half a year after onset of symptoms. HLADRB1*1001 and HLA DQB1*0501 alleles were found in all four patients who were tested.

The Iglon5 antibodies were found to be of the IgG4 type, which was confirmed by immunostaining of rat hippocampus with secondary antibodies specific for IgG4 (40). The antibodies were reactive with rat neuropil throughout the brain and showed increased binding to the molecular layer and the synaptic boutons of the granular layer in the cerebellum. In addition four of the eight patients also showed mild IgG1 reactivity. Moreover, increased levels of hyperphosphorylated tau were seen in neurons of the hypothalamus and dorsal brainstem of two patients. Epitope mapping, passive transfer, or active immunization experiments have to our knowledge not been performed for these antibodies. Furthermore, whether Iglon5 antibodies are a primary cause, a secondary effect, or correlated with this observation is not yet known. The function of Iglon5 is not known. The Iglon protein family is essential for axonal growth cone guidance in the CNS (41). The different Iglons form heterodimers which stabilize cell adhesion. It is likely that IgG4 antibodies binding Iglon5 might affect this function (42). Future experiments should elucidate whether the pathomechanism of Iglon5 autoimmunity is similar to that of MuSK MG and neurofascin 155/CNTN1 antibodies in CIDP.

Limbic encephalitis with antibodies against LGI1

The group of autoimmune synaptic encephalopathies has rapidly expanded over the last few years. In these patients antibodies against CNS synaptic proteins, including the excitatory glutamate NMDA (43) and AMPA receptors (44) and the inhibitory $GABA_B$ or GABAA receptor (45,46) are found. These receptors have

important functions in synaptic transmission and plasticity. A study on 57 patients with limbic encephalitis and antibodies previously attributed to be directed against voltage-gated potassium channels found serum or CSF antibodies against Leucine-rich, glioma inactivated 1 (LGI1) in all patients (47). Patients all have memory loss and often presented with seizures and/or neuropsychiatric symptoms, ranging from alterations in memory, behavior, and cognition, to psychosis. Seizures were observed in 80% of the patients, while myoclonic-like movements or hyponatraemia was found in about half the patients. LGI1 antibodies are of the IgG4 type as demonstrated by subclass specific staining on transiently transfected cells (48). Some patients also had IgG1 and IgG2 LGI1 antibodies. In a cellular overexpression interaction assay the LGI1 antibodies prevent LGI1-ADAM22 interaction (49).

Epitope mapping revealed that LGI1 antibodies are polyclonal and bind to both the N-terminal leucine rich repeat as well as the more distal EPTP domain. Binding of antibodies to the latter domain was responsible for inhibition of the LGI1- ADAM22 protein interaction. In cultured hippocampal neurons the addition of LGI1 antibodies suppressed the clustering of AMPA receptors. LGI1 KO mice show reduced hippocampal AMPA receptor signalling which leads to lethal epilepsy (50). However, how LGI1 is linked to AMPA receptor clustering is not fully known, but it is known that ADAM22 and AMPA receptors are anchored by PSD-95. Ohkawa *et al.* speculate that loss of LGI1-ADAM22 binding to PSD95 might destabilize the binding of AMPA receptors to PSD95 thus increasing the turnover of the receptors and resulting in a loss of clustering (49). Additionally, LGI1 is involved in cell adhesion likely through an interaction with Nogo receptor 1 (51). The effects of patient antibodies on this interaction have not been investigated yet. In future studies it would be interesting to investigate the effects of LGI1 IgG4 in passive transfer experiments.

Neuromyotonia, Morvan syndrome and encephalitis with antibodies against CASPR2

Antibodies against contactin-associated protein-like 2 (CASPR2) are associated with limbic encephalitis, neuromyotonia, and Morvan syndrome, which is hallmarked by the combined symptoms of the first two disorders (52,53). A case series described that out of the 29 patients with neuromyotonia six had antibodies against CASPR2 and 15 had antibodies against both CASPR2 and LGI1 (52). Almost all (27/29) patients were males, as previously reported (53). Three patients had additional antibodies against contactin-2. All patients with CASPR2 antibodies experienced neuromyotonia (i.e. unwanted muscular activity due to peripheral nerve overactivity), dysautonomia, and neuropathic pain. The majority also presented with psychiatric symptoms such as confusion and amnesia. In more than half of the patients with CASPR2 antibodies a thymoma was discovered. Interestingly, 9/21 patients with CASPR2 antibodies also had a history of AChR MG.

A cell based immunofluorescent assay showed that three of seven tested patients had IgG4 CASPR2 antibodies in addition to IgG1 CASPR2 antibodies (52). CASPR2

patient sera can be used to immunostain both the juxtaparanodal region of teased peripheral nerve fibres as well as brain neuropil (52,53). CASPR2 is essential in the CNS and PNS juxtaparanodal region of myelinated axons for clustering voltage-gated potassium channels (VGKCs). Patients with a homozygous recessive deletion mutation of *CNTNAP2*, the gene encoding CASPR2, express truncated non-functional protein (55).

This causes seizures, neuromyotonia, and peripheral neuropathy, indicating that loss of CASPR2 function can underlie the similar phenotype observed in Morvan syndrome. Heterozygous polymorphisms in the same gene have also been associated with epilepsy and schizophrenia (54, 56).

In 1991, Sinha *et al.* performed passive transfer in mice with plasma or purified IgG from a neuromyotonia patient and observed reduced d-tubocurarine sensitivity of neuromuscular synaptic transmission (54). This is compatible with increased neurotransmitter release due to motor axonal action potential broadening following from block of motor axonal and/or presynaptic VGKCs by the patient IgG. More detailed electrophysiological analyses in passive transfer studies using IgG of six additional patients in 1995 confirmed these initial findings (55). Three of the patients were positive in an α-dendrotoxin-based radioimmunoassay, at the time inadvertently considered to test for anti-VGKC antibodies, but now known to test for VGKC-complexed proteins such as CASPR2, LGI1 and contactin (52). It is not known which type of auto-antibodies these patients had, nor whether they were IgG1 or IgG4. Future studies should elucidate the pathogenic importance of CASPR2 antibodies in peripheral nerve function.

However, it appears autoimmunity against a complex of extracellular proteins involved in VGKC clustering and cell adhesion in the (juxta)(para)nodal part of motor neurons is prone to producing IgG4 subclass auto-antibodies.

Non-neurological IgG4-mediated autoimmunities

In 1989 Rock *et al.* published that passive transfer of IgG4 patient antibodies against desmoglein proteins caused skin blistering in BALB/c mice, thereby reproducing the phenotype of pemphigus vulgaris patients (56). Some patients also have IgG1, IgG2, IgA, and IgE antibodies, but the skin lesions mostly contain IgG4 (57,58,56). The IgG4 antibodies bind either desmoglein1 (dsg1), desmoglein3 (dsg3), or both. The antibody profile is important as dsg1 antibodies are associated with skin blistering whereas dsg3 antibodies more commonly cause mucosal lesions (59). Desmogleins are transmembrane glycoprotein cadherins responsible for intercellular adhesion of epidermal keratinocytes. The antibodies cause disease by interfering in cellular adhesion resulting in acantholysis. The HLA association in these patients depends on their origin. Jewish patients have a strong association with HLA DR4-DQ3 haplotype, whereas the majority of non-Jewish patients have a HLA DR14-DQ5 haplotype similar to that of MuSK MG. Interestingly, the lower prevalence of pemphigus at higher latitudes is similar to that seen in MuSK MG (60).

