The two faces of MuSK antibody pathogenicity and their cause and consequences in myasthenia gravis

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General Discussion

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Muscle-specific kinase (MuSK) myasthenia gravis (MG) is an autoimmune disease (AID) where the neuromuscular junction (NMJ) protein MuSK is targeted by antibodies. MuSK is a key regulator of establishing and actively maintaining skeletal muscle NMJs [1]. Disruption of its function by antibodies impairs neurotransmission, resulting in fatigable muscle weakness [2, 3]. MuSK MG is part of the expanding group of IgG4-AIDs, because the majority of MuSK antibodies in these patients are IgG4 and these are sufficient to cause disease [3-8]. IgG4 is considered anti-inflammatory due to its inability to activate complement and antibody-dependent cytotoxicity [9]. In addition, IgG4 has the unique ability to exchange half-molecules in a stochastic process called Fab-arm exchange [10]. The majority of IgG4 will undergo Fab-arm exchange within one day [11, 12]. Consequently, these antibodies become bispecific and functionally monovalent in antigen-binding capacity, losing their ability to internalize antigens through cross-linking. Why MuSK antibodies are predominantly of the IgG4 subclass and whether that matters for their pathogenicity was still largely unknown. The studies in this thesis aim to elucidate the cause and consequences of MuSK antibody pathogenicity related to antibody subclass and characteristics. Here, we will discuss what may be predisposing factors for developing an IgG4 response in IgG4-AIDs, how antibody valency influences the pathogenicity of IgG4 autoantibodies, what we have learned on the pathophysiology of MuSK MG and how this knowledge is contributing to development of novel treatments for neuromuscular disorders. In this discussion, the term "monovalent antibodies" will be used for bispecific antibodies with functionally monovalent antigen-binding capacity and "bivalent antibodies" for monospecific antibodies with functionally bivalent antigen-binding capacity.

Drivers of the IgG4 response in IgG4-AIDs

The dominance of IgG4 in autoimmune diseases is rather surprising given the anti-inflammatory characteristics of IgG4 and its relative low abundance in serum. Several hypotheses may explain the onset of an IgG4 response in autoimmune disease. We used MuSK MG as a model IgG4-AID and hypothesized that MuSK MG patients may have a general predisposition to generate IgG4 responses. Therefore, in **chapter 2** we investigated if these patients have generally elevated serum IgG4 levels. MuSK MG patients (not under recent immunosuppression) have higher serum IgG4 compared to healthy donors and acetylcholine receptor (AChR) MG patients, but for most patients the IgG4 levels fall within

the normal range. Serum IgG4 is also enriched in pemphigus patients, another IgG4-AID [13]. In both diseases, the higher IgG4 levels are partly due to autoantigen-specific IgG4 antibodies. Since the IgG4 levels in these patients generally fall within the range of normal, it is unlikely they have a predisposition to develop IgG4 responses to many antigens that also drives the IgG4 response against the autoantigen. Rather, the increase in serum IgG4 levels may be a bystander effect of the cytokines and other immune signaling factors involved in the class-switch to IgG4 of the autoantigen-specific B-cells, such as IL-10 [9]. In support of this, (trends for) elevated IL-10 levels have been found in MuSK MG, and also in the IgG4-AIDs pemphigus and thrombotic thrombocytopenic purpura [14-17]. In conclusion, other factors likely contribute to the development of IgG4 responses in IgG4-AIDs, such as genetic variants in human leukocyte antigen (HLA) genes, antigen characteristics and environmental triggers.

HLA

Many autoimmune diseases have been associated with specific HLA class II genetic variants, suggesting a role for these genes in their etiology. HLA class II genes encode the major histocompatibility complex (MHC) II responsible for antigen-presentation to T-helper cells by immune antigen-presenting cells such as B-cells and dendritic cells. A recent meta-analysis including all studies investigating HLA class II associations in known IgG4-AIDs found an increased frequency of HLA-DQB1*05, HLA-DRB1*14 alleles and the HLA-DQB1*05-HLA-DRB1*14 combined haplotype in the IgG4-AIDs combined [18]. For example, the HLA-DQB1*05 allele occurs approximately 2-3 times more often and the HLA-DRB1*14 allele 3-4 times more often in patients with pemphigus or MuSK MG compared to controls [18]. Increased HLA-DQB1*05 has not been found in non-IgG4 autoimmunity and may thus confer a specific genetic predisposition for developing IgG4 autoantibodies [18]. In addition, HLA-DRB1*14 was associated with higher anti-MuSK IgG4 titers and IL-10 in the serum of MuSK MG patients [19]. HLA genes may thus influence the development of an IgG4 response, for example through structural properties which allow a good fit for IgG4-inducing peptides, or through regulation of T-cell response facilitating IgG4 responses [20]. Taken together, from the frequency of certain HLA class II haplotypes in IgG4-AIDs, it is clear that proper antigen presentation is one of the factors permitting the development of an IgG4 (autoimmune) response.

