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The role of epitope specificity in antigen-specific immunotherapy for Myasthenia gravis

by

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Summary

Myasthenia gravis (MG) is an autoimmune disease caused by autoantibodies targeting mainly the muscle nicotinic acetylcholine receptor (nAChR) at the neuromuscular junction (NMJ) of skeletal muscles. Impaired signal transduction due to AChR destruction by the autoantibodies leads to muscle weakness and fatigability. The current treatments for MG are not specific and thus can have side effects. Hence, the development of antigen-specific and tolerance-inducing immunotherapies, targeting only the pathogenic components of the immune system without interfering with its normal function, would be very beneficial for MG patients. Early studies from several groups with experimental autoimmune MG (EAMG) animal models have shown that mucosal administration of disease-relevant peptides could induce tolerance, providing the proof of principle for the therapeutic application of the approach.

Recent studies of the laboratory have achieved improvement of EAMG, when the nAChR hα1 ECD was administered intravenously to EAMG rats. We used a rat EAMG model to investigate the antigen specificity and the contribution of epitope spreading on the therapeutic effect. Our data showed that the administration of an AChR peptide different than the one that the animals are immunized with, does not have a significant therapeutic effect and the epitope spreading phenomenon is not observed between the different subunits of the AChR. On the other hand, administration of a mixture of AChR subunits had a beneficial result irrespective of whether disease was induced by one or more AChR subunits. Although, further investigation of the underlying mechanisms involved is needed, autoantigen-induced tolerization is a promising immunotherapy for MG. Importantly, any findings and advances made for MG could be applied to other related antibody-mediated diseases, increasing the impact of these studies.

Table of Contents

1. Introduction 5
1.1 Myasthenia gravis 5
1.1.1 Characteristics of Myasthenia gravis 5
1.1.2 Epidemiology 5
1.1.3 Clinical features of MG 6
1.2 Myasthenia gravis pathophysiology 7
1.2.1 Normal function of neuromuscular junction (NMJ) 7
1.2.2 Pathology of MG 11
1.2.3 Autoantibodies in MG 12
1.2.3.1 Anti-AChR antibodies 12
1.2.3.2 Anti-MuSK antibodies 14
1.2.3.3 Anti-LRP4 antibodies 16
1.2.4 Other antibody specificities 17
1.2.4.1 Extracellular antigens 17
1.2.4.2 Intracellular antigens 18
1.2.5 The thymus in MG pathogenesis 20
1.3 Diagnosis of Myasthenia gravis 21
1.3.1 Bedside tests 21
1.3.2 Electrophysiological tests 21
1.3.3 Serological tests 22
1.4 Immunopathology in MG 24
1.4.1 Immune tolerance 24
1.4.2 Immunogenetics 24
1.5 Treatment of Myasthenia gravis 25
1.5.1 Symptomatic drug treatment 25
1.5.2 Immunosuppressive drug therapy 25
1.6 Novel therapeutic approaches 28
1.6.1 Experimental Autoimmune MG 28
1.6.2 Tolerance induction as a therapy for MG 30
2. Materials 33
2.1 Laboratory equipment 33
2.2 Consumables 33
2.3 Reagents 34

2.4 Media recipes 35
2.5 Buffer solutions 36
3. Methods 36
3.1 Liquid and solid cultures of yeast *Pichia Pastoris* 36
3.1.1 Protein expression in *P. pastoris* 37
3.1.2 Protein isolation from *P. Pastoris* supernatant 38
3.1.3 Protein purification by metal affinity chromatography with Ni^{+2}-NTA agarose column 39
3.1.4 Protein quantification with Bradford colometric method 39
3.2 Animals and EAMG induction 40
3.3 RIPA for the detection of anti-hα1 ECD antibodies 41
3.4 ELISA for the detection of anti-MuSK and anti-OVA antibodies 42
3.5 Statistical analysis 43
4. Results 44
4.1 Evaluation of antigen specificity 44
4.2 Assessment of the contribution of epitope spreading 50
5. Discussion 55
Bibliography 63

1. Introduction

1.1 Myasthenia gravis

1.1.1 Characteristics of Myasthenia gravis

Myasthenia gravis (MG) is an autoimmune disease, characterized by muscle weakness and fatigability. It is caused by autoantibodies targeting components of the neuromuscular junction (NMJ) of skeletal muscles. This results in damage of the postsynaptic muscle membrane and impaired neuromuscular transition [1]. In the majority of patients (about 85%), the antibodies are directed against the acetylcholine receptor (AChR) in the post-synaptic membrane, whilst in some patients the autoantibodies target the muscle-specific kinase (MuSK) or the low-density lipoprotein-receptor-related protein 4 (LRP4). Interestingly, in about 10% of MG patients, no autoantibodies can be found, so these patients are characterized as seronegative. Probably, in these cases the patients have autoantibodies against an unknown target protein or the available diagnostic test is not sensitive enough [2].

1.1.2 Epidemiology

MG is a rare disease that has a worldwide prevalence of 40-180 per million people and an annual incidence of 4-12 per million people. MG prevalence has increased over the years, mostly due to more efficient diagnosis [3]–[5]. MG before the age of 50 (early-onset MG), shows high predominance in women (60-70%), which is typical for an autoimmune disease. On the other hand, after the age of 50 (late-onset MG) is slightly more frequent in men. Overall, MG prevalence shows geographical variation, with juvenile MG being very rare in Europe and North America, whilst being more frequent in East Asia and representing approximately 50% of MG patients in China [6], [7]. Additionally, neonatal MG (NMG) can be triggered in embryos born from myasthenic mothers, due to transplacental transmission of maternal anti-AChR antibodies. It is observed that the disease disappears in few days

(usually 2 to 3 weeks) and the clinical improvement is associated with reduction of the antibodies. However, only a part of infants are infected despite the transfer of maternal anti-AChR antibodies, with no proved correlation between the severity of the disease in mother and the newborn, even though they are exposed to the same antibodies [7]. Overall, MG in childhood is extremely rare, and almost never occurs before 1 year of age [8].

1.1.3 Clinical features of MG

The main symptom in Myasthenia gravis is fluctuating muscle weakness and fatigability of skeletal muscles. The most commonly affected ones are the extraocular, bulbar, limb and axial muscles. Weakness worsens upon unremitting exercise, and improves after rest. In general, patients can be classified into subgroups, according to the implicated muscles and clinical symptoms (Table 1). Ocular myasthenia is characterized by weakness of the outer ocular muscles and occurs in approximately 85% of patients at the time of disease onset. Ocular weakness is usually presented by fluctuating asymmetric ptosis and binocular diplopia, which results in difficulty of the eye closure and double vision. Generally, patients report discomfort with bright lights and during reading. Approximately, in 15-20% of patients the disease is restricted to ocular myasthenia, whilst in the remaining the disease progresses to generalized myasthenia within 2 years of disease onset. The development of generalized myasthenia includes involvement of different muscle groups in addition to the extraocular ones. Patients with generalized myasthenia may display muscle weakness affecting the axial, limb, bulbar, facial and respiratory muscles. The involvement of bulbar muscles can result in dysarthria, dysphagia and dysphonia, whilst the involvement of facial muscles makes the patient seem expressionless or depressed. Additionally, some patients may face the "dropped head syndrome" and exertional dyspnea, due to weakness of axial and respiratory muscles respectively. Involvement of limb muscles seems to affect more often the arms than the legs. Overall, progress of MG varies, but usually maximum severity is reached within two years of disease onset [9]–[13].

Table 1: MGFA Clinical Classification

Class	Clinical symptoms
I	Any ocular weakness
II	Mild Weakness. May also have ocular muscle weakness of any severity
II A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
II B	Predominantly affecting orорpharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
III	Moderate weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
III A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
III B	Predominantly affecting orорpharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
IV	Severe weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
IV A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
IV B	Predominantly affecting orорpharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
V	Defined by intubation, with or without mechanical ventilation, except when employed during routine postoperative management

Modified from Heldal et al. 2014

1.2 Myasthenia gravis pathophysiology

1.2.1 Normal function of neuromuscular junction (NMJ)

The role of the NMJ is the transmission of the signal from the axon terminals of motor neurons to the muscle, translating the neuron action potential into muscle contraction. This process depends on the release of the chemical transmitter acetylcholine (ACh) by the motor neuron, as well as on the acetylcholine receptors (AChR) on the muscle cell membrane. In order to understand the pathophysiology of MG, it is of a great importance to describe a normal neuromuscular transmission and the anatomy of the NMJ. The NMJ (Fig. 1) consists of three basic components: i) the presynaptic motor nerve terminal, where ACh is synthesized, stored in synaptic vesicles and released, ii) the synaptic cleft, which is the space between the nerve terminal and muscle membrane and iii) the postsynaptic muscle

membrane, which contains the AChRs and the enzyme acetylcholinesterase (AChE), that degrades ACh [14]–[16].

Presynaptic motor nerve terminal

During development when a motor neuron approaches a muscle, it branches and innervates many muscle fibers, providing a single unmyelinated nerve terminal to each of the fibers. The presynaptic motor nerve terminal is sheathed by the Schwann cell, except for the part that faces the postsynaptic membrane. As it was mentioned above, each presynaptic nerve terminal contains a plethora of synaptic vesicles, which store, release and uptake the neurotransmitter acetylcholine. ACh is synthesized in the nerve terminal from acetyl CoA and choline with the presence of the enzyme choline transferase and it is packaged in synaptic vesicles. These vesicles merge with the presynaptic membrane at active zones, where calcium channels are arranged [17]. When an action potential reaches the nerve terminal, presynaptic P/Q type voltage-gated calcium channels open, and calcium enters into the presynaptic terminal. The increase of intracellular Ca^{2+} triggers the fusion of the synaptic vesicle with the presynaptic nerve cell membrane, and the release of vesicle content into the synaptic cleft, a process named exocytosis. This procedure requires conformational changes of several proteins on both vesicle membrane and plasma membrane of the nerve terminal [16]. Before exocytosis, synaptic vesicles undergo a procedure which is called "docking", in which they come into close proximity with the nerve terminal membrane and then undergo priming that allows them to respond to the calcium signal. The "docking complex" contains syntaxin and SNAP25 (plasma membrane proteins) and synaprobrevin (synaptic vesicle membrane) [18]. Since vesicles release their content, their membrane is recycled by a clathrin-mediated mechanism, which translocates them into the interior where they merge with endosomes.