Table 1. Overview of the thirteen proteins currently known to be the antigenic target in IgG4-mediated autoimmun

Antigen	Disease	Anatomical (sub) cellular site	Location epitope	Epitope domain
Peripheral Nervous System				
MuSK	Myasthenia Gravis	Neuromuscular junction	N-terminal top	Ig-like 1 domain
Neurofascin155	CIDP & Guillain Barré syndrome	Juxtaparanode of Ranvier/hippocampal neurons	N-terminal top	Ig-like domain
Contactin-1	CIDP & Guillain Barré syndrome	Juxtaparanode of Ranvier	N-terminal top	Ig-like domain dependent on N-glycans
CASPR1	CIDP	Juxtaparanode of Ranvier	N terminal top	
Central Nervous System				
IgLON5	Non-REM and REM parasomnia with sleep breathing dysfunction and a tauopathy	Brain neuropil, prominent in granular layer cerebellum		Not know but antigen contains three Ig-like domains
LGI1	Limbic Encephalitis	Hippocampal neurons/ cell adhesion	Polyclonal response to extracellular domain	EPTP repeat and LRR domain (quite polyclonal)
CASPR2	Limbic Encephalitis, neuromyotonia and Morvan syndrome	Juxtaparanode of Ranvier/ hippocampal neurons		

disorders.

	Passive transfer effective	Protein complex interacting partners	Physiological function antigen	HLA	Key references
	Yes	Lrp4, Dok7 intracellular	Mediates AChR clustering	DQ5, (DR14),DR16	Hoch 2001 Nature med, Niks 2006 Neurology, Cole 2008 Ann Neurol, Klooster 2012 Brain, Huijbers 2013 PNAS
		Contactin, CASPR1	Cell adhesion, maintaining paranodal junction		Labasque 2014 J Biol Chem, Ng 2012 Neurology, Labasque 2011 J Biol Chem, Mathey 2007 JEM
		Contactin-CASPR NF155	Cell adhesion, maintaining paranodal junction		Labasque 2014 J Biol Chem, Querol 2013 Ann Neurol
	Yes	Contactin-CASPR NF155	Cell adhesion, maintaining paranodal junction		Menegoz 1997 Neuron, Labasque 2014 J Biol Chem, Querol 2013 Ann Neurol, Manso 2016 Brain
		IgLON5	Involved in neuronal cell adhesion	HLA-DRB1*1001 and HLA-DQB1*0501 alleles	Sabater L 2014 Lancet Neurol
	Yes	ADAM22 interaction is inhibited at EPTP repeat of Lgi1 with ADAM22	Maintains AMPAr clustering		Sinha 1991 Lancet, shillito 1995 Ann Neurol Lai & Huijbers 2010 Lancet Neurol, Ohkawa 2013 J Neuroscie
	Yes	Neurofascin155 CNTN1	Interacts with Kv1.1 Kv1.2		Sinha 1991 Lancet, shillito 1995 Ann Neurol, Poliak 1999 Neuron, Lancaster 2011 Ann Neurol, Irani 2012 Ann Neurol

Table 1. (continue)

Antigen	Disease	Anatomical (sub) cellular site	Location epitope	Epitope domain
Non neurological diseases				
Desmoglein1	Phemphigus	Skin cell adhesion junction	N-terminal top	Cadherin like domain
Desmoglein3	Phemphigus	Skin cell adhesion junction	N-terminal top	Cadherin like domain
PLA2R1	Membranous nephropathy	Podocytes (Kidney)	N-terminal top	Cystein rich ricin domain
Collagen IV	Good pasture disease	Glomerular basement membrane (Kidney)		NCI-domain (alpha 3 chain)
ADAMTS13	Thrombotic thrombocytopenic purpura	Metalloproteinase in vasculature	Halfway the protein	Cysteine rich spacer domain (quite polyclonal)
THSDA7A	Membranous nephropathy	Podocytes foot processes (Kidney)		

Other currently known IgG4 autoimmune diseases include membranous nephropathy caused by IgG4 antibodies which bind the M-type phospholipase A2 receptor 1 (PLA2R1) or Thrombospondin type-1 domain-containing 7A (THSD7A) on kidney podocytes, Goodpasture syndrome caused by IgG4 antibodies to type IV collagen in the kidney, and thrombotic thrombocytopenic purpura caused by IgG4 antibodies against the metalloprotease ADAMTS13 (61,62,63,64,65). In the first

Passive transfer effective	Protein complex interacting partners	Physiological function antigen	HLA	Key references
Yes	Desmosomes, Ca2+is cofactor	Mediates cell adhesion	HLA-DR4-DQ3 in Jewish patients and HLA-DR14-DQ5 in non-Jewish patients	Mahoney 1999 J Clin Inv, Futei Y 2011 J Dermat Scien, Zhu 2011 J Clin Immunol, Oktarina 2011 Br J Dermatol
Yes	Desmosomes Ca2+is cofactor	Mediates cell adhesion	HLA-DR4-DQ3 in Jewish patients and HLA-DR14-DQ5 in non-Jewish patients	Mahoney 1999 J Clin Inv, Sitaru 2007 Arch Dermatol Res, Zhu 2011 J Clin Immunol, Futei Y 2011 J Dermat Scien
		Mediates podocytes adhesion to collagen IV	Not significant DQA1	Beck 2009 N Engl J Med, Fresquet 2014 J Am Soc Nephrol, Skoberne 2014 Eur J Clin Invest
	PLA2R1 and other collagens	Matrix formation, membrane stability		Ohlsson 2014 Am J Kidney Dis
	Von Willebrand factor	Cleaves von Willebrand factor	HLA-DRB1*11	Tsai 1998 N Engl J Med, Ferrari 2009 J Thromb Haemost, Zheng 2010 Haematlogica, Coppo 2010 J Thromb Haemost, Yamaguchi 2011 Thromb Res Tomas 2014 N Engl J Med.

case cellular adhesion of the podocytes is inhibited, whereas the IgG4 antibodies in the latter case inhibit the disappearance rate of platelet strings. Passive transfer experiments for these diseases have not yet been carried out, which would allow confirmation of the disease mechanism and causality of these auto-antibodies. Table 1 summarizes the present clinical and pathomechanistical knowledge on these diseases.