Antigen characteristics

Class-switch to IgG4 does not occur in response to all antigens. Rather IgG4 responses have been linked to specific types of antigens associated

with food, therapeutic proteins, autoantigens and helminth infections [9]. Furthermore, the IgG4 response often only develops upon prolonged exposure to these antigens and is thought to be driven by cytokines related to a Th2 response [9]. Active immunization with MuSK in mice results in mouse IgG1 (mouse equivalent of IgG4) antibodies against MuSK with an increase in Th2 cytokines, suggesting the autoantigen itself may drive the Th2-like response [21]. Extracellular autoantigens are generally exposed and accessible to antibodies. After initiation of an autoantibody response this might contribute to prolonged chronic exposure to the immune system. However, not all (partly) extracellular autoantigens causing antibody-mediated autoimmunity induce IgG4 responses. For example, the AChR also has a prominent extracellular part, but AChR MG is mainly mediated by IgG1 AChR antibodies. Interestingly, the autoantigens in IgG4-AIDs are all soluble or single-pass membrane proteins [7,8]. In contrast, the antigens targeted in autoimmune diseases with a striking IgG1/3 dominance (e.g., AChR MG, neuromyelitis optica or systemic lupus erythematosus) are receptors spanning the membrane multiple times or located in the nucleus. This suggests specific characteristics related to such proteins may be needed in combination with chronic exposure and permissive HLA variants. Such characteristics may be of biochemical nature e.g., charge, hydrophobicity or functional groups. Similar biochemical properties may be the result from different combinations of post-translational modifications, amino acid sequences or tertiary structures. Commonalities may thus not lie in specific sequences or modifications, but in the combined properties they have. Therefore, it will be interesting to compare the different IgG4 autoantigens from a high-level structural biochemical perspective to identify commonalities. Alternatively, it may be the way the immune system is exposed to these types of antigens, for example mostly in the absence of infection or damage that may ultimately lead to an anti-inflammatory IgG4 response [9]. Taken together, how antigen characteristics and the immune response against them interact and influence each other may be an important factor contributing to IgG4 responses in AIDs.

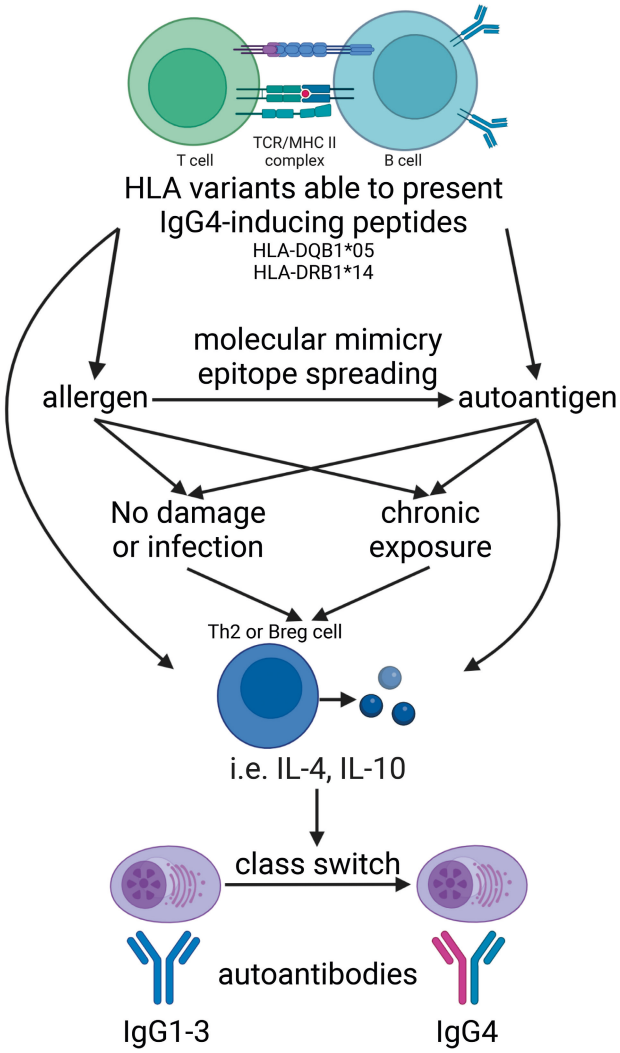

Figure 1. Factors contributing to the IgG4 response in IgG4-AID. The class-switch to IgG4 autoantibodies in IgG4-AIDs likely requires a combination of patient and antigen-dependent stimulating and permissive factors. The structure of the MHC class II complex is determined by genetic HLA variants and needs to match the (auto)antigen peptide. This can either be an environmental antigen such as allergens, which through molecular mimicry or epitope spreading can lead to an antibody response against the autoantigen, or the autoantigen itself. Chronic exposure and a no-damage or infection environment combined with (yet unknown) characteristics of the (auto)antigen and the HLA variants can stimulate cytokines secretion (such as IL-4 or IL-10) that induce the class switch to IgG4 autoantibodies. Created with BioRender.com.