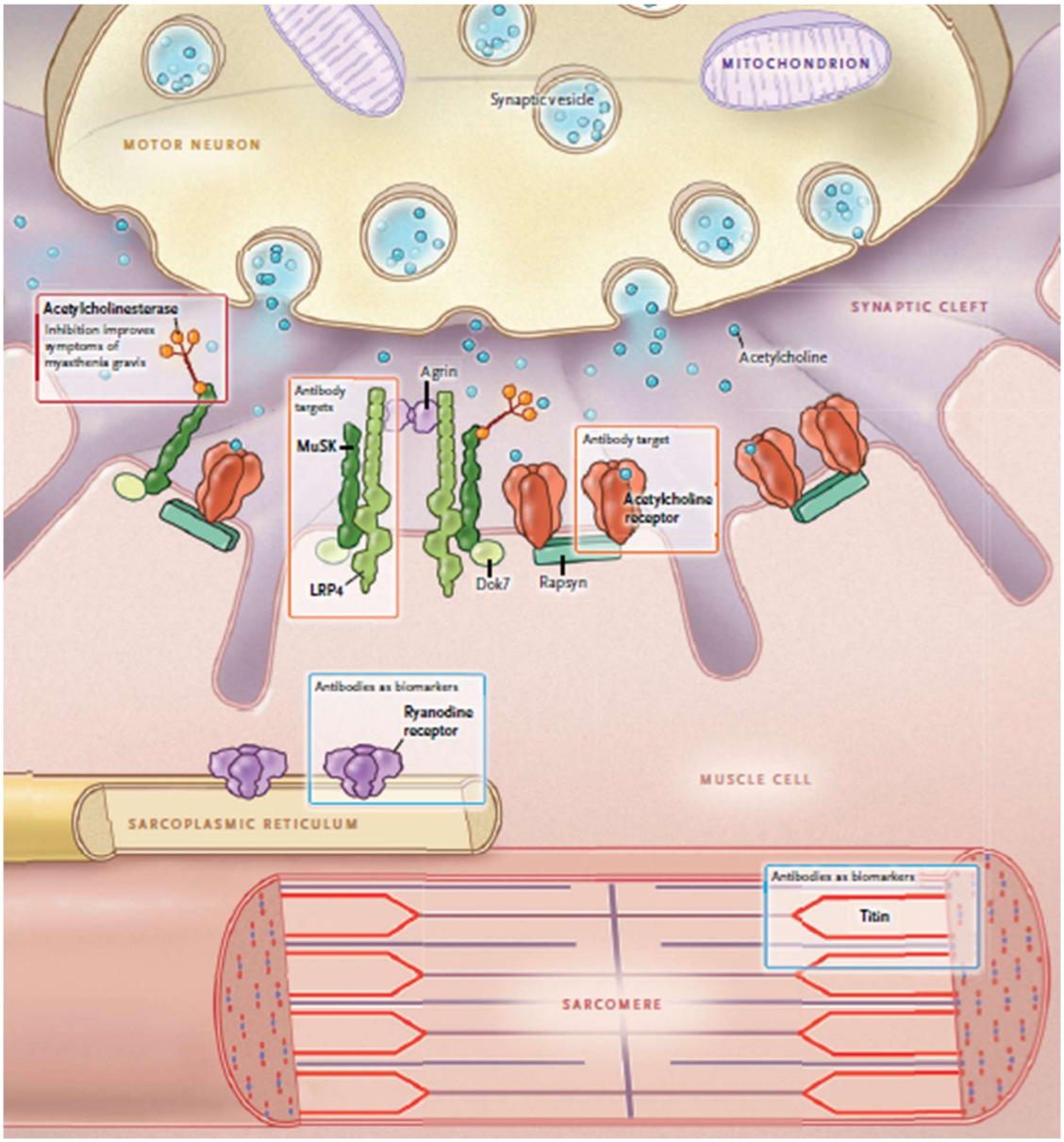


Figure 1. Neuromuscular Junction and Key Elements for the Pathogenesis of Myasthenia Gravis.

Neuromuscular transmission involves release of presynaptic acetylcholine, which binds to acetylcholine receptors in the postsynaptic membrane. The receptors interact with several other proteins in the membrane, including Dok7 and rapsyn. Mutant Dok7 and rapsyn are important in the development of congenital myasthenia. Antibodies against acetylcholine receptors, as well as antibodies against muscle-specific kinase (MuSK) and lipoprotein receptor–related peptide 4 (LRP4), induce myasthenic weakness. Antibodies against the intramuscular proteins titin and ryanodine receptor are relevant biomarkers in some subgroups of myasthenia gravis. Acetylcholine is degraded by local acetylcholinesterase, and acetylcholinesterase inhibition leads to symptomatic improvement in patients with myasthenia gravis.

Adapted from Nils E. Gilhus, the New Engalnd Journal of Medicine, 2016

Synaptic cleft

Once exocytosis takes place, ACh is released rapidly across the synaptic cleft, before it reaches the AChR at the postsynaptic membrane. Synaptic clefts are divided into primary and secondary. The primary cleft is the space of ~70nm between the presynaptic nerve membrane and the postsynaptic muscle membrane and is comprised of basal lamina. Basal lamina is composed of collagen IV, laminin, fibronectin, entractin, perlecan, agrin and plays an important role in NMJ innervation, development and regeneration [19]. Furthermore, AChE, the enzyme that hydrolyses acetylcholine and terminates the transition action, is associated with the basal lamina [20]. The secondary clefts are the spaces between the junctional folds of the postsynaptic membrane and they communicate with the primary cleft.

Postsynaptic muscle membrane

Postsynaptic region is organized in deep infoldings, the secondary synaptic folds or junctional folds, which increase the surface area of postsynaptic membrane. Junctional sarcoplasm contains several organoids such as mitochondria, Golgi apparatus, and intermediate filaments, in order to meet the high metabolic and structural needs of the postsynaptic region. A junctional fold normally has a slender stalk and a terminal expansion, the crest, where the AChRs are anchored [21]. The AChRs are connected to the cytoskeleton elements by α- and β-dystroglycans, which form the core of a larger protein complex that operates to maintain muscle structure and signaling function [22]. Motor nerve terminals organize postsynaptic differentiation by releasing a proteoglycan called agrin, which acts by activating a postsynaptic transmembrane kinase on the myotube surface, the muscle-specific kinase (MuSK). The activation of MuSK leads to clustering of AChRs and other postsynaptic components through association with a cytoplasmic linker protein, rapsyn. Through this process, the AChRs of the postsynaptic muscle protein are clustered and stabilized [23].

Once ACh crosses the synaptic cleft it binds to AChRs that are concentrated on the crests of the postsynaptic membrane, creating a local depolarization potential, which is called endplate potential (EPP). Under resting conditions, AChR are impermeable to Cl^- ions and permeable to Na^{2+}, K^+ ions and to a lesser extent to Ca^{2+} and Mg^{2+} ions. The binding of ACh to its receptor results in opening of the AChR ion channel and the entry of cations, especially Na^{2+}, leading to EPP generation. When a certain threshold depolarization is achieved, voltage-gated sodium channels open, allowing the influx of more Na^{2+} and the efflux of K^+, generating the muscle action potential and contraction [24]. The resting potential of a muscle membrane is -95mV, and when an action potential is triggered, it raises the membrane potential to the threshold of about -50mV. After the generation of an action potential, the efflux of K^+ restores the resting membrane potential within 1-2 mS (refractory period), and during the 3-10mS (latency) period, ACh in the synaptic cleft is hydrolyzed by acetylcholinesterase and sodium channels close [25].

The main abnormalities of the NMJ in MG include i) reduced number of the AChRs, ii) shortening of the synaptic folds due to destruction of the crests, and iii) widening of the synaptic clefts due to the shortening of the junctional folds. These abnormalities come as a consequence of the autoimmune attack on the postsynaptic membrane.

1.2.2 Pathology of MG

Myasthenia gravis is an antibody-mediated disease, caused by autoantibodies targeting NMJ proteins. Antibodies against the nicotinic acetylcholine receptor (nAChR) are detected in approximately 85% of the patients with generalized MG. Approximately 6% of the patients have antibodies against MuSK, whilst antibodies against LRP4 are found in about 2% of the MG patients. Patients without detectable antibodies against any known targets account for about 10% of all MG patients (seronegative MG, SN-MG) [26]–[28].

1.2.3 Autoantibodies in MG

1.2.3.1 Anti-AChR antibodies

The nicotinic AChR (nAChR) belongs to a superfamily of ligand-gated ion channels (LGIC), known as Cys-loop receptors. This superfamily also includes the 5-hydroxytryptamine type 3 (5-HT_3) or serotonin, glycine, γ-aminobutyric acid type A ($GABA_A$) and $GABA_C$ receptors. The main characteristic of Cys-loop receptors is a conserved pair of disulphide-bonded cysteines that are separated by 13 residues, in their amino-terminal domain [26], [29], [30]. The nAChR is composed of five homologous protein subunits with the stoichiometry of $\alpha_2\beta\delta\varepsilon$ in adult muscle, whereas in fetal or adult denervated muscles the composition is $\alpha_2\beta\gamma\delta$ [31]. During the development of the muscle, the fetal AChR is expressed along the entire fiber surface producing spontaneous contraction, a crucial procedure for the development of the fetus [32]. In some cases, MG patients reexpress the fetal AChR, in order to prevent the lethality of some mutations of AChR subunit genes [32]. In the muscle AChR the subunits are arranged in a circular order of αγαβδ, like barrel staves around a central channel (**Fig. 2b**) [33]. Each subunit has a N-terminal extracellular domain (ECD), 4 transmembrane domains (M1-M4), an intracellular domain (ICD) between the M3 and M4, and a small extracellular tail (C-terminal end) after M4 (**Fig. 2a**) [34]. The majority of antigenic epitopes involved in MG are located on the ECDs, subunits, whilst more than half of the autoantibodies target an area which is called main immunogenic region (MIR). The MIR is formed by overlapping epitopes located on the α1 subunit ECD, whose central core lies between amino acids 67-76 [35]. In addition, the autoantibodies against the α subunit are more pathogenic than those against the other subunits. [36]. The main reasons behind that are, firstly, two α subunits and thus two MIRs are present on each AChR, secondly, the MIR is exposed at an angle that allows antibody binding to two adjacent AChRs, and thirdly, the MIR is involved in AChR sensitivity to activation by ACh [37]. However,

autoantibodies against all five subunits, including the γ subunit of the fetal AChR, can be detected in MG patients, even in the same patient.