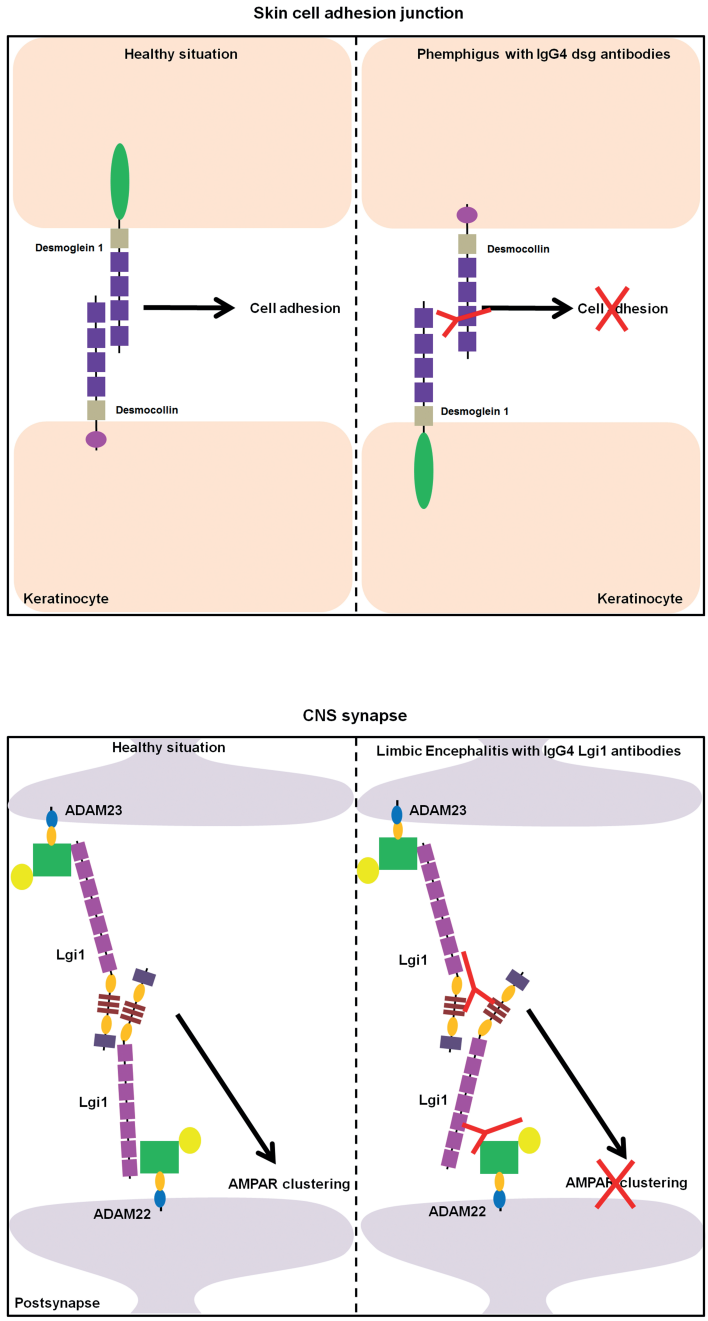
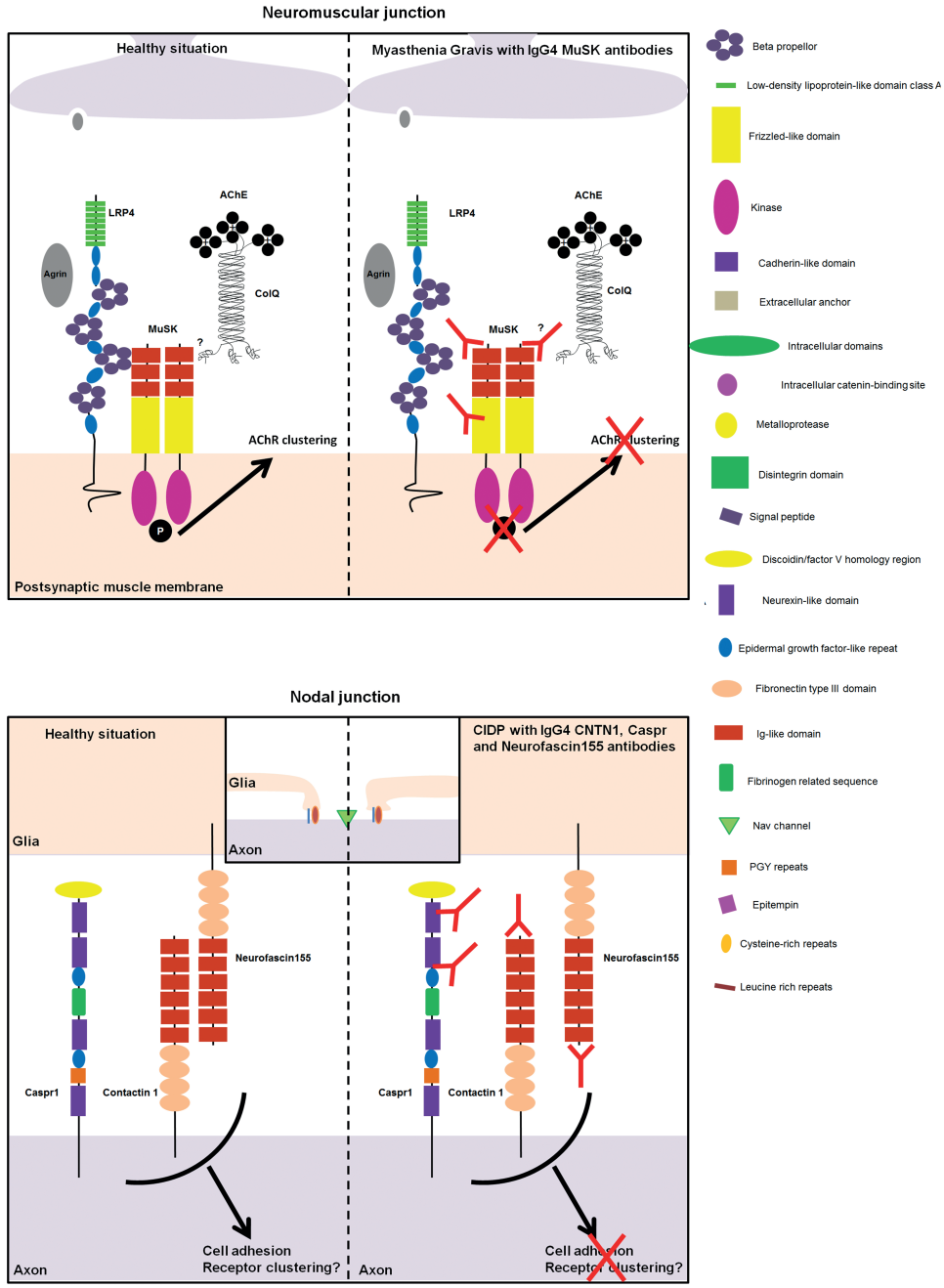


Figure 1: Schematic representation of the cellular locations, protein interactions and auto-antibody effects in the healthy and diseased situation, related to four IgG4-mediated autoimmune diseases. Specific protein domains are indicated in the legend. An important similarity between



these extracellular antigenic proteins is that they are essential for protein-protein interactions which facilitate cell adhesion and associated receptor stabilization. These essential functions are inhibited by IgG4 auto-antibodies and thereby cause severe disease.

The unique features of IgG4

IgG4 is a relatively rare antibody subclass with unique properties. The concentration of IgG4 in healthy adult blood is 0.08-1.4 g/L, which represents approximately 5% of the total IgG pool. The presence of IgG4 antibodies is usually associated with an IgE-mediated allergy, and derives from a Th2 IL10 assisted immune response. IgG4 antibodies are most often observed after prolonged immunization. Antibody class switches from IgG1/IgE to IgG4 are associated with inhibition of inflammation and improvement of the allergic symptoms.

IgG4 differs from other IgG subclasses in several structural and functional aspects: 1) IgG4 antibodies continuously undergo half-antibody exchange and can thus be functionally hetero-bispecific, 2) IgG4 has low affinity for Fcγ receptors, 3) IgG4 cannot activate complement because it is unable to bind the first complement cascade component C1q, 4) IgG4 generally develops as an anti-inflammatory response after prolonged antigenic exposure, 5) IgG4 has very high affinity for its antigen as it has undergone a higher number of somatic hypermutations and 6) IgG4 can interact through its Fc domain with other IgGs like the IgG rheumatoid factor (66,67,68,69).

IgG subtypes are highly homologous, however, variations in several residues enable IgG4 to have different effector functions. These include having low affinity for the Fcγ receptors and the C1q complement factor, a property derived from several residues (mostly P331 and L234) in the CH2 and hinge region (70,71). In addition, IgG4 molecules consist of two heavy chains and two light chains joined by non-covalent bonds. This is in contrast to IgG1, IgG2, and IgG3 molecules where the heavy chains are joined through their lysine 409 residue in the constant heavy chain three domain (CH3) and proline 228 in the hinge region, which facilitates inter-heavy chain sulphide and hydrogen bridges. In IgG4 molecules half-antibody exchange is caused by serine 228 and arginine 409 making them more prone to cause intra-heavy chain disulphide bonds rendering the molecule monovalent (72,73,74,75). The ability of IgG4 to continuously undergo half-antibody exchange theoretically implies that only one arm of the antibody is recognizing the antigen. Therefore the density at which these antibodies bind might not allow for a high concentration of antigen-bound antibodies and thus they might be less effective in forming cross-linked immune complexes, which in turn would be internalized and degraded (76,77).