Environmental triggers

Sometimes environmental antigens trigger an autoimmune reaction through molecular mimicry or epitope spreading. Sand-fly saliva or walnut antigens have been identified as potential environmental antigens triggering different forms of pemphigus, which is mediated by antibodies against skin proteins desmoglein (dsg) 1 or 3 [22, 23]. Both environmental antigens are allergens, fitting with other known IgG4-inducing antigens. Healthy individuals in regions with endemic forms of pemphigus were found to have (IgG1) antibodies against non-blocking epitopes of dsg-1 and cross-reactive with sandfly antigens, while pemphigus patients in these regions have high levels of IgG4 antibodies against blocking epitopes of dsg-1 [22, 24]. Molecular mimicry and epitope spreading both between the environmental and autoantigen, and within the autoantigens appears to be involved in the evolution to pathogenic dsg-1 or -3 antibodies [22]. Taken together, this suggests the environmental antigen may be the initial trigger of the autoantibodies, but symptoms will only develop upon epitope spreading to blocking epitopes driven by the autoantigen. It will be exciting to investigate whether for other IgG4-AIDs cross-reactive allergens can be identified. This would suggest that IgG4-inducing environmental factors may play a critical role in the etiology of IgG4-AIDs.

In sum, the IgG4 response in IgG4-AIDs is likely driven by a combination of patient, environmental and antigen-dependent characteristics. Since IgG4-AIDs are rare, a multiple-hit model for the etiology of the IgG4 response in IgG4-AIDs can be hypothesized. This model may include a permissive HLA class II to properly present an (environmental) antigen with IgG4-inducing properties. Antibodies against the environmental antigen may cross-react with an autoantigen or epitope spreading will generate auto-reactive antibodies. Continuous exposure to the autoantigen may further shape an autoimmune response dominated by IgG4. Some of the critical steps currently hypothesized to contribute to the development of an IgG4 autoimmune response are summarized in Figure 1. It is unlikely these are the only factors driving the IgG4 (autoimmune) response, because not all individuals carrying these HLA variants develop IgG4-AID after exposure to specific environmental antigens. It is thus expected the model will become more complex in the future.

Role of antibody valency in MuSK MG

Several observations from the clinic suggest MuSK antibodies of the IgG4 subclass are critical contributors to disease: 1. IgG4, but not IgG1,

MuSK antibody titers correlate with disease severity in MuSK MG patients [25, 2]. In one MuSK MG patient clinical remission was associated with a switch of serum MuSK antibodies from predominantly IgG4 to IgG1 [25, 3]. Symptomatic MuSK MG patients without anti-MuSK IgG4 have not been described until date. In addition, passive transfer of MuSK MG patient IgG4 caused muscle weakness in mice, while IgG1-3 fractions of the same patients did not [3]. Monovalent antigen binding is unique to IgG4, and it has been estimated that up to 99% of anti-MuSK IgG4 in the serum is monovalent [26]. Functional monovalency of IgG4 MuSK antibodies may thus contribute to their clinical relevance in MuSK MG.

To investigate the role of MuSK antibody valency in MuSK MG, we generated patient-derived monoclonal MuSK antibodies from B-cell receptor sequences (BCR) from individual MuSK-positive memory B-cells (**Chapter 3** [27]). We discovered that monovalent MuSK Fab fragments are antagonists of agrin-induced MuSK activation, while their bivalent equivalents are (partial) agonists. This supports the previously found valency-dependent effects of anti-MuSK rabbit IgG and Fab fragments also apply to MuSK antibodies from patients [28, 29]. Next, we adapted the controlled Fab-arm exchange method to IgG4 to generate pure and stable monovalent MuSK antibodies (**chapter 4** [30-32]). Passive transfer of monovalent MuSK antibodies caused rapid-onset muscle weakness, while their bivalent equivalents did not cause muscle weakness or muscle weakness with later onset and slower progression. Functional monovalency can thus exacerbate the pathogenicity of MuSK antibodies, but bivalent MuSK antibodies can be pathogenic in a clone-dependent manner. Isotype switching to IgG4 and subsequent Fab-arm exchange-induced functional monovalency of IgG4 is thus clinically relevant for the development of MuSK MG.

Depending on valency, MuSK antibodies have opposing effects on MuSK. Dimerization of MuSK upon agrin and Lrp4 binding is crucial for MuSK autophosphorylation, initiating further intracellular signaling. Monovalent MuSK antibodies are thought to block the interaction between MuSK and Lrp4, thereby preventing MuSK dimerization, intracellular signaling, AChR clustering and finally neuromuscular transmission [6, 33, 34]. Since bivalent MuSK antibodies can bind two MuSK molecules simultaneously, it is hypothesized that bivalent binding to MuSK can force dimerization, initiating autophosphorylation of MuSK independent of Lrp4 and thereby maintaining MuSK functioning (**chapter 3** [27]). The fact that dimerization of MuSK is a crucial part of its function is thus likely responsible for the amplified pathogenicity of functionally monovalent MuSK antibodies.