AChR antibodies result in pathogenicity by three effector mechanisms. Firstly, since they belong to the IgG1 and IgG3 subclasses, they can activate the complement and as a result the formation of the membrane attack complex (MAC), causing the destruction of the postsynaptic membrane [38], [39]. Secondly, as the antibodies are bivalent, it is possible to cross-link receptors, causing their endocytosis and degradation by a process called antigenic modulation, leading to cutback of functional AChRs in the postsynaptic membrane [40]. Finally, some AChR antibodies target the AChR binding side of the receptor, interfering with receptor activation by ACh and thus, blocking the signal transduction [36].

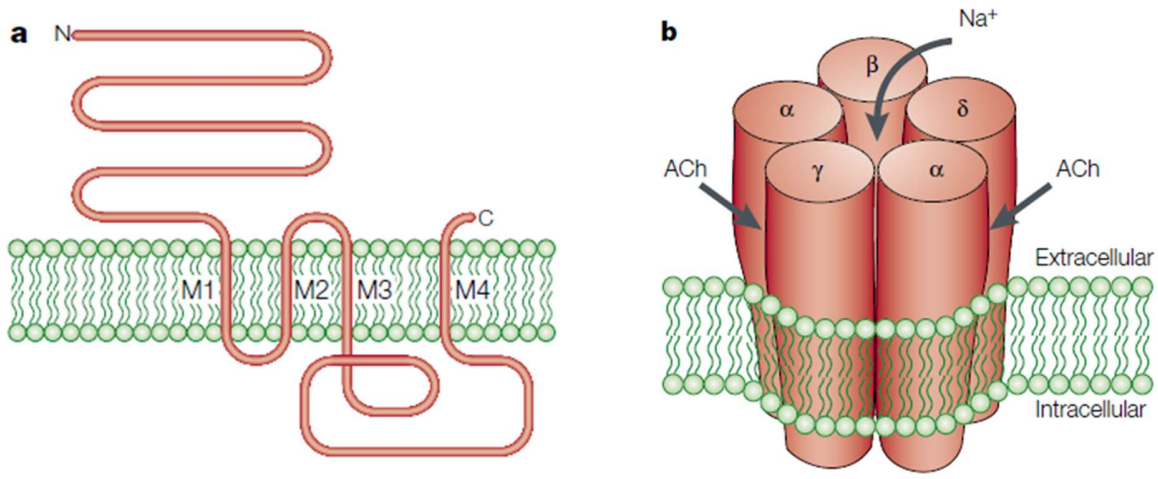


Figure 2. Structure of the nicotinic acetylcholine receptors. a. The threading pattern of receptor subunits through the membrane. b. A schematic representation of the quaternary structure, showing the arrangement of the subunits in the muscle-type receptor, the location of the two acetylcholine (ACh)-binding sites (between an α- and a γ-subunit, and an α- and a δ-subunit), and the axial cation-conducting channel.
Adapted from Arthur Karlin, Nature Reviews, Neuroscience, 2002

In general, there is no correlation between the total AChR antibody concentration and symptom severity, although such a correlation has been described at an individual level [41]. MG patients with anti-AChR antibodies can be categorized according to their clinical

symptoms and disease pathogenesis in early onset MG, late onset MG and thymomatous MG. In early onset MG, the first symptoms show up before the age of 50 years, with female patients being three times more than the male ones. It is estimated that more than 80% of the patients have thymic follicular hyperplasia, containing lymphocytic infiltrates and germinal centers that could be sites of B-cell responses against AChR [42]. Additionally, early onset MG has an association with HLA-DR3, HLA-B8 and other autoimmune risk genes that are known to influence immune disorders [43], [44]. On the other hand, in late onset myasthenia, patients rarely have thymic hyperplasia or thymoma. It is reported that the majority of patients are male, and the onset of their first symptom is after the age of 50 years [45]. Finally, thymoma-associated MG is a paraneoplastic disease, with 10-15% of MG patients, mostly the elderly, having a thymoma [46].

1.2.3.2. Anti-Musk Antibodies

The muscle specific kinase (MuSK), is a single-pass transmembrane glycoprotein of ~120 kDa. The extracellular domain carries three Ig-like domains (Ig1-3), and one cysteine-rich domain (CRD), also called Frizzled (FZ)-like domain. The transmembrane region is followed by a short juxtamembrane region and a tyrosine kinase domain [47]. MuSK is a central component of the postsynaptic signaling complex that coordinates the formation and maintenance of NMJs. MuSK activation leads to i) clustering and anchoring AChRs and additional critical muscle proteins, ii) transcriptional upregulation of synapse-specific genes by subsynaptic nuclei and iii) induction of a retrogate signal leading to presynaptic differentiation [48].

Since MuSK is a tyrosine kinase receptor, it interacts with several proteins that regulate its activity or activate downstream pathways (**Fig. 3**) [49]. One of these proteins is agrin, a heparansulphate proteoglycan, responsible for AChR clustering. When agrin is released by the axon terminal, it binds to LRP4 in muscles and phosphorylates MuSK. The formation of an agrin-LRP4 tetrameric complex (two agrin and two LRP4 molecules) is critical for MuSK

activation. Activated MuSK jointly with Dok-7, a muscle cytoplasmic protein, stimulates rapsyn, a scaffolding protein, to concentrate and anchor the AChRs at the postsynaptic membrane. Furthermore, MuSK is involved in tethering AChE, via interactions with collagen Q (ColQ) [27], [50]–[52]

MuSK antibodies belong to the IgG4 isotype, which does not activate the complement and have low affinity for Fc receptors of the immune cells [53]. In addition, IgG4 can undergo Fab-arm exchange, which is the exchange of IgG4 half-molecules and the generation of bi-

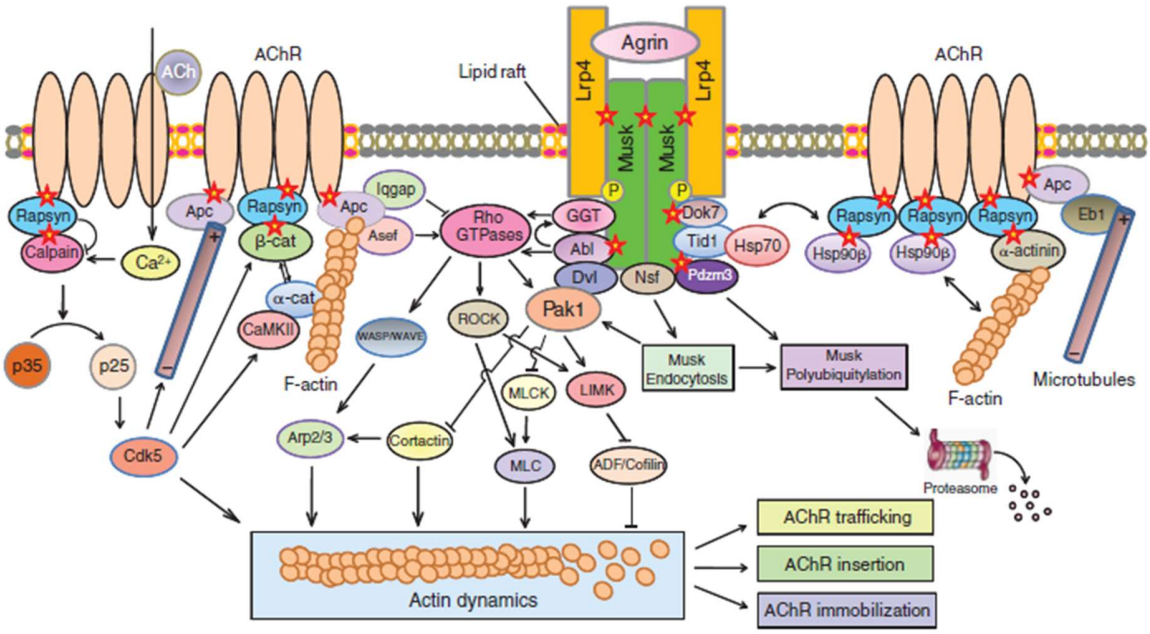


Figure 3. Intracellular pathways activated by agrin for AChR clustering. Agrin interacts with Lrp4 to increase its interaction with Musk and the dimerization of Musk and thus Musk activation. Subsequently, interactions between the kinase and distinct proteins, such as Dok7, which are crucial for its catalytic activity and for downstream signaling, are increased. Agrin also stimulates the association of AChR with rapsyn and Apc, which link the receptor directly or indirectly to the cytoskeleton. Rapsyn stability is increased at the synapse by the chaperone Hsp90b, and rapsyn interacts with and inhibits calpain, and thus antagonizes the AChR cluster-dispersing effect of ACh. Agrin signaling is regulated by Musk endocytosis, the E3 ubiquitin ligase Pdzrn3, lipid microdomains and intracellular calcium. Many pathways illustrated here have been identified in cultured muscle and non-muscle cells, and their role in vivo remains to be studied. For example, mice lacking Shp2, a cytoplasmic tyrosine phosphatase, are viable and form normal NMJs, although many in vitro studies suggest a crucial role in AChR clustering.. Stars indicate protein-protein interactions that are increased by agrin. Red lipid bilayers indicate lipid rafts.
Adapted from Haitao Wu et al. Development 137, The Company of Biologists, 2010

specific antibodies [54]. MuSK antibodies recognize epitopes within the first two Ig-like domains, and in approximately 30% of the patients they also recognize the CRD domain. The predominant target is the first Ig-like domain [55], which has two crucial functions. Firstly, the external part of the domain is required for the interaction with LRP4, which then binds to neuronal agrin, activating downstream pathways, which lead to AChR clustering. Secondly, the opposite medial side of the domain mediates MuSK dimerization [56], [57]. So, MuSK antibodies can induce MG through two mechanisms: i) inhibition of MuSK dimerization and ii) blockage of MuSK binding partners. Both mechanisms cause defects in the maintenance of postsynaptic AChR clustering, thereby impairing neuromuscular transmission [55]. Despite the predominance of IgG4 antibodies, IgG1 and IgG3 MuSK antibodies can be detected too in patient's sera. These antibodies can contribute to the disease mechanisms, since they are bivalent and complement activating, but their activity remains still unclear [58].