Together these aspects of the IgG4 molecule render it highly inadequate for activating a cellular or complement mediated immune response. In IgG4 autoimmune diseases it is therefore likely that the antibodies cause pathology by directly interfering in the function of the antigen, such as mechanical blocking of ligand-receptor interactions. Indeed it appears that IgG4 antibody-mediated autoimmune diseases share a specific/common disease mechanism, which is different from IgG1-3 mediated autoimmune diseases. IgG1-3 auto-antibodies induce cross-linking and internalization of the antigen as seen for instance in AChR myasthenia gravis, and NMDA or AMPA receptor limbic encephalitis (78,44). Thus far IgG4 auto-antibodies

have been demonstrated to cause inhibition of protein-protein interaction which prevents cell adhesion and loss of connectivity (Figure 1).

What is the aetiology of IgG4-mediated autoimmune diseases?

An overview of the hypothetical causes of IgG4 autoimmunity is summarized in figure 2. Interestingly, all antigens in the currently known IgG4-mediated autoimmune diseases are N-linked extracellular glycoproteins involved in maintaining cell-cell interactions. In some cases glycosylation of the antigen is also essential for patient antibody binding (35). Moreover, an overlap between HLA haplotype associations is observed with DQ5 and DR14 in some conditions. This suggests that there might be a common underlying aetiology in all these IgG4 autoimmune diseases. It is tempting to speculate that decreased tolerance to post-translationally modified proteins in combination with a certain HLA haplotype makes the patients more susceptible for developing IgG4-mediated autoimmunity against glycosylated antigens. IgG4 antibodies are usually only observed upon exposure to certain worms or foods or after prolonged immunization (79,66,80). This is related to the way in which class switch is induced to cause IgG4 production. Both IgE and IgG4 result from a Th2 immune response requiring IL-4/IL-13 to induce a switch to these Ig types (81,82,83). Due to these similarities antigens that induce (allergic) IgE response often are also capable of inducing an IgG4 response. Furthermore, IL-10 might be responsible for supporting an IgG4 predominated response (84). The 'modified Th2 response' is known for the predominance of IgG4 and hallmarked by the absence of IgE (85). This response is often observed in bee-keepers whose symptoms have improved after prolonged exposure to bee venom. Alternatively, allergic responses to grass pollen and dust mites usually consist of both IgG4 and IgE antibodies against the antigens (86,87). It will be interesting to investigate whether MuSK MG patients also have IgE antibodies to MuSK, or whether the immune response against MuSK emulates the modified Th2 response. In addition, identifying the trigger of this response would be a fascinating line of research. The property of an allergen responsible for dictating what type of Th response occurs is to date not known. One hypothesis derived from these observations is that the exposure to such an IgG4 inducing allergic trigger results in cross reactivity with an antigen like MuSK, ultimately resulting in IgG4-autoimmunity. In accordance with this hypothesis MuSK MG patients seem to have a Th2 shifted IgG subclass distribution (unpublished observation). Moreover, serum IgG4 levels correlate with the level of IgG4 positive B cells (88). In concordance with this we observed increased clonal expansion of three IgG4 B cell clones in a MuSK MG patient whom had not received immunosuppression yet (unpublished data). Whether these clones are responsible for IgG4 anti-MuSK production is yet to be confirmed.

One other question that remains is whether bivalent IgG1 and IgG3 antibodies binding the same antigenic target as the pathogenic IgG4, are also pathogenic. Both IgG4 and IgG1-3 from MuSK MG patients affected AChR cluster stability in myotube cultures, albeit through different mechanisms (24). In contrast, a MuSK MG

patient was described to undergo a class switch from IgG4 antibodies to IgG1 MuSK antibodies while entering stable remission (9). If IgG1-3 antibodies in MuSK MG or other IgG4-associated autoimmune diseases are less pathogenic this could form an alternative explanation for the presence of IgG4 antibodies. The prolonged exposure to the antigen could have induced a class switch to pathogenic IgG4 antibodies, which then cause clinical manifestations of the disease. From immunotherapy it's known that IgG4 responses usually take months of repeated antigen exposure before an IgG4 response becomes evident (89). MuSK MG patients might then have been exposed to the trigger for an extended period, but only start experiencing disease symptoms when the class switch to IgG4 is induced.

Lastly, one could postulate polymorphic variations in the regulatory network of the immune system enabling an IgG4-dominated response. Whether or not MuSK MG patients are predisposed to develop an IgG4 response against a certain antigen is not known.

The presence of IgG4 antibodies can be either beneficial or detrimental to an individual depending on the target antigen and the effect of this antibody binding. IgG4 binding to bee venom in beekeepers or peanuts in allergic patients for example inhibits IgE and IgG1 inflammatory responses while not affecting the endogenous protein (89,90). However, in cases of IgG4-autoimmunity, the target antigen, although perhaps not the initial trigger, remains present and inhibits its essential function resulting in prolonged autoimmunity and severe pathology.

IgG4-autoimmunity unifying treatment strategies

The discovery of the IgG4-mediated autoimmune disease niche may provide opportunities towards specific treatment for all diseases within this class. In allergy the class switch from IgG1 to IgG4 antibodies generally alleviates symptoms and is applied as a treatment for allergies. The reverse might be true for IgG4-mediated autoimmune diseases as observed in a patient with MuSK MG who went into remission after a class switch to IgG1 and coninciding reduction in IgG4 anti-MuSK levels (9). Whether and/or how this observation could form a therapeutic strategy would be interesting to further explore.

Specific deletion of IgG4-producing B-cells could form another treatment option. Rituximab, a monoclonal antibody targeting CD20 on pro-B cells, has been shown to be a remarkably effective drug in MuSK MG, CIDP, pemphigus, and LGI1 limbic encephalitis (91,92,93,94). The exact effect of this drug in these autoimmune diseases is unknown, but it appears the destruction of pro-B cells reduces antibody titres and significantly alleviates symptoms.

Since the titres of the IgG4 antibodies appear to directly correlate with disease severity, the lowering of antibody titres (like with plasmapheresis) should result in a reduction of symptoms. This is likely to be true with the exemption that the pathology has not resulted in irreversible damage. In many antibody-mediated autoimmune diseases IvIg has proven an effective treatment, although its exact mechanism is not