Relevance of antibody valency in other IgG4-AIDs

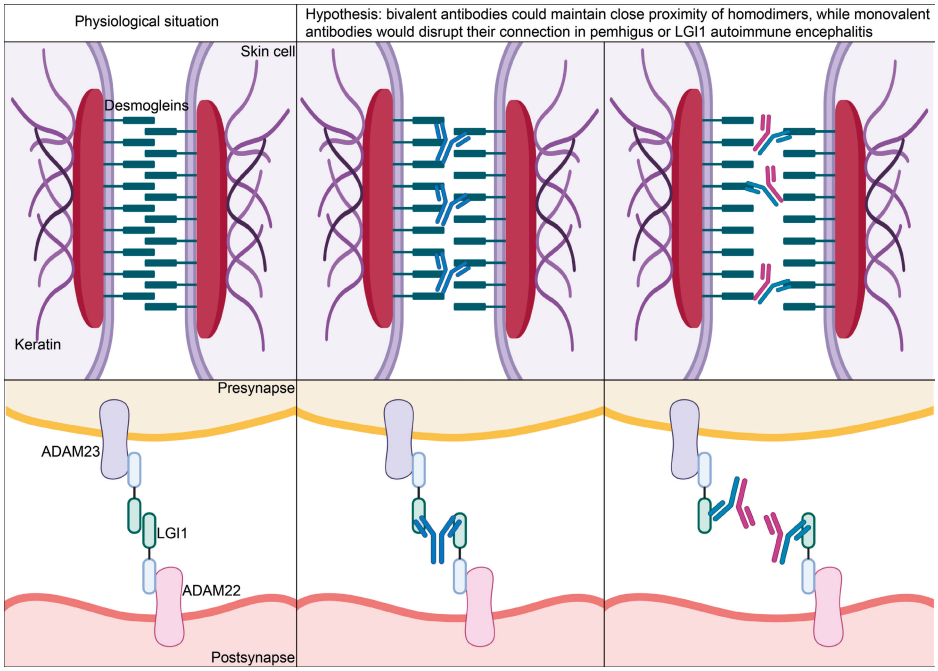

Figure 2: Hypothesis of theoretical valency-dependent effects in IgG4-AIDs where the antigens form a homodimer. Top: in pemphigus homodimers of desmogleins contribute to the cell-cell contact between epithelial cells in the skin. Bottom: in LGI1 autoimmune encephalitis homodimers of LGI1 connect ADAM23 at the pre-synapse to ADAM22 at the post-synapse. Bivalent antibody binding might maintain the close proximity of the homodimers, while monovalent antibody binding might completely disrupt the interaction. Created with BioRender.com.

Functional monovalency of IgG4 autoantibodies may also amplify pathogenicity for other IgG4-AIDs if dimerization of the antigen is part of its normal physiological function like in MuSK MG. In most IgG4-AIDs, the IgG4 autoantibodies block the interaction between two different proteins, making valency-dependent effects unlikely [7, 8]. However, in pemphigus, dsg-1 and dsg-3 can make homodimers between skin cells to ensure skin integrity. In leucine-rich, glioma inactivated 1 (LGI1) autoimmune encephalitis, LGI1 homodimers connect ADAM23 and ADAM22 on the pre- and post-synapse respectively. Dsg-1, dsg-3 and LGI1 make homodimers between cells in a trans-manner, in contrast to MuSK in which both MuSK molecules are in the same cellular membrane. Still, one could hypothesize that a bivalent antibody against these antigens maintains the close proximity of the antigens by binding both molecules, while monovalent antibodies would just interfere with the interaction

(Figure 2). If bivalent antibodies maintain the function of the antigen sufficiently, one expects valency-dependent effect on pathogenicity.

In an endemic form of pemphigus, healthy individuals and pemphigus patients in remission can have IgG1 dsg-1 antibodies, while patients with active disease have very high anti-dsg-1 IgG4 titers, supporting the important role of IgG4 in pemphigus similar to MuSK MG [24]. Both monovalent and bivalent dsg-1 or -3 antibodies can cause skin lesions in mice or *in vitro* human models [35-39]. Fabs from patient-purified anti-dsg-1 IgG caused skin lesions much quicker than the undigested patient-purified IgG, suggesting valency-dependent effects [40]. However, this was not the case for Fabs of patient-purified anti-dsg-3 IgG [41]. Most autoantibodies in patient-purified IgG may already be Fab-arm exchanged monovalent IgG4, possibly explaining a lack of effect upon digestion into Fab fragments. Furthermore, not all bivalent dsg-1 or -3 antibodies may be able to maintain sufficient dimerization, depending on the epitope recognized. Differences in epitopes targeted between patients may also explain differences in valency-dependent effects between patient-derived antibody preparations. So far, the evidence for valency-dependent effects in pemphigus thus remains inconclusive. To determine if functional monovalency exacerbates pathogenicity of autoantibodies in pemphigus, studies using a panel of pure (monoclonal) bivalent or monovalent dsg-1 or -3 antibodies side by side would be needed.