MuSK-associated MG is usually reported in adult women, and rarely in the elderly or children [53]. In contrast to patients with AChR MG, MuSK MG is characterized by predominant involvement of the cranial, bulbar and respiratory muscles, with ocular weakness and thymoma, being less common [53]. MuSK MG patients are more severely affected, with frequent myasthenic crises. Also, there is a positive correlation with symptom severity and the anti-MuSK antibodies concentration [58].

1.2.3.3 Anti-LRP4 Antibodies

The low-density lipoprotein receptor-related protein 4 (LRP4) is a member of the low-density lipoprotein receptor (LDLR) family, expressed in skeletal muscles and in motor neurons in the brain. It is a single-pass transmembrane protein, with a large extracellular N-terminal region that contains eight low-density lipoprotein receptor domains class A (LDLa) repeats, two EGF-like domains, and four β-propeller (BP) domains, each of which is fused together with an EGF-like domain, a transmembrane domain and a short C-terminal region

[57] In the muscles, LRP4 binds neural agrin released from the nerve terminals and, their interaction is critical for MuSK activation, AChR clustering and NMJ formation. In addition, LRP4 is an inhibitor of the Wnt signaling pathway, making it a really important protein in the formation of skeleton and kidney [59].

LRP4 antibodies belong mostly to the IgG1 subclass, and are capable of activating the complement. However, the contribution of complement in their pathogenicity is still not certain. LRP4 autoantibodies interfere with the interaction with MuSK and agrin. The anti-LRP4 antibodies are found in approximately 2% of all MG patients, and ~19% of SNMG patients. In most cases, LRP4 antibodies are more common in women than men [60]. They have also been reported in 10-23% of amyotrophic lateral sclerosis (ALS) patients [61] and in 3,6% of patients with other neurological diseases. The patients with LRP4-MG have milder symptoms and LRP4-MG can manifest purely as ocular MG [60].

1.2.4 Other antibody specificities

In addition to the main MG antibody specificities that are discussed above, a number of both extracellular and intracellular autoantibodies have emerged in MG patients. Even though their pathogenicity is not clear yet, their identification as disease biomarkers can be of a great importance for MG diagnosis.

1.2.4.1 Extracellular antigens

Agrin is a ~200 kDa extracellular protein with multiple binding domains that is released from the motor nerve terminal. The N-terminal of the protein is required for its anchorage to the basal lamina, whilst the carboxy-terminal is essential for AChR clustering [62]. Agrin antibodies have been detected in 2-15% of MG patients, mostly with antibodies against AChR, MuSK or LRP4. Patients with agrin antibodies have mild to severe symptoms and moderate response to treatment. Agrin antibodies appear to be pathogenic since they are

capable of inhibiting agrin-induced MuSK activation and AChR clustering in *in vitro* experiments [63], [64].

Voltage gated potassium channel a-subunit Kv1.4 is a 73kDa protein, widely expressed in the central nervous system, especially in axonal and presynaptic membrane. In addition, it also appears in NMJ and in the endocardium. Kv1.4 antibodies are present in 10-20% of MG patients and seem to cross-react with voltage gated K^+ channels in the heart muscle [65]–[67].

Collagen Q is a protein located in the extracellular matrix at the NMJ, where it mediates the anchoring of AChE. Antibodies against ColQ are found in almost 3% of SNMG patients, but there is no evidence to be MG specific and pathogenic yet [68].

AChE is an enzyme localized close to the postsynaptic membrane, where it is anchored to MuSK via molecules of ColQ. AChE catalyzes the breakdown of ACh to choline and acetate, thus terminating its action. Antibodies against AChE are found in 5-50% of MG patients, with no significant MG-specificity, since AChE antibodies are found in patients with several autoimmune diseases [69], [70].

Collagex XIII antibodies have been detected in the serum of approximately 7% of AChR-MG patients and 16% of SNMG, but the presence of antibodies against this transmembrane collagen do not correlate with symptom severity and are not MG specific [71].

1.2.4.2 Intracellular antigens

Titin is the largest known protein, found in muscle, where it extends the entire length of the sarcomere, giving elasticity and flexibility to the muscle. Even though it is a molecule with a molecular weight between 3,000 and 4,200 kDa, titin antibodies bind to a 30 kDa domain, called MGT30 (myasthenia gravis titin-30) which is located near the A/I-band junction [72], [73]. Titin antibodies are found in 20-40% of MG patients with AChR antibodies, mainly those with thymoma-associated disease, or late-onset MG. These antibodies are rarely found in early-onset or ocular MG. Since titin is located intracellular, titin antibodies

should not interfere with muscle function. However, they appear to be prognostic of more severe form of MG. Additionaly, titin antibodies are a marker of thymoma in patients with MG, with disease onset before the age of 50 years [74].

The ryanodine receptor (RyR) is a calcium channel with a molecular weight of ~565 kDa and it is located in the sarcoplasmic reticulum membrane, opening upon sarcolemma depolarization and releasing Ca^{2+} into the cytoplasm, causing muscle contraction. There are two forms of RyR, the skeletal one (RyR1) and the cardiac one (RyR2). It is composed of four homologous subunits that build a tetramer with a central channel. It is expressed mainly in striated muscle tissue, but it can also be found in epithelium and neurons [74]. The RyR antibodies in MG patients interact with both RyR1 and RyR2. The RyR antibodies cause allosteric inhibition of RyR function, inhibiting Ca^{2+} release from the sarcoplasmic reticulum [75], [76]. RyR antibodies are present in up to 75% of MG patients with a thymoma and in approximately 14% of patients with late-onset AChR-MG. Overall, their presence has been correlated with more severe disease manifestation [77].

Cortactin is a cytoplasmic protein involved in actin assembly that also acts as a signaling protein for AChR clustering mediated by the agrin-Musk complex [27]. Cortactin antibodies are present in up 20% of MG patients without AChR or MuSK antibodies, but also in about 9,5% of seropositive MG [78], [79]. However, cortactin antibodies have been found in up to 13% of patients with various autoimmune disorders including myositis [79]. Therefore, their contribution to pathogenesis and MG diagnosis is still not clear.

Rapsyn is a scaffolding protein, which plays an important role in AChR clustering, by linking the intracellular domains of the receptors [80]. Antibodies against rapsyn have been found in about 15% of MG patients, including among SNMG [81], However, rapsyn antibodies have also been detected in various other autoimmune diseases, decreasing their diagnostic potential, whilst no correlation with disease severity has been identified.

Although the pathogenicity of the above antibodies is not clear yet, their detection can be valuable for MG diagnosis, especially for the seronegative patients. The identification of

these antibodies as markers of disease severity, can also play an important role on the efficient therapy of the disease.

1.2.5 The thymus in MG pathogenesis

The thymus is the primary lymphoid organ for T lymphocyte maturation which plays an important role in tolerance induction to self-antigens and in responsiveness of lymphocytes to foreign antigens. During development, precursor T-cells migrate from the bone marrow to the thymic subcapsular epithelium, where a random process of gene rearrangements occurs in the regions that will code for the T-cell antigen receptor and will develop into double-positive $CD4^+CD8^+$ thymocytes. The immature T cells undergo either positive or negative selection and $CD4^+$ or $CD8^+$ single-positive T cells located in the thymic medulla, eventually are released to the periphery. Autoreactive T cells are eliminated during interactions between the developing thymocytes and thymic stromal cells (epithelial cells, mesenchymal cells, dendritic cells and a few myeloid cells), a process called negative selection [82], [83]. In healthy individuals, the thymus undergoes a progressive reduction in size and a decrease in thymopoieses, which results in the decrease in the output of newly developed T cells in the periphery.

Regarding MG, there is a strong association between thymic pathology and MG disease manifestation, since the majority of MG patients have lymphoid follicular hyperplasia or thymomas. In 10-15% of MG patients a thymoma is present and up to 50% of thymona patients develop MG [84]. The hyperplastic thymus includes all the components of the anti-AChR response: loss of AChR expression, B cells producing anti-AChR antibodies and anti-AChR autoreactive T cells [85], [86]. The immunopathogenesis of thymoma-related autoimmunity, is that autoreactive T cells are positively selected and released to the periphery, where they are activated in order to help the antibody-production by B cells [84], [85].

1.3 Diagnosis of Myasthenia gravis

The clinical diagnosis of MG can be achieved with three different categories of tests. These include the i) bedside tests, such as edrophonium or ice-pack test, ii) electrophysiological tests, such as repetitive nerve stimulation or single-fibre electromyography and iii) serological tests, such as detection of autoantibodies against AChR, MuSK, LRP4, titin and RyR.

1.3.1 Bedside tests

Edrophonium is short-acting AChE inhibitor that extends the duration of action of ACh in the NMJ, increasing both the amplitude and duration of the EPP. The edrophonium test works within 30 seconds and its effect lasts for approximately 5 minutes. It is administered intravenously and the patient is observed for an improvement in muscle strength, particularly the eyelid ptosis or extraocular muscle movement. The sensitivity of this diagnostic test is around 71.5-95% for generalized disease. Since it is associated with a low, but serious risk of bradycardia and/or hypertension, cardiac monitoring during the procedure is suggested [87], [88].