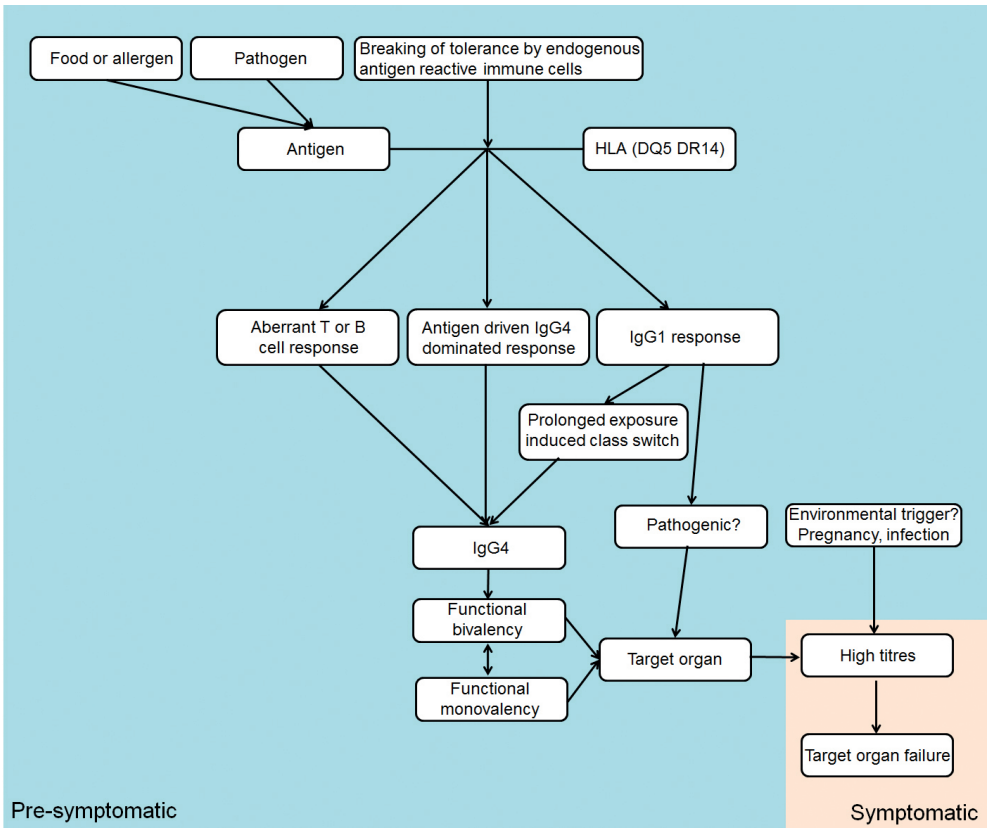


Figure 2. A unifying hypothesis for IgG4 autoimmunity. Several stages during the development of an antibody response could be causative for the formation of pathogenic IgG4 auto-antibodies. First, due to failure in negative selection in the thymus tolerance could be broken by endogenous antigen reactive immune cells. Second, specific pathogens (worms, allergens) and foods are known to elicit an IgG4 antibody response. Such a response could be responsible for the development of auto-antibodies that cross react with MuSK. The combination of an antigen with a specific HLA haplotype might play an essential role in this. Alternatively, the endogenous T and B cell interaction in IgG4-mediated autoimmunity patients might be inclined to steer the immune response in an IgG4 predominated response thereby diverting from IgG1 predominated auto-immunity. It might also be possible that IgG1-3 antibodies are less pathogenic than functionally monovalent IgG4 auto-antibodies, but after prolonged exposure to a certain antigen the switch to IgG4 is made which ultimately results in IgG4-mediated autoimmunity. All of these changes are likely to occur in a presymptomatic stage, but upon increase of the antibody titres become symptomatic. The increase in antibody titres might be caused by an additional environmental trigger like pregnancy. Whether functionally monovalent or bivalent IgG4 antibodies are equally pathogenic is not known. One could imagen that an increase in IgG4 titres also increases the functional bivalent numbers of auto-reactive antibodies which causes them to be pathogenic. Then the lack of IgG1 induced phenotype in passive transfer experiments might be related to the low titre of these antibodies rather then their pathogenicity. Alternatively, IgG4 auto-antibodies might be pathogenic as they physically obstruct protein-protein interactions that normally require dimerization for their functionality. The nature of the antigen might then be important in the sensitivity to IgG4 auto-antibodies.

yet completely understood (95). One of the hypothesis regarding its effectiveness is that IvIg might increase the catabolism of auto-antibodies through the FcRn (96). FcRn-deficient mice show increased catabolism of serum IgG (97). In 2005 Vaccaro *et al*. described a monoclonal antibody that has an increased affinity for the FcRn at neutral pH (98). The binding of this "Abdeg" (for antibodies that enhance IgG degradation) antibody prevented binding of endogenous IgG to the FcRn, blocking the normal recycling pathway of IgG in endothelial cells, thus directly lowering circulating IgG levels. This approach has proven successful in animal models for arthritis, autoimmune encephalomyelitis, and AChR MG (99,100,101). For AChR MG, both with active and passive immunization protocols, exposure to an anti-rat FcRn H chain antibody resulted in a reduction of the disease symptoms. Furthermore, it was found to be equally effective as 25 to 50 fold higher doses of IvIg (100). We expect that this approach could prove to be effective as an acute treatment for all IgG4-mediated autoimmune disease exacerbations. Moreover, it would be less invasive for the patients than plasmapheresis.

IgG4, as mentioned earlier, is produced by B cells stimulated by IL-4 and IL-13. Aversa and colleagues described a mutant mouse version of the IL-4 protein (hIL-4.Y124D) that antagonizes IL-4, thereby inhibiting IgG4 and IgE production in stimulated cultured B cells (102). The effect of this protein on the production of other Ig subclasses was not discussed. One could imagine that exposure of patients with IgG4-mediated autoimmune diseases to such a protein might alleviate their symptoms and improve their health.

Lastly, IgG4 specific apheresis would allow for removal of the pathogenic antibody containing fraction while maintaining the residual antibody repertoire present in the patient. This would imply that the patient is also deprived of IgG4 antibodies that might play an important role in suppressing other ongoing inflammatory processes. What the effect of this would be in each individual patient is difficult to predict. In many adults low or absent IgG4 levels have been detected without associated illnesses, thus suggesting it might not form a problem. Alternatively, one could consider replacement with a healthy IgG4 pool.

The downside of the here described therapeutic approaches is the fact that they all have a systemic effect which might result in side effects that harm the patient. An antigen specific approach would therefore form a more attractive approach.

MuSK myasthenia gravis specific treatment strategies

Ideally, one should always aim to maintain homeostasis while removing either the source of the pathogenic factor, the pathogenic factor itself, or prevent the pathological effect from occurring. This suggests that the goal should be to either specifically eliminate the initial antigenic trigger (if still present), the plasma cells responsible for producing MuSK antibodies, or remove the MuSK auto-antibodies or prevent their inhibitory effect on MuSK. An overview of these disease specific intervention strategies is given in figure 3.

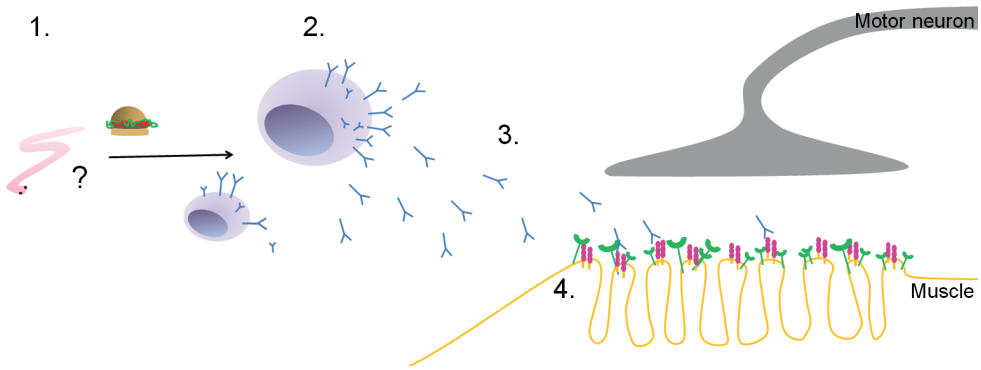


Figure 3: Overview of the different stages during MuSK autoimmunity that could form a target for disease specific treatment strategies. Autoimmunity is caused by an initial trigger (1). Upon removal of this trigger the incentive for the IgG4 immune response might be removed thus dissolving the (cross-reacting) immune response. This trigger initiated an inflammatory response resulting in antibody production by B cells. These auto-antibody producing B cells can be recognized by the B cell receptor they carry on their cell-surface which is unique for each antibody. The B cell receptor forms an interesting target for specific depletion of MuSK auto-antibody producing B cells (2). Alternatively, the MuSK auto-antibodies could specifically be depleted preventing they reach their target and cause disease (3). Lastly, MuSK auto-antibodies cause myasthenia by inhibiting its signaling cascade. A treatment that would overrule this effect, activating the MuSK signaling cascade, might proof an effective therapy (4).

The removal of the initial trigger and the by standing co-stimulatory immune cells is a challenging approach at this point as we do not currently know whether there is an initial exogenous trigger and how this results in MuSK auto-antibodies. This is an interesting line of investigation, but one that will require a long term plan for it to be feasible. The advantage of pursuing this research would be that there is the potential to prevent the onset of MuSK MG.