Two important functional domains of LGI1 are the leucine-rich repeat (LRR) domain and the epitempin-repeat (EPTP) domain. Two LGI1 molecules interact through the LRR domain, while LGI1 interacts with ADAM22 or 23 through the EPTP domain. LGI1 antibodies binding the EPTP domain block the interaction between two different proteins (LGI1 and ADAM22 or 23) [42]. Amplified pathogenicity by monovalency is thus not expected for anti-EPTP domain antibodies. Both bivalent monoclonal LGI1 antibodies and monovalent Fabs binding the LRR domain internalize the LGI1-ADAM22 complex in HEK293 cells, which is recapitulated by patient-purified IgG4 and to a lesser extent patient-purified IgG1-3 [42]. In addition, passive transfer of bivalent mAbs and patient purified IgG both caused impairments in the hippocampus, indicating both monovalent and bivalent LGI1 antibodies are pathogenic *in vivo* [42-44]. The pathogenic effects of LGI1 antibodies binding the LRR domain thus also do not seem to be amplified by functional monovalency.

Taken together, until now the functional monovalency exacerbating pathogenicity of IgG4 autoantibodies is rather unique to MuSK MG and perhaps pemphigus, but an unlikely pathogenic mechanism in most IgG4-AIDs. Importantly, functional monovalency of anti-neurofascin 155 (anti-NF155) IgG4 even reduced their pathogenicity in chronic inflammatory demyelinating polyneuropathy (CIDP), because cross-linking is an important part of antibody pathogenicity in this disease [45]. This suggests the valency-dependent effects depend both on the disease-specific pathogenic mechanism and the function of the antigen.

The study on valency-dependent effects in CIDP furthermore shows significant individual variation in the percentage of bivalent anti-NF155 IgG4 among all IgG4 NF155 antibodies [45]. Between 10 and 20% bivalent anti-NF155 IgG4 was common among these patients and the patient with the highest anti-NF155 titers even had 78% bivalent anti-NF155 IgG4. Bivalent anti-MuSK IgG4s were also detected in MuSK MG patients, but the method did not allow for the quantification of the proportion of total anti-MuSK IgG4 [26]. It thus appears that although the majority of IgG4 undergoes Fab-arm exchange, a clinically relevant amount of bivalent IgG4 autoantibodies may be present. The relative amounts of bivalent and monovalent IgG4 autoantibodies may vary between individuals due to differences in the reducing environment permitting Fab-arm exchange, or the polyclonality of the total IgG4 pool. One can imagine that if a large proportion of total IgG4 are the IgG4 autoantibodies, Fab-arm exchange between IgG4 autoantibodies targeting the same or different epitopes on one autoantigen becomes more likely. The resulting "bispecific" IgG4 autoantibody may then still be able to bind the autoantigen in a functionally bivalent manner after Fab-arm exchange. For IgG4-AIDs with valency-dependent pathogenicity, the net pathogenic effect of IgG4 autoantibodies will thus depend on the complex mixture and relative amounts of functionally monovalent and bivalent autoantibodies present in the patient.

The clinical relevance of the IgG4 subclass in IgG4-AIDs may be mediated by other factors associated with the class-switch to IgG4, such as higher titers and high affinity antibodies due to further affinity maturation [9]. These processes occur in IgG4-AIDs: in MuSK MG, pemphigus and LGI1 autoimmune encephalitis, the IgG4 autoantibody titers are much higher than the IgG1 autoantibody titers, and the BCR repertoire and patient-derived isolated clones show a large amount of mutations suggestive of affinity maturation (**chapter 3**, [4-6, 24, 25, 27, 42, 46, 47]). Furthermore, *in vitro* pathogenicity of monovalent MuSK antibodies depended on affinity

maturation due to monovalent binding. The pathogenic bivalent anti-MuSK clone in **chapter 4** also had a higher affinity than the non-pathogenic clone (unpublished observation), suggesting that high affinity might also contribute to the *in vivo* pathogenicity of bivalent MuSK antibodies. The class-switch to IgG4 may thus drive symptom manifestation in most IgG4-AIDs by inducing higher titers of high affinity autoantibodies, but in MuSK MG (and potentially pemphigus) Fab-arm exchange induced monovalency adds significantly to this pathogenicity.