1.3.2 Electrophysiological tests

Repetitive nerve stimulation tests the neuromuscular transmission and it is performed by stimulating the nerve at a frequency of 2-5 Hz. This nerve stimulation produces a progressive reduction in the amplitude of the compound muscle action potential from the fourth stimulation. When this reduction is ≥10% the test is considered positive. This test is virtually always positive in generalized MG but may be negative in nearly 50% cases of ocular MG. [89], [90]

Single fiber electromyography (SFEMG) is the most sensitive diagnostic test (>95%) in MG. For this test, a specially constructed concentric needle electrode allows the identification of action potentials from individual muscle fibers. This special needle is recording

simultaneously the action potentials of two muscle fibers that are innervated by the same motor axon. The variability in time of the second action potential relative to the first is called jitter and normally is less than 55μsec. In MG, jitter is increased and is usually >100μsec, due to low and reductive EPPs. Even though this diagnostic test is highly sensitive, increased jitter is not specific for primary NMJ disease. However, it is specific for a disorder of neuromuscular transmission when no other abnormalities are seen in standard needle electromyography examination [91]–[94].

1.3.3 Serological tests

Serological tests play an important role in MG diagnosis, since they can detect the autoantibodies in a serum sample, in a minimally invasive way. Even though the final diagnosis is the result of several tests that are mentioned above, the high specificity of many MG antibody assays facilitate diagnosis [95].

Radioimmunoprecipitation assay (RIPA) is one of the most efficient and sensitive serological test in MG diagnosis. RIPAs are widely applied for the detection of AChR, MuSK, and less frequently other antigens. The AChR antibody assay is based on indirect labelling of solubilized AChR (both adult and fetal) with ^{125}I-α-bungarotoxin, a highly specific AChR antagonist [96], [97]. RIPA assay is highly sensitive and its specificity can reach 99% on positive results, which amounts to ~85% among GMG patients and ~50% for OMG [98]. RIPA is also used for the detection of MuSK antibodies, using ^{125}I-labelled MuSK, with high specificity for MG [99]. The detection of both AChR and MuSK antibodies in the same patient by RIPA is rare [100], [101]. RIPA tests are very important to patients with MuSK-MG, because as it was mentioned above, the concentration of anti-MuSK antibodies correlate with disease severity, so any changes in antibody titers can reflect disease activity. A recently developed RIPA can also be used for the detection of titin antibodies with ^{123}I-labeled MGT30. Titin antibodies have been found in 13,4% of SNMG patients and even though they are not

predictive of more severe disease, they are a valuable biomarker for the diagnosis of SNMG patients [102].

Enzyme-linked immunosorbent assays (ELISAs) are used for the detection of AChR and MuSK antibodies but with lower sensitivity than RIPA. The advantage of ELISA over the RIPA is that it does not involve radioactivity and can be performed with standard equipment in the laboratory [103], [104]. ELISA is widely used for the detection of titin, RyR and cortactin antibodies [75].

Cell based assays (CBA) are being used for MG diagnosis the recent years. This method involves the transient or stable expression of the target antigen in a cell line, followed by incubation of the cells with test serum and the detection of autoantibody binding by fluorescence microscopy using labelled secondary or tertiary antibodies [105]. In the case of AChR antibody CBA, AChR molecules are clustered on the membranes of cultured test cells, permitting the detection of antibodies that bind only to high density AChRs, mimicking their clustering at the NMJ [106]. Hence, the CBA is a semi-quantitative method that cannot provide the accurate antibody titers [107]. CBAs are very important for the detection of MuSK and LRP4 antibodies in previously SNMG patients [108]. Using the MuSK CBA test, about 13% of SNMG patients have been found positive for MuSK antibodies, with the variation ranging from 5- to 22% among countries. Furthermore, this test has allowed the detection of antibodies in SN-OMG patients, which is not common with RIPA [109]. In the case of LRP4 CBA, ~19% of SNMG patients were found positive for LRP4 antibodies, with a variability of 7-33% [60]. The presence of antibodies only detectable from CBA is associated with milder symptoms and better response to treatment although these antibodies are proven to be pathogenic [106].

1.4 Immunopathology in MG

1.4.1 Immune tolerance

MG is a CD4+ T cell–dependent autoimmune disease, so it is of a great importance to investigate the production and function of T cells. As it was mentioned before, the T cell development and maturation takes place in the thymus, which is responsible for the early production of CD4+ T cells. T cells depending on the factors they express can be either conventional T (Tconv) cells (CD4+CD25-) that differentiate into effector cells during immune responses, or T regulatory (Treg) cells (CD4+CD25+) that downregulate the immune response [110]. The development of autoimmune diseases involves a breakdown in the mechanisms that control self-reactive lymphocytes. The primary mechanism that generally maintains self-tolerance is thymic deletion of autoreactive T cells with high affinity for self-antigens. However, this mechanism is not perfect and autoreactive T cells do escape to the periphery. Treg cells are essential for the maintenance of immunologic self-tolerance by suppressing potential autoreactive T cells in the periphery. In MG patients a normal Treg number is observed but with a severe functional defect in their regulatory activity together with a decreased expression of the transcription factor Foxp3, which is essential for T-cell regulatory function. Indeed, transduction of Foxp3 converts naive CD4+CD25- T cells into CD25+ regulatory cells with suppressive activity [111]–[113].

1.4.2 Immunogenetics

Genetic factors partly contribute to MG susceptibility, with the human leukocyte antigen (HLA) locus remaining the most strongly associated risk factor for the disease. In early-onset MG there is a clear association with HLA-DR3 and B8 alleles, which is usually associated with thymic follicular hyperplasia, whilst late-onset MG is less strongly associated with HLA-DR2 and B7 [114] [115]. HLA-DR3 and DR7 seem to have opposing effects on MG phenotype, DR3 having a positive association with early-onset MG and a negative association with late-onset MG, while DR7 has the opposite effects [116]. Other, non-HLA susceptibility genes have been

found to play a role in the pathogenesis of autoimmune MG. These include CTLA-4, PTPN22, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-β [117]. Some of these are also associated with other autoimmune diseases and might thus represent a non-specific susceptibility to autoimmunity [117]. In addition, in MUSK-associated MG there is an HLA association with DR14-DQ5 [2].

1.5 Treatment of myasthenia gravis

1.5.1 Symptomatic drug treatment

The first-line treatment in MG patients involves the acetylcholinesterase inhibitors, which increase the amount of ACh available for binding in the NMJ. Pyridostigmine bromide is the most commonly used drug of this category, with the onset of effect within 15-30 minutes [77]. Neostigmine and ambenonium chloride are also AChE inhibitors, less effective than pyridostigmine. Inhibitors rarely induce complete relief from symptoms, have no effect on the underlying immune response and do not affect disease progression. In MUSK-associated MG, AChE inhibitors are often insufficient and may induce side-effects [118]. Juvenile MG often has an excellent response to pyridostigmine [119].

1.5.2 Immunosuppressive drug treatment

Immunosuppressive therapy is essential for almost all patients with late-onset MG, thymoma MG and MuSK-MG in order to suppress the autoantibody production and reduce the activity of the immune system.

Oral corticosteroids are first-choice immunosuppressive drugs for the treatment of MG. Prednisone is a steroid drug used when symptoms of MG are not adequately controlled by AChE inhibitors alone. Prednisone improves muscle strength in all MG subgroups.) The improvement usually begins in 2-4 weeks, with maximal benefit after 6-12 months or more [120]. The side effects of chronic steroids include weight gain, hypertension, hyperglycemia, osteoporosis, aseptic necrosis of the hip and cataracts [120].

Azathrioprine (AZA) is an effective drug for all MG subgroups that can be combined with prednisolone for higher effectiveness. This combination is often a first-line choice for MG treatmen [121]. AZA is a purine antimetabolite that interferes with T and B cells proliferation, with leukopenia and hepatotoxicity being some of the main side-effects [122]. The AZA effect is seen usually after 4-10 months, with reported improvement for up to 24 months [123], [124].

Cyclosporine inhibits T-cell proliferation by disruption of calcineurin signaling, which blocks the synthesis of IL-2 and other essential proteins for the function of CD4+ T cells. It is mainly used in patients who do not tolerate or find insufficient AZA [125]. Some of the side effects are tremor, gum hyperplasia and aneamia, but the most serious are hypertension and nephrotoxicity [126].

Tacrolimus acts with a similar mechanism with cyclosporine. Sustained benefit has been reported in anti-RyR-positive patients that it is considered to be due to enhancement of ryanodine-receptor-related calcium release from the sarcoplasmic reticulum [127].

Cyclophosphamide is an alkylating agent that inhibits DNA replication. It acts mainly in B-cells by suppressing their activity and antibody synthesis, and has a dose dependent effect on T-cells. In general, cyclophosphamide is used in chemotherapies and it is very toxic, with side effects such as bladder and gonadal toxicity, teratogenicity, myelotoxicity, hair loss and opportunistic infections [128]–[130].

Methotrexate inhibits the synthesis of purine and pyrimidine, leading to impaired DNA, RNA, protein synthesis and reduced lymphocytes. It has similar efficacy and tolerability to AZA but with very serious side effects. Some of them are leucopenia, thrombopenia, aneamia, infections, hepatotoxicity, stomatitis, gastrointestinal symptoms, arthralgia, osteoporosis, pulmonary disorders, nephrotoxicity and malignancies. Furthermore, it has teratogenic potential, so it is not recommended in young women or during pregnancy. It is also possible that it reduces female fertility [131], [132].

