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The pathophysiology of MuSK myasthenia gravis

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Citation
Huijbers, M. G. M. (2016, July 6). *The pathophysiology of MuSK myasthenia gravis*. Retrieved from https://hdl.handle.net/1887/41483

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Note: To cite this publication please use the final published version (if applicable).

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Title: The pathophysiology of MuSK myasthenia gravis
Issue Date: 2016-07-06

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.

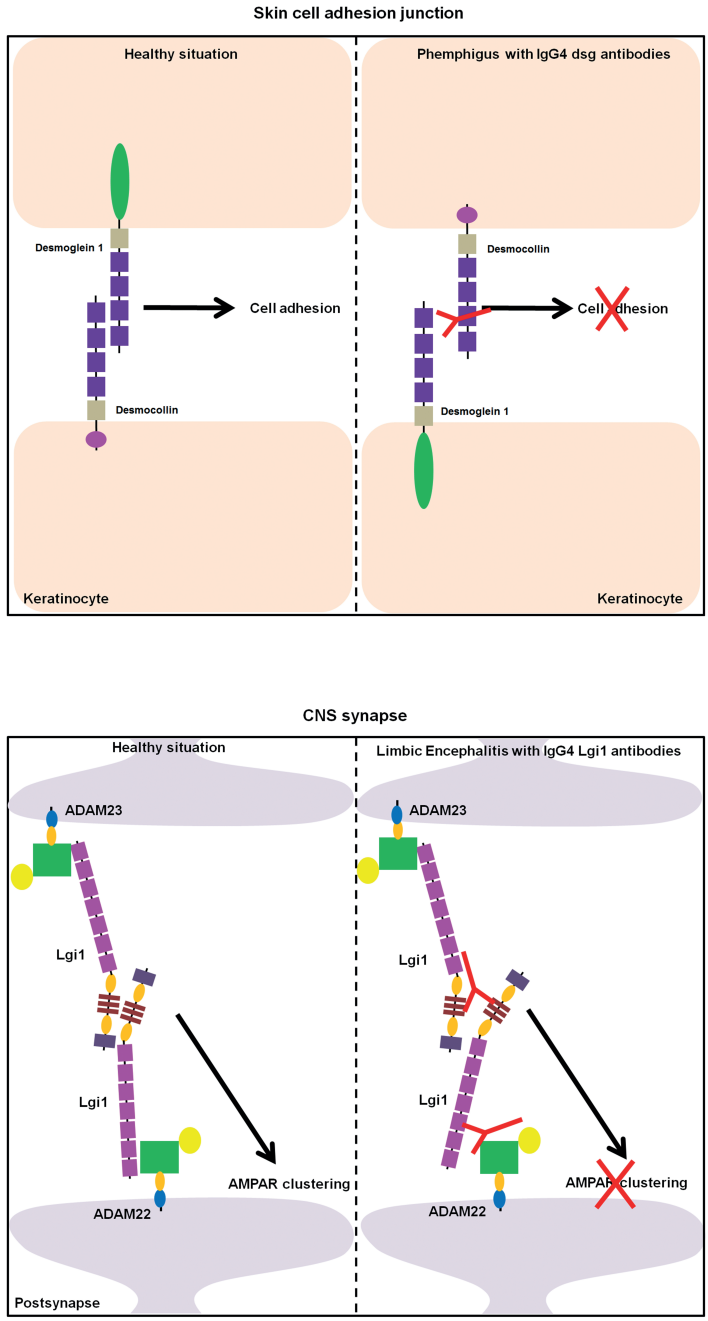
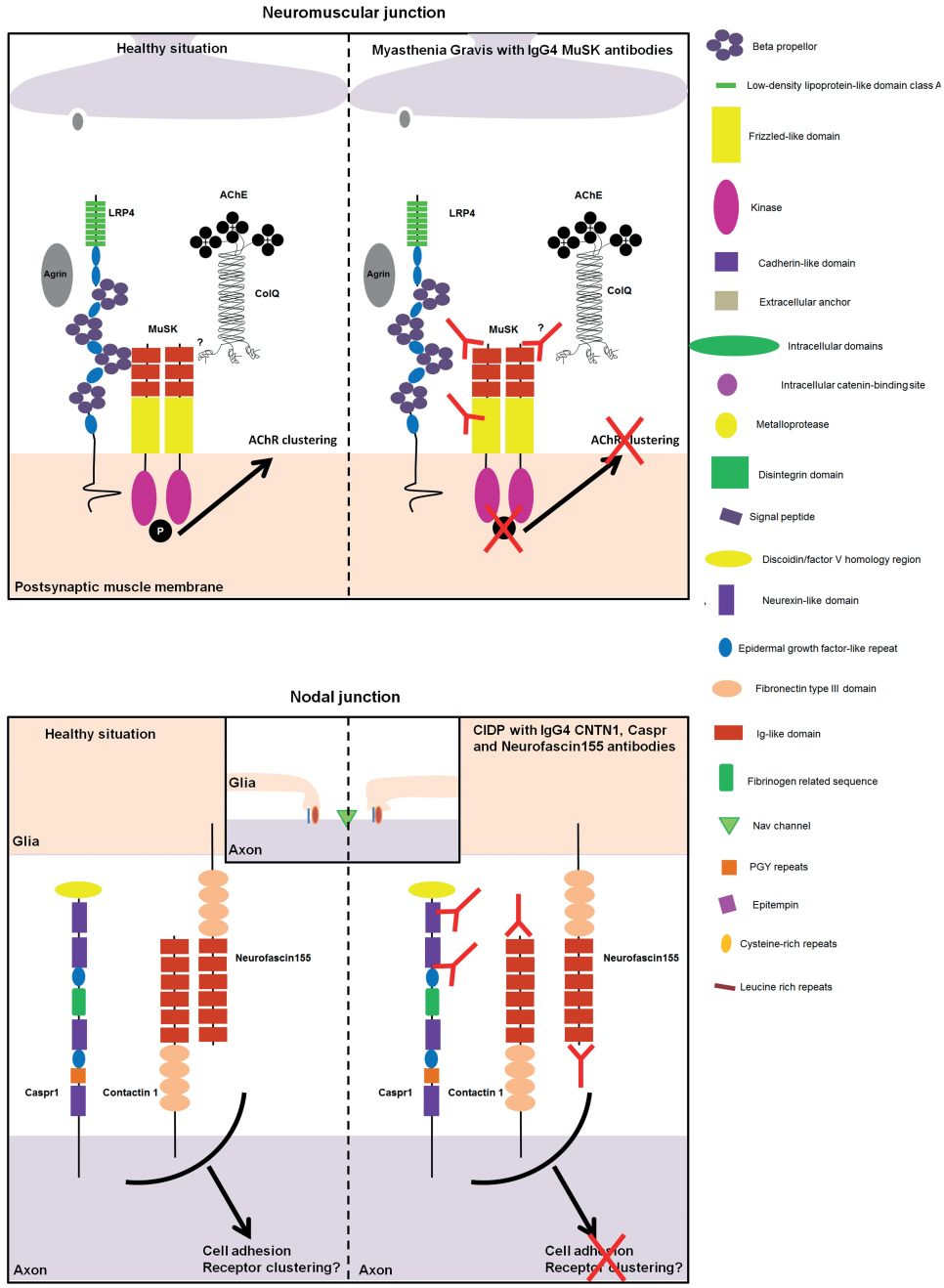