The elimination of MuSK antibody producing B cells is a challenging but theoretically effective strategy. It would include the identification of these immune cells by their B cell receptor and a neutralizing approach. The B cell receptor is unique as are the antibodies they produce. Thus identifying the sequence of the variable region of these B cell receptors and generating either a biological or a neutralizing antibody that is able to bind the B cell receptor would enable the

inhibition of these B cells. A similar approach was taken for a demyelinating autoimmune disease by coupling the myelin oligodendrocyte glycoprotein (MOG) antigen to an Fc IgG1 tail. The MOG-Fc was able to bind to antigen specific B cells *in vitro* and *in vivo* and induce MOG specific B cell cytotoxicity (103). Additionally to treat type 1 diabetes insulin reactive B-cells were depleted using a monoclonal antibody that specifically bound the combination of insulin bound to the B cell receptor (104). In both cases depletion was incomplete, but did result in improvement of disease symptoms. For AChR MG pathogenic lymphocytes (both T and B cells) were eliminated

using recombinant AChR coupled to ricin (105,106). The ricin induced cytotoxicity which was specific for lymphocytes involved in AChR antibody production. This also prevented the induction of EAMG upon transfer into rats.

Depletion of the MuSK auto-antibodies would be an attractive therapeutic approach as it only eliminates pathogenic antibodies, leaving all other beneficial antibodies present in the patient. Tzartos *et al.* immobilized recombinantly produced MuSK on beads and depleted pathogenic antibodies from MuSK MG patient plasma before infusing rabbits (107). This approach significantly reduced myasthenic symptoms in the rabbits. One could be afraid that some of the immobilized protein would be eluted from the column and could cause the induction of an immune response against the protein in the patient. Moreover, it might interfere in endogenous MuSK function as there is the possibility for it to bind to LRP4 and prevent signalling. A mutant form of MuSK could form a solution for such a depletion approach. We have shown that the I96A version of MuSK does bind patient auto-antibodies while this protein is unable to bind to LRP4 (23,22). Thereby using this variant of MuSK would form less of a threat for endogenous MuSK signalling while binding the pathogenic auto-antibodies.

MuSK antibodies do not result in the depletion of MuSK nor interfere in dimerization of MuSK, we expect that activating MuSK should be sufficient to overcome the effect of the pathogenic antibodies (23,23,24). In 1997 such activating antibodies were described to stimulate MuSK tyrosine phosphorylation and AChR clustering in cultured myotubes (108). It will be exciting to see if these antibodies can overcome the inhibiting effects of patient MuSK auto-antibodies *in vivo*. Lastly, as described in **chapter 1,** Lindstrom and collegues have shown that vaccination of EAMG rats with the intracellular domain of the AChR subunits is safe and resulted in reduced pathogenic antibodies, improved their health and prevented the onset of reinduced EAMG (109). The authors hypothesize that this approach is effective as it diverts the immune response away from the pathogenic epitope and induces a class switch from complement fixing antibodies to non-complement fixing antibodies. Antibodies against the intracellular domain of MuSK where not detected in three different cohorts (**chapter 4**). We do not know whether they would be pathogenic, but it's exciting to hypothesize that this approach might prove beneficial for patients with MuSK MG and other forms of autoimmunity as well.

CONCLUSION

Recently a group of (neurological) autoimmune diseases, hallmarked by the predominant involvement of antigen-specific pathogenic IgG4 auto-antibodies, has been identified. IgG4 antibodies previously considered to be benign and anti-inflammatory in these cases cause severe disease symptoms. The occurrence of strong HLA-DR14 and or DQ5 associations in some of these diseases suggests a common underlying aetiology. These diseases form a new niche in antibody-mediated autoimmunities. Future research should aim at elucidating the cause of

the predominant IgG4 response in these autoimmune diseases and investigate similarities in order to develop a treatment that might prove effective for several of these illnesses.

This thesis has aimed to contribute to this future prospect by critically characterizing MuSK MG auto-antibodies and their pathogenic effects. MuSK MG might form a good model for all IgG4-mediated autoimmune diseases as the antigen is well characterized, easily obtainable, and the patients have been extensively characterized.

Chapter 2 describes passive transfer studies in NOD/SCID mice. These studies confirmed that MuSK auto-antibodies are of the IgG4 subclass and can cause MG independent from aid of the innate and adaptive immune system. Exposure to MuSK auto-antibodies results in both pre and post synaptic defects. AChR's disassemble and move away from the synaptic zone, while presynaptic input is lost and compensatory upregulation of ACh release is reduced. This suggests that MuSK is not only essential in establishing post-synaptic stability, but also for retrograde signaling.

Chapter 3 shows that the IgG4 auto-antibodies against MuSK inhibit MuSK-LRP4 signaling by physically hindering the MuSK-LRP4 interaction, thereby preventing AChR clustering and NMJ stabilization. The antibodies do not interfere in MuSK dimerization nor do they cause MuSK cell surface depletion.

Chapter 2 and 3 both confirm the auto-antibody dose-dependent nature of muscle weakness *in vitro* and in mice.

Chapter 4 investigates a potential role for epitope spreading throughout the course of disease in MuSK MG patients. This study proves that the immune response against MuSK remains largely focused on a main immunogenic region, and that epitope spreading is rare and does not contribute to disease severity or treatment responsiveness.

Chapter 5 details studies that used our experience in detecting MuSK auto-antibodies in patients with ALS. The clinical phenotype of MuSK MG can have a striking overlap with that of ALS. Some case reports show that the diagnosis of MuSK MG and ALS can be confused. We found that patients with ALS are diagnosed correctly in the majority of cases. However, in the case of a long disease trajectory with occasional stable periods of disease as well as the presence of ocular and/or bulbar muscle weakness, a MuSK MG diagnosis should be considered.

Chapter 6 concludes that studying rare IgG4-mediated autoimmune diseases like MuSK MG, not only provides insight in the disease mechanism, but also generates new understanding in normal physiology and might provide therapeutic applications that surpass the initial application.

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APPENDIX

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ENGLISH SUMMARY

Muscle-specific kinase (MuSK) myasthenia gravis (MG) is a neuromuscular autoimmune disease that is hallmarked by fatigable muscle weakness of in particular the cranial and bulbar muscles and a high frequency of respiratory crises. MuSK regulates neuromuscular junction formation and maintenance by relaying the neuronal agrin-Lrp4 signal through binding with Lrp4 and activating intracellular signaling cascades facilitating acetylcholine receptor (AChR) clustering. The extracellular portion of MuSK consists of three Ig-like domains and a Frizzled-like (Fz) domain. Interaction with Lrp4 is facilitated through the Ig-like 1 domain, which also harbors the main immunogenic region (MIR) for MuSK auto-antibodies in MuSK MG. The other domains have been implicated in interactions with other proteins like ColQ and biglycan as well as a role as a trophic Wnt receptor.

Although the cause of the presence of the auto-antibodies is not known in MuSK MG, earlier studies have shown that auto-antibodies of the IgG4 subclass correlate with disease severity. In this thesis we have provided evidence for the pathogenicity of the IgG4 subclass antibodies by selectively purifying them from plasmapheresis material from patients and infusing them in NOD/SCID mice. These passive transfer experiments in mice lacking a functional innate and adaptive immune system, demonstrated progressive body weight loss and severe myasthenia. These effects could be attributed to declustering of the AChRs and dysregulation of neuromuscular transmission. Mice that received control IgG4 or IgG1,2,3 fractions from the same patients did not show these effects.

To further understand how the IgG4 auto-antibodies against MuSK cause myasthenia we tested the effects of these antibodies on the naturally occurring agrin-dependent MuSK-Lrp4 interaction *in vitro*. IgG4 MuSK patients' antibodies compete dose-dependently with the interaction between MuSK and Lrp4. Again, this was unique to the IgG4 subclass antibodies of patients only, and was not observed for the other antibody subclasses. Patient IgG4 antibodies did not cause inhibition of MuSK dimerization or MuSK internalization. As agrin-dependent MuSK-Lrp4 interaction is essential for maintaining the neuromuscular junction and its functionality, we concluded that loss of this interaction is the main pathomechanism by which the auto-antibodies cause myasthenia. This has now been confirmed by several independent studies.