Rituximab is a chimeric murine-human IgG1 kappa monoclonal antibody that depletes all types of B lymphocytes through specific binding to the transmembrane CD20 antigen. Initially, it was developed for the treatment of B-cell lymphoma, but is also used as a treatment for autoimmune diseases. This drug should be an alternative for severe cases that do not respond to first-line immunosuppressive treatment [133], [134]. Meta-analysis studies indicate that patients with MuSK-MG have a favorable response, which is really important, since these patients have lower response to first-line immunosuppressive drugs [135]–[137]. Concerns regarding rituximab are infections, the risk of precipitating additional autoimmune diseases and JC virus-related progressive multifocal leukoencephalopathy [86], [138].

Intravenous immunoglobulin (IVIG) is an immunomodulatory treatment used for patients with exacerbating MG. Among potential mechanisms that operate at the NMJ are the anti-AChR antibody activity decrease by idiotypic-anti-idiotypic interactions, ACh-AChR interaction increase, reduction of complement binding to post-synaptic neuromuscular membrane, reduction of antibody-dependent phagocytosis. Among potential mechanisms operating at intravascular and lymphatic organ levels are reduction of anti-AChR antibody synthesis, modification of serum complement level, suppression of pathogenic cytokines, reduction of superantigen-induced immune system activation, competition of antigen recognition, inhibition of suppressor T cell function and modulation of IgG Fc-related cell function [139], [140]. The treatment effect usually appears within 3-10 days and the response appears to last for about 45 days due to recurring autoantibody production [141]. Some nonspecific side effects of IVIG may appear after few hours of infusion including facial rash, angina pectoris, nausea, shivering, back pain, fever, sweating, headache and hypotension [142]. More severe side effects develop in approximately 1-2.5% of the IVIG-treated patients and include allergic reactions, infections, pulmonary edema, acute renal failure, venous thrombosis, stroke, myocardial infraction, hemolysis and aseptic meningitis [132].

Plasmapheresis is the most direct way to remove pathogenic antibodies from the blood and can be remarkably effective as a short-term treatment. It is mainly used in severe cases,

or in order to treat MG crises [143]. In plasma exchange, plasma is separated from corpuscular blood components, and is usually replaced by a supplement which consists of a solution of human albumin and crystalloids. In addition to antibodies, other plasma components, such as cytokines, coagulation factors and complement are also removed, a fact that could lead to side effects. Typically one exchange, removing one to two plasma volumes is done every other day, up to a total of four to six times [144]. An alternative to plasmapheresis, immunoadsorption is more selective for removing only IgG antibodies, by binding to a specific matrix (protein A or tryptophan) [145]. These aphaeretic approaches are generally a temporary treatment since the antibodies usually recover within around 6 weeks. Common side effects include hypertension and paresthesias from citrate-induced hypocalcemia, Also, infections and thrombotic complications related to venous access have been reported [146], [147].

Thymectomy is the surgical removal of the thymus gland. It is mandatory in patients with thymoma, since the tumor should be removed. Thymectomy is also recommended as an option for patients with EOMG without a thymoma and in particular those with AChR antibodies [148], [149]. There is no evidence yet that it is beneficial for patients with MuSK-MG and LRP4-MG and is not recommended for patients with ocular MG [150]. The advantage of thymectomy is a significant reduction in symptoms and the ongoing chronic medical treatments [77].

1.6 Novel therapeutic approaches

1.6.1 Experimental Autoimmune MG

Experimental autoimmune MG (EAMG) is an invaluable tool for the development of novel therapies against the disease because EAMG is usually induced in vertabrates by active immunization with *Torpedo californica electroplax* nAChR (T-AChR) in Complete Freund's Adjuvant (CFA), as well as by passive transfer of MG sera or anti-AChR antibodies [151]. The most studied and used models are rats (65%) and mice (35%), with the incidence of clinical

EAMG being higher in rats than mice [152]. EAMG mimics MG in its clinical and immunological manifestations and is a reliable model to investigate the molecular mechanism and therapeutic approaches for the treatment of human MG.

EAMG is usually induced in 6-8 weeks old Lewis rats with a single administration of TAChR in CFA. The susceptibility to EAMG is influenced by sex, age and genetic backround of the different rats. In each case, the rats mount an active immune response against the injected antigen. However, only ~1% of the produced antibodies cross-reacts with the animal's own muscle AChR and this subset is responsible for the disease [151]. The induced EAMG is characterized by two distinct phases: i) a transient acute phase with a mild muscular weakness, beginning approximately 8-10 days post immunization and recovering after 3-4 days and ii) a severe, progressive chronic phase starting approximately 25-30 days post immunization, ending often in death [153].

The immunization with TAChR results in the generation of anti-rat nAChR antibodies and as a result in the activation of the complement cascade which leads to the degradation of the muscle end-plate and loss of nAChRs and ultimately to the impairment of the neuromuscular signal transduction. In more detail, IgM directed to TAChR are readily detectable in rat serum during the acute phase of EAMG, while more than half of the antibody repertoire belong to IgG subtype, which practically means that a switch from IgM to IgG production occurs very early in the process. During the chronic phase, 35 days post immunization all detectable anti-TAChR antibodies belong to the IgG class. These late-IgGs contain both T-AChR and R-AChR antibodies and cross-react with the R-AChR with high affinity [153]. As EAMG progresses into its chronic phase, the titer of cross-reacting antibodies continues to increase the concentration of nAChRs on the muscle membrane is greatly reduced. The development of the chronic phase is associated with dramatic increase in titer of serum antibody against muscle nAChR, (approximately 30% compared to normal muscles) and there is a decrease of post-synaptic membrane area and simplification of its folded structure. The impairment of neuromuscular transmission could result from

decreased muscle nAChR content as the amount of receptor extracted from the muscles of myasthenic animals is significantly reduced (approximately 30% compared to normal muscles). Moreover, in chronic EAMG a large portion of the remaining nAChR is bound by antibodies and their function is partially or completely impaired [154]

The course of EAMG and disease severity, is evaluated by monitoring the loss of body weight and muscular strength of the immunized animals. Myasthenic symptoms, assessed prior or after exercise (repetitive paw grips in the cage grid for 30 seconds), include tremor, hunched posture, muscle weakness and fatigue. Grading of the symptoms (clinical scoring) is as follows [155]:

0: no symptoms;

1: no symptoms observed at rest but only after exercise (repetitive grasping of a rack for 30 s);

2: symptoms present before exercise;

3: very severe symptoms, hind limb paralysis;

4: moribund, death.

1.6.2 Tolerance induction as a therapy in MG

The current treatments for MG are not specific and can thus be accompanied by adverse side effects. Furthermore, the long-term immunosuppression that accompanies these treatments, increases the risk of infections or neoplasia whilst some patients remain unresponsive to them [156]. The development of antigen-specific therapies targeting only the pathogenic components of the immune system without interfering with its general function, would be very beneficial for MG patients.

An approach for antigen-specific therapy is the induction of immunosuppression or immune tolerance in a targeted manner. The impact of this treatment is not immediate, but it can achieve a long-lasting or even permanent effect [157]–[159]. Indeed, studies have shown that EAMG symptoms can be prevented or ameliorated by oral or nasal

administration of AChR. In more detail, nasal and oral administration of *Torpedo* AChR [160], [161] or the human acetylcholine α-subunit as well as oral administration of AChR-derived recombinant fragments [162] have been shown to ameliorate or even prevent the clinical manifestations of the disease. Induction of oral tolerance results in decrease in AChR sperific T cell proliferative response and IL-2 production. The immune response is shifted from Th1 toward Th2/Th3 and co-stimulatory factors are down-regulated. The underlying mechanism for the mucosal tolerance induced by the AChR fragments is shown to be active suppression and not clonal anergy. Improvement of the animals' clinical condition is accompanied from reduction of anti-nAChR serum antibodies and improved muscle nAChR content. In addition, it is shown that nasal administration of the recombinant fragment hα1-205 is effective in suppressing EAMG, and induce nasal tolerance by active suppression involving a shift from Th1 to a Th2/Th3 type. This is accompanied with a downregulation of the expressed co-stimulatory molecules such as CTLA4, B7-1 and B7-2 [163], [164]. Furthermore, it has been shown that in MuSK immunized mice, the oral administration of recombinant rat MuSK protein results in a significant therapeutic effect and lower disease severity [159]. In more detail, it is suggested that administration of low doses of MuSK leads to induction of antigen specific Tregs and consequently to immune tolerance. Oral tolerance has an impact on the numbers and function of Treg cells, on the secretion of pro and anti-inflammatory cytokines and on Th1/Th2 effector cells [159]. Moreover, it is demonstrated that intravenous administration of solubilized MHC II complexed with an immunodominant peptide of the AChR (AChRα 100-116) is toloregenic and improves the survival of EAMG rats that are treated [165]. The mechanism of induced tolerance is complex and involves multiple immunoregulatory pathways. There is evidence that mechanisms of both active suppression and anergy are involved, and that relatively low doses predispose to suppression, whereas high doses favor anergy [166]. In contrast with *Torpedo* AChR, nasal administration of peptides of α and δ subunit of TAChR (α 61–76, α 100–116, α 146–162, δ 354–367, and α 261–277), did not induce tolerance to EAMG rats, neither alone or in combination. It is suggested

that a single or multiple peptides of TAChR hardly induce tolerance against the whole AChR protein, probably due to epitope spreading [167]. On the other hand, oral administration of the T-cell epitope α 146-162 of the TAChR suppresses T-cell responses to AChR and ameliorates the disease in EAMG mice. It is suggested that tolerance is achieved through T-cell anergy, since the production of IFN-γ, IL-2 and IL-10 is reduced [168]. Some studies, have proved that oral administration of a mixture of subunit constructs containing both extracellular and cytoplasmic domains of human AChR α1, β1, γ, δ, and ε (2: 1: 1: 1: 1 analogy) prevents the induction of EAMG in rats and reduces the severity of the disease. The nasal administration of this mixture prevents EAMG but is inefficient at treating ongoing EAMG [157].

Despite these promising results, no such treatment has been established yet, mostly due to problems encountered with the reproducibility of the animal models and the strenuous procedures needed to acquire the necessary amounts of the treatment antigen.