Mechanisms of MuSK antibody-mediated pathogenicity at the NMJ in MuSK MG

The overview of prior MuSK antibody research in **chapter** 1 highlights the importance of investigating how intrinsic MuSK antibody characteristics influence the consequences they have on the NMJ, to fully understand the mechanisms of disease in MuSK MG. Monoclonal MuSK antibodies are useful tools to link antibody characteristics to consequence. The MuSK clones we isolated from a MuSK MG patient in **chapter 3** bind the Ig-like 1 domain [27]. Another group generated monoclonal MuSK antibodies binding the Ig-like 2 domain of MuSK from MuSK MG patients [48]. In addition, several monoclonal antibodies binding the Fz domain of MuSK were identified in phage-display screens [49-51]. The functional effects of patient-derived monoclonal antibodies on muscles have been tested by others [47, 48, 52] and by us *in vitro* and *in vivo* in this thesis (**chapter 3, 4 & 5** [27, 31]), and are summarized in Table 1.

Table 1: overview of consequences of MuSK antibodies on the NMJ, based on monoclonal MuSK antibodies with differences in pathogenicity, epitope and valency.

	Agrin	Pathogenic MuSK antibodies		Nonpathogenic MuSK antibodies	
Characteristics*					
Functional valency		monovalent	bivalent	monovalent	bivalent
Epitope		Ig-like 1 Ig-like 2[#]	Ig-like 1	Frizzled	Ig-like 1 Frizzled[&]
C2C12 myotubes					
MuSK-P activation	+++	----	++++	=	+++
AChR clustering	+++	----	+	=	+
MuSK internalization	=	=	=	=	=
Dok7-MuSK interaction	+	-	+	=	+
Dok7 protein level	-	=	--		-/--
(NMJ) gene expression	=	=	=		=
NOD/SCID mice					
In vivo Muscle strength		----	---		=
In vivo Muscle fatigability		----	--		=
EMG decrement		--	--		=
Postsynaptic NMJ morphology		--	----		-
Presynaptic NMJ morphology		=	=		=
Safety factor		--	---		-
NMJ gene expression					
Musk		+	+		=
Lrp4		=/-	=		=
Dok7		=/-	=		=
Rapsn		=	=		=
Chrne		---	+		=
Chrng		=	++++		++++
Chrna1		=	++		=
Ache		----	+		=
Colq		----	+		+

Table 1 Continued
*from known and tested monoclonal antibodies
[#] tested on AChR clustering only [47]
[&] tested on MuSK-P activation, AChR clustering, Dok7-MuSK interaction, Dok7 protein levels, *in vivo* muscle fatigability, EMG decrement, postsynaptic pathology (**chapter 5**, [52])

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Scale from decreasing/impairing (-) to increasing/activating (+) relative to healthy physiological processes. No change is denoted with the equal sign (=). The amount of – or + signs is an indication of the order of the relative effect sizes.

Antibody valency

By comparing the consequences of pathogenic bivalent and monovalent MuSK antibodies side by side, extensive differences become apparent. The valency-dependent effects on *in vitro* MuSK activation were discussed earlier. *In vivo*, it took longer for signs of muscle weakness to develop and muscle fatigability was not progressive to complete loss upon the pathogenic bivalent MuSK antibody compared to its monovalent equivalent (**chapter 4** [31]). In addition, bivalent and monovalent MuSK antibodies binding the Ig-like 1 domain of MuSK affected the expression of NMJ genes in the masseter muscle differently. Monovalent MuSK antibodies reduced the gene expression levels of the AChR epsilon subunit (*Chrne*), Collagen Q (*Colq*) and acetylcholine esterase (*Ache*), while the pathogenic bivalent MuSK clone slightly increased the expression of these genes (Table 1, **chapter 5**). In contrast, bivalent MuSK antibodies binding the Ig-like 1 domain increased the expression of the gamma subunit of the AChR (*Chrng*) regardless of pathogenicity. Although pathogenic bivalent and monovalent MuSK antibodies both ultimately lead to failure of neurotransmission, the processes leading up to that appear strikingly different. These different pathomechanisms may also contribute the observed differences in onset, progression and severity of muscle fatigability.

These differential pathogenic mechanisms between monovalent and bivalent MuSK antibodies have consequences for the model systems used to study MuSK MG. Active immunization models of MuSK MG in rodents will only produce bivalent MuSK antibodies. The disease course of the pathogenic bivalent MuSK antibody is more similar to the development of weakness upon the antigen boost in active immunization models of MuSK [21, 53-56]. In addition, the NMJ gene expression changes seen upon active immunization match those seen upon passive transfer of pathogenic

bivalent MuSK antibodies, but not monovalent MuSK antibodies [54]. Active immunization models of MuSK MG thus only recapitulate a part of the disease mechanisms of MuSK MG in patients. Passive transfer of patient-purified IgG4 likely represents the dominant disease mechanism of monovalent MuSK antibodies. However, patient-purified total IgG models contain the entire complex antibody pool present in patients and thus likely have the best translational value as a pre-clinical model for (therapeutic agents targeting) the molecular mechanisms of disease in muscles.