As the different domains of MuSK have been implicated to support different roles in neuromuscular junction maintenance, and patients sometimes experience adverse effects of the first-line treatment of acetylcholine esterase inhibitors (AChEi), we studied how the immune response against MuSK patients changes over time and whether potential changes in the auto-immune repertoire may contribute to the AChEi hypersensitivity. Epitope mapping experiments showed a dominant response in all patients against the N-terminal Ig-like domain, which correlated tightly with disease severity both within and amongst patients. Epitopes outside this domain were identified in many patients, but epitope spreading was seen in only 19% of

the patients. Reactivity against other domains did not correlate with disease severity, nor did epitope specificity correlate to treatment responsiveness. These observations suggest that auto-antibodies against the first Ig-like domain are crucial determinants in the disease and support the idea that functional inhibition of MuSK-Lrp4 interaction is the predominant pathomechanism.

Because of the predominant bulbar weakness, MuSK patients can sometimes be misdiagnosed as amyotrophic lateral sclerosis (ALS) patients. We identified four patients that were initially diagnosed with having a bulbar form of ALS based on clinical examination, but later on were recognized to actually suffer from MuSK myasthenia. This misdiagnosis is rare as we did not find any other MuSK MG patients amongst a cohort of 256 ALS patients.

In addition to MuSK MG, 13 other neurological and non-neurological diseases have been identified to be associated predominantly with IgG4 auto-antibodies. From the diseases of which the pathomechanism has been studied, exciting parallels can be drawn to MuSK MG: (1) In all these diseases the antigens involve proteins responsible for maintaining cell-cell interaction and are often stabilizing essential receptors, and (2) the antibodies cause disease by interfering in protein-protein interaction, thereby inhibiting cell-cell interaction, resulting in severe disease. These observations suggest the presence of a specific sub-group in antibody-mediated auto-immune diseases. MuSK MG might therefore represent a promising model to study the cause and consequence of IgG4-mediated auto-immunity.

NEDERLANDSE SAMENVATTING

Muscle-specific kinase (MuSK) myasthenia gravis (MG) is een neuromusculaire auto-immuunziekte die wordt gekenmerkt door vermoeibare spierzwakte van in het bijzonder de craniale en bulbaire spieren en het vaak voorkomen van respiratoire crises. MuSK regelt de vorming en instandhouding van de neuromusculaire synaps door het doorgeven van het neuronale agrine-Lrp4 signaal. Door binding van Lrp4 aan MuSK activeren intracellulaire signalering cascades die acetylcholine receptor (AChR) clustering faciliteren. Het extracellulaire domein van MuSK bestaat uit drie Ig-achtige domeinen en een Frizzled-achtig (Fz) domein. Interactie met LRP4 wordt bewerkstelligt door het Ig1 domein, dat ook de belangrijkste immunogene regio (MIR) herbergt. De andere domeinen zijn betrokken bij interacties met andere eiwitten zoals ColQ en biglycan of heeft een rol als een trofisch Wnt receptor.

Hoewel de oorzaak van de aanwezigheid van auto-antilichamen in MuSK MG niet bekend is, heeft onderzoek aangetoond dat de auto-antilichamen van de IgG4 subklasse correleren met de ernst van de ziekte. In dit proefschrift hebben we de pathogeniteit van de IgG4 subklasse antilichamen onderzocht door ze selectief uit plasmaferese materiaal van patiënten te zuiveren en in NOD/SCID muizen te injecteren. Deze muizen, die een functioneel aangeboren en adaptief immuunsysteem missen, ontwikkelden gewichtsverlies en ernstige spierzwakte. Deze effecten werden veroorzaakt door het verlies van AChR clusters en de resulterende ontregeling van neuromusculaire transmissie. Muizen die IgG4 van gezonde controles of IgG1,2,3 fracties van dezelfde MuSK MG patiënten ontvingen ontwikkelden geen myasthenie.

Om beter te begrijpen hoe de IgG4 antistoffen tegen MuSK, myasthenie veroorzaken, hebben we de effecten van deze antilichamen op agrine-afhankelijke MuSK-Lrp4 interactie getest *in vitro*. IgG4 van MuSK MG patiënten veroorzaakte dosis-afhankelijke verlies van MuSK-Lrp4 interactie. Ook dit was uniek voor de IgG4 antilichamen van patiënten. De patiënt IgG4-antilichamen veroorzaakten geen inhibitie van MuSK dimerisatie of MuSK internalisatie. Aangezien agrine-afhankelijke MuSK-Lrp4 interactie essentieel is voor de stabiliteit en functionaliteit van de neuromusculaire junctie, hebben we geconcludeerd dat het verlies van deze interactie het belangrijkste mechanisme is waardoor de auto-antilichamen myasthenie veroorzaken. Dit is inmiddels bevestigd door verschillende andere onafhankelijke studies.

Omdat de diverse domeinen van MuSK verschillende functies hebben in de neuromusculaire junctie en patiënten soms nadelige effecten van de eerstelijnsbehandeling met acetylcholine-esterase-remmers (AChEi) ondervinden, hebben we onderzocht hoe de immuunrespons tegen MuSK varieert in de tijd en of een mogelijke verandering in het auto-immuun repertoire bijdraagt aan de AChEi overgevoeligheid. Epitoop mapping experimenten toonden een dominante respons bij alle patiënten tegen het N-terminale Ig1 domein dat nauw correleerd met de ernst van de ziekte binnen en tussen patiënten. Epitopen buiten dit domein komen voor bij veel patiënten, maar actieve epitoop spreiding werd waargenomen

bij slechts 19% van de patiënten. Reactiviteit tegen de andere domeinen van MuSK correleert niet met de ernst van de ziekte noch correleert epitoop specificiteit met de response op AChEi. Deze waarnemingen suggereren dat auto-antilichamen tegen het eerste Ig domein van cruciaal belang zijn in de ziekte en het ondersteunt het idee dat de functionele remming van de MuSK-Lrp4 interactie de hoofdoorzaak van de myasthenie is.

Door de dominante bulbaire spierzwakte, worden MuSK MG patiënten soms verward met patiënten met amyotrofe laterale sclerose (ALS). We hebben vier patiënten geïdentificeerd waarbij een verkeerde diagnose gesteld werd. Deze patiënten werden primair gediagnostiseerd met een bulbaire vorm van ALS, terwijl ze eigenlijk MuSK MG hadden. Het stellen van een verkeerde diagnose is zeldzaam aangezien we geen andere MuSK MG patiënten hebben gevonden in een cohort van 256 ALS patiënten.

Naast MuSK MG zijn er tot nu toe 13 neurologische en niet-neurologische ziekten geïdentificeerd die worden geassocieerd met auto-antilichamen van het IgG4 subtype. Van de ziekten van wie het ziekte mechanisme is onderzocht, kunnen duidelijke parallellen worden getrokken met MuSK MG: 1) In al deze ziekten zijn de antigenen verantwoordelijk voor cel-cel interactie, en zijn ze vaak betrokken bij de stabilisatie van essentiële receptoren. 2) De auto-antilichamen remmen eiwit-eiwit interacties, waarbij cel-cel interacties verloren gaan, wat resulteert in ernstige ziekte. Deze waarnemingen suggereren dat het hier gaat om een specifieke subgroep onder de antilichaam-gemedieerde auto-immuunziekten. MuSK MG kan een veelbelovend model zijn om de oorzaak en gevolgen van IgG4-gemedieerde auto-immuniteit te bestuderen.