We have recently identified intravenous antigen administration as a potent method for disease amelioration in experimental animals. We have also established a protocol for the reproducible emergence of robust experimental autoimmune MG (EAMG) in rats. Therefore, we now aim to further explore aspects of the novel therapeutic approach. In more detail we: 1. investigated the effect of antigen-specific therapy in different concentrations and time points post immunization 2. examined the effect of antigen-specific therapy in comparison with antigen specificity (for example: therapeutic potency of hβ1 ECD, MuSK and OVA in hα1 ECD immunized Lewis rats) and 3. Determined the potential effect of epitope spreading on the therapeutic effect (for example: therapeutic potency of hα1 ECD in rats immunized with a mixture of AChR subunit ECDs).

2. Materials

2.1 Laboratory equipment

The necessary instruments and devices are listed below:

• Ultra-pure water production system, MilliQ Direct 8, Millipore

• Incubator for yeast cultures in agitation GALLENKAMP, MRC

• Water baths Memmert and Digiterm 3000542

• Sterilizing oven REYPA Steam Sterilizer

• Centrifuge KUBOTA 7780 (heads AG-580CA and AG-5006 and plastic centrifuge tubes and glasses suitable for centrifugation at high turns)

• Refrigerated microcentrifuge Heittich

• Solution microfiltration system Millipore

• Solution ultrafiltration system PALL Corp. Ultrasette 10K (includes membranes that block molecules with a molecular mass >10kDa)

• Peristaltic pump Millipore

• Electronic precision scale for measuring small quantities, Mettler, model AESO

• Electronic scale KERN

• Magnetic stirrers HEIDOLPH

• Mixers, Vortex-GENIE 2

• Spyramix, Phoenix Instruments

• Ph-meter, HANNA

• γ-counter, 1470 Wizard, Perkin Elmer

• Anesthesia machine, Parkland Scientific

• Tissue homogenizer, Grainger

2.2 Consumables

• Petri dishes for solid cultures of yeast, GreinerBio-One

• Conical flasks 500mL, 1L, 2L ISOLAB

- Plastic centrifuge tubes 250mL, 500mL, Corning
- Plastic centrifuge tubes 250mL, 500mL KUBOTA
- Disposable polypropylene test tubes 15 and 50mL, Greiner
- Disposable plastic tubes of 1.5mL GreinerBio-One
- Plastic tips, and plastic pipettes 1, 2, 5, 10 and 25 mL, Costar
- Plastic tips for repeating pipettes, Eppendorf
- Disposable plastic cells, SARSTEDT
- Filters with pore diameter 0.22 and 0.45 µm, Millex, Millipore
- Filters with pore diameter 0.22µm, NALGENE
- Filtering paper 3MM, Whatmann
- Chromatography columns, BIORAD
- Syringes 2, 5, 10 mL, Becton, Dickinson and Company
- Needles 25G, 30G and 22G Becton, Dickinson and Company
- Insulin syringes 1mL, 27G Becton, Dickinson and Company
- Surgical scalpel blades, Swann-Morton

2.3 Reagents

- Agarose, Sigma
- Bacto-peptone, Bacto-tryptone, Bacto-yeast extract, Becton, Dickinson and Company
- Yeast Nitrogen Base (YNB), Sigma
- Imidazole, Sigma
- Glycerol, Applichem
- Ni2+-NTA agarose beads, Qiagen
- Na125I for α-bungarotoxin (α-btx) and ha1 ECD radiolabeling
- Albumin from bovine serum (BSA), Applichem
- Iodoacetamide, Applichem
- Hepes, Applichem
- EDTA, Applichem
- Phenylmethylsulfonyl fluoride (PMSF), Sigma

- Sodium Chloride, Applichem
- Potassium di-hydrogen, Applichem
- Di-Potassium hydrogen, Applichem
- Triton X-100, Applichem
- Rabbit anti-rat IgG, DakoCytomation
- Complete Fraud's Adjuvant, Becton, Dickinson and Company
- Isoflurane, IsoFluo, ESTEVE

2.4 Media Recipes

The culture media for Pichia pastoris cultures were used after liquid sterilization (120oC, 20 minutes, 2 Atm) or after filtration with a sterile filter with pore diameter 0.22μm and were comprised of:

YPD	1% (w / v) yeast extract, 2% (w / v) peptone, 2% (w / v) dextrose
YPS-agar	YPD containing 1.5% (w / v) agar
RDB-agar	1M sorbitol, 2% (w / v) dextrose, 1.342% (w / v) YNB, 4x10-5 (w / v) biotin, 0.005% (w / v) for each amino acids L-glutamic acid, L-lysine, L-methionine, L-leucine, L-isoleucine, 2% (w / v) agar
BMGY	1% (w / v) yeast extract, 2% (w / v) peptone, 100 Mm phosphate buffer, pH 7.0, 1.34 (w / v) YNB, 4x10-5 (w / v) biotin, 1% (v / v) glycerol
BMMY	1% (w / v) yeast extract, 2% (w / v) peptone, 100 Mm phosphate buffer, pH 7.0, 1.34 (w / v) YNB, 4x10-5 (w / v) biotin, 0.5% (v / v) methanol

2.5 Buffer solutions

PBS 10X	60 mM phosphate buffer pH 7.4, 137 mM NaCl, 2.7 mM KCl
Radioimmunoassay Wash Solution	PBS 1X + 0.5% Triton x-100
Phosphate Buffer (PB)	1M K2HPO4, 1M KH2PO4
Protein isolation dialysis buffer	50mM PB, pH=8.0, 300mM NaCL, 2% Glycerol

3. Methods

3.1 Liquid and solid cultures of yeast *Pichia pastoris*

P. pastoris is a single-cell methylotropic yeast which is used for heterologous expression of proteins from higher eukaryotic organisms. As a eukaryotic organism, yeast has the advantages of higher eukaryotic expression systems and therefore can produce soluble, properly folded recombinant proteins that have undergone the necessary post-translational modifications (glycosylation, phosphorylation, disulfide bond formation). Compared to other eukaryotic systems used for heterologous expression *P. pastoris* is faster, easier and less costly, and usually gives higher levels of heterologous protein expression.

P. pastoris has a potent promoter, which induces protein expression after the addition of methanol to the medium, allowing controlled expression of heterologous proteins. *P. pastoris* can grow with methanol as the sole source of carbon. The first step, to methanol's catabolism is its oxidation to formaldehyde and peroxide hydrogen, a reaction catalyzed by the enzyme alcoholic oxidase. There are two alcohol oxidase genes with high homology, the AOXI and AOX2, with AOXI being responsible for most of the alcohol oxidase's activity in the cell. Because *P. pastoris* secretes very few of its own proteins, isolation of heterologously expressed secreted proteins is easy.

For the current thesis project we used transformed yeast strains, containing plasmids with our proteins of interest, namely the human AChR subunit α1 extracellular domain (hα1 ECD), hβ1 ECD, hδ ECD and hε ECD. These constructs have their wild-type Cys-loop exchanged with the Cys-loop from the acetylcholine binding protein (AChBP) from the snail Lymnaea stagnalis. AChBP is a soluble protein, composed of five identical subunits, which share an overall 20-24% identity with the ECDs of the AChR. This mutation results in much better solubility and high expression yields. Moreover, the constructs contain an N-terminal Flag tag as well as a 6xHis tag at the C-terminus used for their purification with antibody or metal affinity chromatography respectively.

3.1.1 Protein expression in *P. pastoris*

First, *P. pastoris* cells from stock stored in -80°C were streaked on RDB medium plates, lacking histidine, and were incubated in 30°C. Colonies are usually developed after 48-72h. Individual colonies of transformed *P. pastoris* cells containing one of the plasmids with our protein of interest, were transferred to 200ml (x2) of BMGY medium and were incubated for 16-20h, at 30°C under stirring at 200rpm. After the o/n incubation when the culture had reached $O.D._{600nm}$= 1.0, we transferred 50 ml of yeast culture from the BMGY flasks to each of 8 flasks of BMMY medium (500ml each) and were incubated for 3 days at 22°C under shaking at 200rpm. In the BMMY medium glycerol had been replaced by 0.5% (v/v) methanol in order to induce protein expression. As the *P. pastoris* cells require good ventilation for growth, the 500ml of BBMY were placed in flasks with volume capacity of 2L. At day 4, as induced protein expression had been completed, the BMMY liquid culture's supernatant containing the secreted protein was collected, by centrifugation at 7.000 rpm, for 15 min at 4°C, using a KUBOTA centrifuge.

3.1.2 Protein isolation from *P. pastoris* supernatant

The process for the protein isolation consists of two steps:

a) Microfiltration

The yeast culture supernatant was transferred to a metal sterilized cauldron, which was hermetically sealed. The vessel was connected to a nitrogen gas bottle, under pressure, through a hose which was attached at a specific point on the top of the can. Opposite to the hose's connection site, a metal microfiltration device (Millipore), was connected via a rubber on the top of the can. In the microfiltration device were placed in a horizontal arrangement from top to bottom, Whatman 3M filter paper, gauze, filter with pore diameter 0.45μm, gauze and filter with pore diameter 0.2μm. At the bottom of the microfiltration device was attached a rubber tube, which ended up in conical flask, with volume capacity 10L. In this large flask the filtered supernatant was collected, after the passage of pressurized nitrogen gas ~1Atm, in the metal can.

b) Ultrafiltration and dialysis

After the process of microfiltration the supernatant was condensed through a tangential flow device, which contained a membrane that excluded molecules with molecular weight >10kDa, while smaller molecules pass through the membrane with the solution. Prior to use, the device was washed with 2L of ddH2O for 20 minutes without recycling in order to remove the 0.1% azide solution in which the device was required to be stored. Thereafter, the flow of the protein solution was applied through the ultrafiltration device by recycling with a peristaltic pump of adjustable volumetric flow. At the end of the condensation process, the protein buffer was exchanged by adding 1L of 50mM PB buffer, 300mM NaCl and 2% Glycerol pH 8.0 for each liter of initial supernatant. The solution was then again condensed. By maintaining a constant volumetric outlet flow rate of 35ml/min, 1L of protein volume was condensed to a final volume of 100ml over 25 minutes.