Epitope

So far, only one patient-derived MuSK antibody was found to be pathogenic *in vivo* in bivalent form [31]. What drives the pathogenicity of bivalent MuSK antibodies such as the 13-3B5 clone is still unclear, because the cellular consequences of pathogenic and nonpathogenic bivalent MuSK antibodies investigated so far are quite similar to each other and agrin (Table 1). It can be hypothesized that pathogenicity of bivalent MuSK antibodies may be explained by its specific binding epitope, because that will determine how MuSK is forced to dimerize. The binding-site of pathogenic MuSK antibodies may induce such an unnatural conformation that it precludes the interaction with proteins binding to MuSK both extra- and intracellularly, beyond the antibody-binding site. In line with this, the pathogenic and nonpathogenic bivalent MuSK clones have different binding sites on the Ig-like 1 domain of MuSK, because they do not compete for binding [52]. So different binding sites, even within the same functional domain of MuSK, appear to have different consequences. In **chapter 5**, we found that Dok7, considered one of MuSK's most important downstream interactors, can bind upon forced dimerization by a pathogenic bivalent antibody. However, a growing number of interactors for MuSK is being identified with functions in downstream signaling, scaffolding and gene expression [57]. To give insights into what drives the pathogenicity of bivalent MuSK antibodies, it would thus be very interesting to 1) resolve the conformation of MuSK in complex with different MuSK antibodies, and 2) map MuSK's extracellular and intracellular interaction partners upon bivalent antibody binding.

Complement

The mechanisms of disease in MuSK MG investigated in this thesis are all independent of immune system interactions, such as with the complement system. Complement is not considered to play a large role in MuSK MG due to the predominance of IgG4 autoantibodies. However, co-occurring MuSK IgG1-3 antibodies may in theory bind complement.

Bivalent MuSK IgG1-3 antibodies, that are non-pathogenic in the absence of complement, might thus cause damage if complement can bind. In addition, IgG4 can activate the lectin complement pathway in a glycosylation-dependent manner in another IgG4-AID [58]. Although complement is certainly not required to develop MuSK MG, it may have a disease modifying effect for a subset of autoantibodies. So far, there is no support for complement activation in muscles of MuSK MG patients [59]. However, it is important to realize that complement deposition has been investigated on very limited human muscles. Serum analyses do suggest an active alternative complement pathway in MuSK MG patients [60]. To better understand if complement activation has any relevance in MuSK MG, it will be interesting to investigate whether MuSK IgG4 antibodies have glycosylation patterns compatible with the lectin pathway and if bivalent MuSK IgG1 antibodies can activate complement on muscles [61].

From monoclonal back to polyclonal

In this thesis, monoclonal antibodies were used to interrogate the mechanisms of disease in MuSK MG. However, MuSK MG patients have a pool of MuSK antibodies with different characteristics. Most patients have MuSK antibodies predominantly of the IgG4 subclass (mostly monovalent) and targeting the Ig-like 1 domain of MuSK, but co-occurring MuSK antibodies of other IgG subclasses (bivalent) and targeting other domains of MuSK are regularly found [4-6, 62]. In patients, different mechanisms thus likely contribute to their disease. Passive transfer of MuSK MG patient-purified IgG(4) induces *in vivo* muscle weakness similar to monovalent MuSK antibodies. This supports the idea that binding of monovalent IgG4 MuSK antibodies to MuSK is the most relevant pathological mechanism in patients with MuSK MG [3, 63-67]. However, in some patients purified total IgG can activate MuSK phosphorylation or induce some AChR clustering *in vitro*, suggesting bivalent MuSK antibodies can play a role [68, 69]. The unique composition and relative abundance of MuSK antibodies with different characteristics will thus determine the net effect and pathogenic mechanisms in an individual patient.

To understand the complexity of the anti-MuSK response, it will be interesting to decompose the polyclonal MuSK antibody pool in individual patients to determine if it consists of a few dominant clones or many different clones. Monoclonal MuSK antibodies can then be used to investigate how the effects of different clones influence each other. Co-occurring pathogenic bivalent MuSK antibodies may contribute to the disease pathophysiology of monovalent MuSK antibodies, while nonpathogenic bivalent MuSK antibodies may counteract pathogenic

effects of both monovalent and bivalent MuSK antibodies. In addition, it can be hypothesized that multiple antibodies binding to MuSK simultaneously may work synergistically to disrupt more processes due to steric hindrance or conformational changes. In line with this, some polyclonal preparations of bivalent MuSK antibodies could completely inhibit AChR clustering, while this was generally not the case for monoclonal bivalent MuSK antibodies [6, 27-29, 31, 47-49]. Although monoclonal antibodies are a great tool to systematically investigate the consequence of specific antibody characteristics, we ultimately need to combine them again to generate a more complex model based on multiple MuSK antibody characteristics. Such a model may be of better predictive value for disease severity and therapeutic response and thereby benefit clinical management.