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CURRICULUM VITAE

Maartje Huijbers was born on the 16th of March 1985 in Eindhoven. In 2003 she graduated from the Dr. Knippenbergcollege in Helmond and started the undergraduate program of Medicine at Maastricht University. After successfully completing the first two years, she decided to take a short leave to start a bachelor in Molecular Life Sciences at the same university. After she finished her bachelor's degree in Maastricht, she started a master's programme in Biomedical Sciences at Leiden University. Her first internship "Investigating new targets in seronegative myasthenia gravis" under the supervision of dr. Rinse Klooster and prof. Jan Verschuuren sparked her enthusiasm for the neuromuscular autoimmune field. She continued in this field by investigating peripheral and central nervous system autoimmune diseases during an 11 month internship in the prof. Josep Dalmau lab at the University of Pennsylvania in Philadelphia. This led to two publications which currently have been cited over 550 times.

After graduating in 2011 she started a PhD at the departments of Neurology and Human Genetics of the LUMC under supervision of Prof. Jan Verschuuren and Prof. Silvère van der Maarel investigating the pathomechanism of MuSK myasthenia gravis. The results of this PhD are outlined in this thesis. In collaboration with Prof. Steven Burden at New York University she unravelled the main pathomechanism by which MuSK auto-antibodies cause myasthenia. These results also led to a patent application and prizes for "Best Content" at the Annual Neurology Science Day at the LUMC as well as the Myasthenia Gravis Young Investigator Travel Fellowship from the New York Academy of Sciences.

Her enthusiasm for science reaches further than research alone. She guided ten students, has been an engaged member of Student and PhD candidate representation committees, organized several conferences and is a member of the Boerhaave Committee (responsible for post-academic education).

Currently, she is employed as a post-doctoral researcher at the departments of Neurology and Human Genetics of the LUMC.

DANKWOORD

Graag wil ik mijn promotoren Prof. Dr. Jan Verschuuren en Prof. Dr. Silvère van der Maarel danken voor hun vertrouwen, support en enthousiasme. Het samenspel tussen de klinische blik van Jan en de praktische technische kennis van Silvère, tezamen met jullie creativiteit hebben mij veel geleerd en maken het een feest om te mogen werken "op de brug van het LUMC".

Naast supervisie van mijn promotoren zijn er ook twee bijzondere mensen die mij de fijne kneepjes van het vak hebben geleerd. Jaap, dank voor al je adviezen, kritische blik en steun in het proefdieronderzoek. Daarnaast Rinse. Ik heb vrijwel alles wat ik hier in het lab weet van jou geleerd. Ik ben je daarvoor, en voor onze fijne persoonlijke samenwerking, erg dankbaar.

I had the incredible opportunity of studying abroad twice. First I would like to thank Josep, Myrna and Rita for welcoming me in their labs in Philadelphia, for being my American family, taking care of me when I was sick and for teaching me so much about autoimmunity and performing research. The second trip was to Steve in New York. Thank you and Ruth for all your advice, the awesome Skirball apartment and enabling any idea I had to be translated into practise.

Ik heb het geluk gehad te mogen samenwerken met drie enorm goede analisten. Kirsten, dank voor je hulp! Zelfs toen ik aan de andere kant van de oceaan zat was je nooit te beroerd om me met of aan data te helpen. Yvonne, wat is het fijn dat we jou in ons team hebben. Je bent ongelofelijk hardwerkend en aanstekelijk enthousiast. Bedankt voor de steun zowel wetenschappelijk als privé. Daarnaast Inge. Dank voor je bereidheid om altijd te helpen, jouw organisatietalent en mooie data en/of snoepjes om me door het 4 uur moment heen te helpen.

Het prettige aan de afdeling Humane Genetica is het feit dat je vrijwel iedereen aan kan spreken voor hulp, antilichamen, apparaten of advies. Daarom eerst een groot DANK! Voor de hele afdeling en iedereen die me feedback of hulp heeft gegeven.

Daarnaast natuurlijk mijn directe collegae. Aan de medische kant: Ellen, Erik, Maarten en Sander. Elke keer weer leer ik van jullie door de klinische kant te verweven met onze wetenschappelijke kennis. Bedankt ook voor de leuke congressen en de onbeperkte supply aan patiënteninformatie. Ingrid, hartelijk dank voor jouw steun bij het verkrijgen en opslaan van patiëntensera en materialen en in het bijzonder jouw flexibiliteit bij de "last minute" verzoekjes.

Dan mijn directe collega's. Lief en leed heb ik met velen van jullie gedeeld, waarvoor dank. In de wetenschap zijn de tegenslagen vaak omvangrijker dan de meevallers, maar wat je wel met elkaar in de hand hebt is of je die tegenslagen met plezier tegemoet treed. Mijn kamergenootjes, de FSHD collegae, de oude proteomics groep en in het bijzonder Amanda M, Amanda P, Bianca, Klaus (Maarten), Laure, Lucia, Marlinde, Thomas, en Yvonne K hebben dit voor mij zeker bewerkstelligd. Daarnaast uiteraard een groot dank voor de mooie (praktische) grappen, de jaarlijkse culinaire experimenten en bovenal de mentale steun, Peter.

In de afgelopen jaren zijn er ook een aantal studenten de revue gepasseerd. Ik wil graag Mar, Sophie, Pauline, Natasa, Marieke, Anna-Fleur, Hanna, Robert, Ruben en Rory danken voor jullie vertrouwen in mij en al jullie hulp. Lesgeven is mijns inziens de beste manier van leren en ik heb er met jullie erg van genoten.

Promoveren is denk ik grotendeels een kwestie van uithoudingsvermogen. Een aantal van mijn lieve vrienden hebben extra bijgedragen aan het vergroten hiervan. Graag dank ik mijn studievrienden Leo, Ils, Diahann, Kay, Mich, Marieke en Anneliene, mijn VAO/LAP friends, de frituurclub, mijn fantastische vriendinnen en oudhuisgenootjes Caro en Lisan voor de foute maar ontzettend gezellige avonden die mij er vaak doorheen gesleept hebben. En dan mijn vrienden vanuit de oudheid: Luuk, Bart, Myrthe en de kids, Putri (ook voor de prachtige kaft), Ana en Pauline, Tamara en Claudje. Op afstand, maar niet minder in mijn hart en bereid om me te steunen en op te beuren met gezelligheid! En natuurlijk Smirt, niet voor niets mijn paranimf. Wat heerlijk om de ins en outs van het promoveren samen te delen i.c.m. heel veel fun en fijne gesprekken. Bedankt voor alle jaren ongecompliceerde steun!

Ook mijn familie wil ik danken voor hun voortdurende interesse, de kaartjes overzee en het geduld om op deze ceremonie te wachten. In het bijzonder wil ik ook mijn lieve oma danken, voor haar wijze woorden, koude handen en warme hart die me vaak hebben opgevrolijkt!

Tot slot de mensen die er altijd voor mij waren en hopelijk er altijd zullen zijn. Mama en pappa, dank voor jullie vertrouwen en support. Dat ik altijd op stimulatie kon rekenen en af en toe een kritische vraag. Jullie hebben mij altijd hard werken geleerd in combinatie met goede intenties, zie hier het resultaat! En mijn lieve zussen Sanne, Dorien en Leonie. Het is fantastisch dat we al zo vele jaren succes, plezier, lief en leed met elkaar kunnen delen. Bedankt voor de steun en het luisterend oor. Dit boekje is mede mogelijk gemaakt door jullie ☺.

En dan tot slot mijn lieve vriend Paul. Hoe onwaarschijnlijk was onze ontmoeting en hoe onwaarschijnlijk fijn hebben wij het samen. Dank voor je steun, regelmatige champagne dosissen en jouw (flauwe) grappen die me het licht in de soms donkere tijden geven. Het is een eer en een feest om met jou aan mijn zijde te mogen promoveren en te leven.

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