Figure 1: Schematic representation of the cellular locations, protein interactions and auto-antibody effects in the healthy and diseased situation, related to four IgG4-mediated autoimmune diseases. Specific protein domains are indicated in the legend. An important similarity between



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

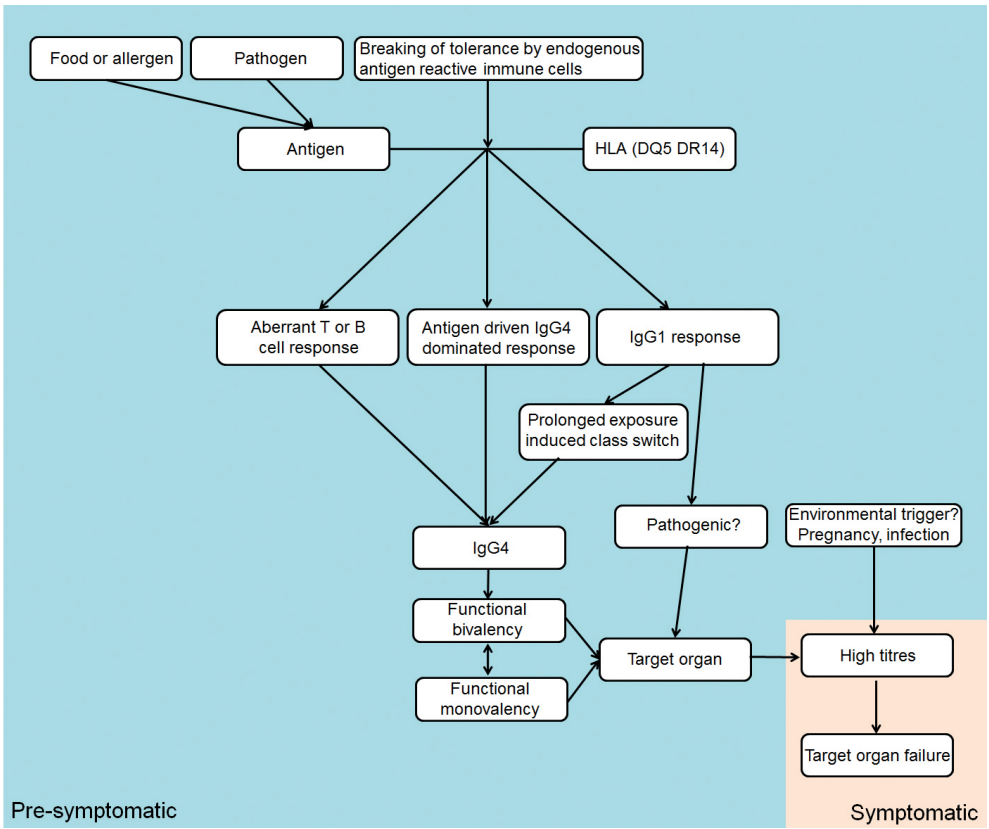


Figure 2. A unifying hypothesis for IgG4 autoimmunity. Several stages during the development of an antibody response could be causative for the formation of pathogenic IgG4 auto-antibodies. First, due to failure in negative selection in the thymus tolerance could be broken by endogenous antigen reactive 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.

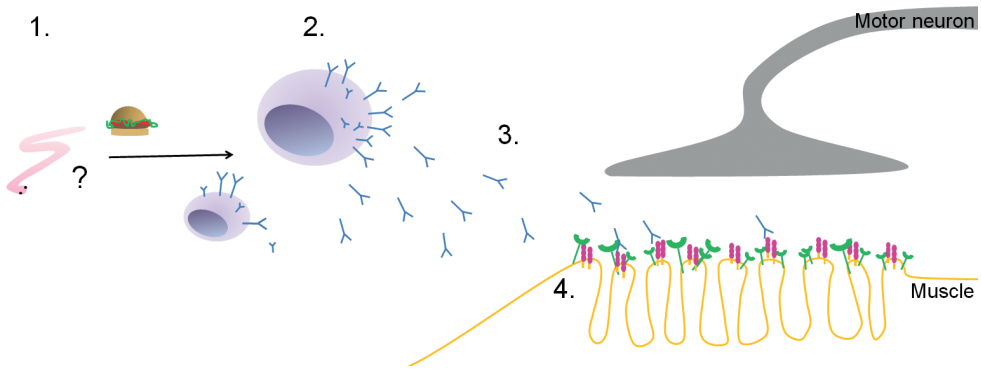


Figure 3: Overview of the different stages during MuSK autoimmunity that could form a target for disease specific treatment strategies. Autoimmunity is caused by an initial trigger (1). Upon removal of this trigger the incentive for the IgG4 immune response might be removed thus dissolving the (cross-reacting) 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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