Therapeutic potential of the MuSK signaling pathway

Since nonpathogenic bivalent (agonistic) MuSK antibodies have the opposite effect of monovalent (antagonistic) MuSK antibodies, it is tempting to speculate they can also have therapeutic potential for MuSK MG by counteracting the effect of pathogenic (monovalent) MuSK antibodies. Agonistic MuSK antibodies against the Ig-like 1 domain of MuSK can partly rescue *in vitro* AChR clustering of monovalent MuSK antibodies and MuSK MG patient-purified IgG, suggesting MuSK agonists can overcome antibody-induced MuSK inhibition [52]. So far, the *in vitro* rescue by MuSK agonists binding the Ig-like 1 domain of MuSK was not able to prevent *in vivo* muscle weakness upon passive transfer of polyclonal MuSK MG patient-purified IgG4. In addition, some male Bl6 mice developed a lethal urologic syndrome. This phenomenon did not occur in NOD/SCID mice and therefore appears mouse strain-specific and may not translate to humans. Nevertheless, it complicates further pre-clinical development of monoclonal antibodies targeting the Ig-like 1 domain of MuSK as a therapeutic [52].

To understand or even anticipate unexpected (detrimental) side-effects of new therapeutics, it is important to know where the target is expressed. In **chapter 6**, we investigated the spatio-temporal expression pattern of eight "MG-related genes" involved in maintaining neurotransmission, which cause (congenital) myasthenia gravis when impaired by autoantibodies or mutations [70]. This revealed that many of these genes are widely expressed in tissues other than skeletal muscle. For example, *DOK7* was prominently expressed in the cerebellum, pituitary gland, testis and heart. The function of these tissues thus warrants monitoring upon

DOK7 targeted therapies. *MUSK* is expressed in the testis, small intestine and bladder. This may guide further studies in light of the urologic syndrome observed in male mice discussed above.

Impaired neurotransmission is not only a problem in MG, but also in neuromuscular disorders such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), muscular dystrophies and sarcopenia. Stimulating AChR clustering through the MuSK signaling pathway may have therapeutic potential to enhance neurotransmission in multiple of these diseases [71]. The MuSK signaling pathway can be activated in different ways. Activating MuSK signaling through *DOK7* gene therapy, Lrp4 overexpression or agrin biologicals has already shown beneficial effects in mouse models for Emery Dreifuss muscular dystrophy, SMA or sarcopenia [72-76]. In addition, activating MuSK directly with agonistic MuSK antibodies binding the Fz domain of MuSK was beneficial in mouse models for ALS and Dok7 congenital myasthenic syndrome, and did not cause unwanted side-effects [50, 51, 77]. Together these studies provide proof-of-concept that stimulating MuSK signaling, e.g. by agonistic MuSK antibodies, has therapeutic potential in neuromuscular disorders.

Concluding remarks

Taken together, the results in this thesis contributed to our understanding of the cause and consequences of (IgG4) MuSK antibodies in MuSK MG. The IgG4 response in MuSK MG is unlikely to be driven by a predisposition to generate IgG4 responses or other abnormalities in the immunoglobulin response. Rather the characteristics of (prolonged exposure to) the autoantigen or an environmental trigger, combined with antigen presentation driven by variations in the HLA genes are likely contributors to the IgG4 autoimmune response. Overlap in for example the HLA associations between different IgG4-AIDs suggest a common underlying mechanism.

The class-switch to IgG4 may be critical for symptom manifestation in MuSK MG because Fab-arm exchange-induced functional monovalency amplifies the pathogenicity of many MuSK antibodies. In addition, an IgG4 response usually consists of high titers of high affinity antibodies. Therefore, an initial IgG1 response against MuSK may not result in clinical MG because of the low titers and their sometimes non-pathogenic agonistic effects. Although the effect of functional monovalency is rather unique to MuSK antibodies, the high titers and affinity of IgG4

autoantibodies may also play a role in symptom manifestation in other IgG4-AIDs.

Monoclonal patient-derived MuSK antibodies have been key in discovering the different consequences contributing to the mechanisms of disease in MuSK MG. We have learned the consequences MuSK antibodies have on the NMJ 1) can range from causing MG by impairing MuSK function or having therapeutic potential by inducing proper MuSK signaling, 2) is determined by how they influence MuSK dimerization and 3) depend on at least a combination of antibody subclass, valency, epitope, affinity, and relative titers. In the future, when we understand the composition of antibody characteristics in individual patients and how the different consequences combine, we can translate the effects of individual MuSK antibodies back to an integrated model of the mechanisms underlying MuSK MG.

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