3.1.3 Protein purification by metal affinity chromatography with Ni^{2+}-NTA agarose column

In an alkaline environment, proteins containing six consecutive histidine residues (6xHis-tag) bind with high affinity to the nickel beads column, because in an alkaline environment the histidine residues are negatively charged, forming a ring surrounding the nickel cations. A specific volume of a nickel suspension solution containing 50% (v/v) Ni^{2+}-NTA agarose beads was centrifuged at 1000rpm, for 5 minutes at 4°C, in a Juan centrifuge, and the supernatant was carefully removed. The Ni^{2+}-NTA beads were equilibrated with 50mM PB buffer, 300mM NaCl pH 8.0 buffer with gentle agitation. The supernatant was removed, each time, by centrifugation at 1000 rpm, for 5 minutes at 4°C. After the dialysis, the condensed *P. pastoris* supernatant (150 ml volume), containing the soluble protein we express, was mixed with the equilibrated Ni^{2+}-NTA agarose beads. 10mM imidazole was added to prevent non-specific binding to the Ni^{2+}-NTA beads. The expressed protein bound to the agarose beads upon o/n incubation at 4°C with gentle agitation. Afterwards, the Ni^{2+}-NTA with the bound protein were placed in a glass chromatography column.

The packed Ni^{2+}-NTA and protein were washed with 10 column volumes of buffer solution consisting of 10mM imidazole, 50mM PB, 300mM NaCl pH 8.0. Gradual elution is performed first with 20mM Imidazole 50mM PB, 300mM NaCl pH 8.0. The purified protein is eluted with 150mM Imidazole, 50mM PB, 300mM NaCl pH 8.0 in one or more fractions.

3.1.4 Protein quantification with Bradford colorimetric method

Coomassie brilliant blue dye binds to protein molecules and more specifically to lysine (K) residues to produce a colored product. The color intensity depends on the concentration of the protein in the solution. In order to be able to quantitate a protein

in a solution, a standard calibration curve is made with BSA at various concentrations. For the standard curve, solutions of 100μl were prepared, with 0.125, 0.250, .0500 or 1.5μg/μl BSA concentrations in 50mM Hepes, 300mM NaCl pH 8.0. In 20μl of the pre-mentioned dilutions we added 1ml of Biorad protein assay solution, which was diluted five times with ddH2O and we measured the solutions' absorbance at 595nm. The same procedure was followed for the unknown sample. From the standard reference curve, which was designed based on the absorbance of the BSA solutions, the protein concentration in the unknown sample was calculated in μg/μl.

3.2 Animals and EAMG induction

6-7 week old female Lewis rats were obtained from the animal breeding unit of the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute. They were maintained in the large rodent unit of the Department. All experiments described were conducted according to the regulations and guidelines for animal care. Before immunization the rats were anaesthetized with 3% isoflurane supplemented with oxygen. A 250μl portion of emulsion containing a 1:1 ratio of antigen/CFA was administered to each rat. Owing to loss of some of the viscous emulsion on the walls of the eppendorf tubes and in the hub of the syringe, excess emulsion was prepared. The rats were immunized either with 80μg hα1 ECD, diluted to the appropriate PBS volume, for a total volume of 125μl or with a mixture of AChR subunit ECD (80μg of each hα1, hβ1, hγ and hε) in CFA. The 125μl of CFA were supplemented with 2mg/ml inactivated Mycobacterium turbeculosis H37RA. The antigen/CFA mixture was emulsified with 20 repeated syringe extrusions, using 2ml syringes with 22G needles.

To proceed to the animals' immunization, we slowly loaded the antigen/CFA emulsified mixture into a 2 ml syringe with a 25G needle, as it is very viscous and easy to form bubbles when transferred. The rats were immunized once in the hind footpads (75μl to each footpad) and at the base of the tail (100μl in two doses).

3.3 RIPA for the detection of anti-hα1 ECD antibodies

RIPA is a sensitive technique for the detection of antibodies against a specific antigen in serum samples. RIPA's principal of function is based on the antibodies' property to specifically bind to their antigen, forming complexes of high molecular weight. The first step is the radio-labeling of the protein-antigen of interest, which is carried out by other members of the laboratory. The second step is the incubation of the radiolabeled antigen with the rat serum sample. If the sample contains antibodies against the protein-antigen, a complex will be formed. Afterwards, incubation with antibodies from other species that bind to the first antibodies results in heavier complexes that are readily precipitated by centrifugation. In the samples in which antibodies against the protein-antigen are present, radioactivity emitting from the labeled protein-antigen will be trapped in the precipitate. The last step involves washing with a suitable detergent-containing solution to remove unbound radioactivity and measure the radioactivity of the sample. The greater the amount of antibodies against the antigen, the more the radioactive counts per minute measured.

In more detail, the followed procedure was: Initially, 50000cpm of labeled antigen (hα1 ECD) were incubated with 2μl rat serum in a final volume of 50μl (with the appropriate volume of PBS-BSA 0.2%) for 2 hours at 4^{o}C. We also added in the reaction 2μl of normal rat serum (NRS) in order to block possible non-specific binding. Subsequently, 10μl of rabbit anti-rat IgG were added and the mixture was incubated for 2 hours at 4^{o}C, so as to create a precipitate. Next, 1ml of washing solution was added and centrifugation at 2500rpm for 15 minutes at 4^{o}C, followed. Thereafter, the precipitated pellet was resuspended and a second wash with subsequent centrifugation followed. The supernatant was removed and the radioactivity bound to the precipitate was measured in γ-counter. The radioactivity of the precipitate was proportional to the amount of anti-hα1 ECD antibodies bound to the labeled hα1 ECD.

3.4 ELISA for the detection of anti-MuSK and anti-OVA antibodies

Enzyme linked immunosorbent assay (ELISA) is a sensitive technique used for the detection of antibodies against an antigen present in serum samples. The first step is the immobilization of the protein-antigen on a 96-well polystyrene microtiter plate (coating solution). Coated plates are covered and incubated overnight at 4^{o}C. The next day, the coating solution is discarded, and the wells are washed three times with PBS 1X + 0.05% Tween (washing buffer). Following wash, the plate is inverted and tapped on absorbent paper in order the excess liquid to be removed. The next step is the blocking of the plate with an irrelevant protein or other molecule in order to cover all unsaturated surface-binding sites of the microplate wells. Blocked plates are covered and incubated with PBS 1X + 0.05% Tween + 5% milk (blocking buffer) for 1h at 4^{o}C. The blocking solution is discarded, and the wells are washed one time with washing buffer. Following wash, the plate is inverted and tapped on absorbent paper in order the excess liquid to be removed. Next, the serum samples are added in each well, and incubated for 1h at 4^{o}C. If the sample contains antibodies against the protein-antigen, a complex will be formed. The plate is washed three times with washing buffer and tapped on absorbent paper. After wash, the secondary antibody is added (total anti-IgG), which is covalently labeled to horseradish peroxidase (HRP) and incubated for 1h at 4^{o}C and the plate is again washed three times with washing buffer and tapped on absorbent paper. Afterwards, TMB substrate solution is added and incubated for 15 to 30min in 37^{o}C under dark conditions. TMB reacts with the enzyme conjugate, and produces a measurable byproduct, which is proportional to the amount of antibodies against the coated antigen. Next, the absorbance signal is measured at 655nm with an ELISA microplate reader. Finally, H_2SO_4 5.61% v/v (stop solution) is added and the absorbance signal is measured at 450nm with the ELISA microplate reader.

3.5 Statistical analysis

The rat clinical scores and serum antibody titers were compared for differences among treatment groups using one-way analysis of variance (ANOVA) with Tukey HSD post-hoc test (GraphPad Prism 6).

4. Results

In MG, the AChR α1 subunit contains the MIR and is considered the most immunogenic, compared to the other AChR subunits [151]. Therefore, the development of an antigen specific therapy based on the α1 subunit ECD appears promising. The main goal of this project was to investigate the antigen specificity of a novel therapeutic approach against the autoimmune disease MG, using immunized rats as an EAMG model. The primary parameter monitored for the evaluation of the progression of EAMG and assessment of the effect of the treatment was the EAMG score of each animal, which is graded as: 0: no symptoms, 1: no symptoms observed at rest but only after exercise (repetitive grasping of a rack for 30s), 2: symptoms present before exercise, 3: very severe symptoms, hind limb paralysis, and 4: moribund, death. The animals' clinical scoring was done once every week for the first 35 days and every day thereafter. Furthermore, hα1 ECD antibody titers we measured in rat sera samples collected at specific time points after treatment, by RIPA with ^{125}I radiolabeled hα1-ECD.

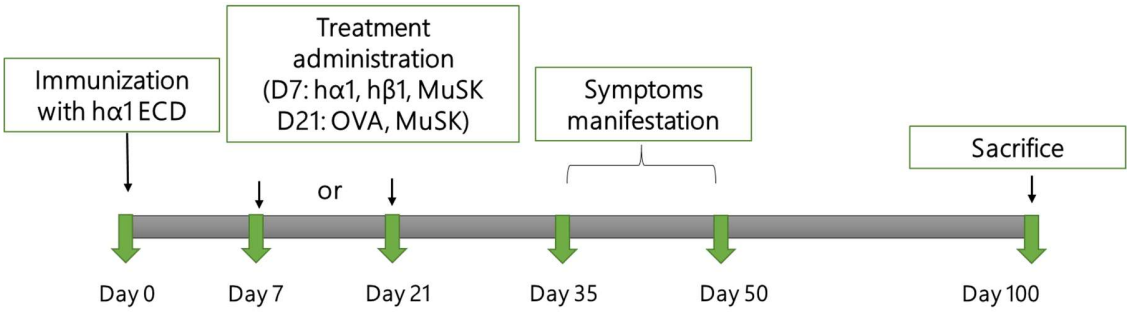
4.1 Evaluation of antigen specificity

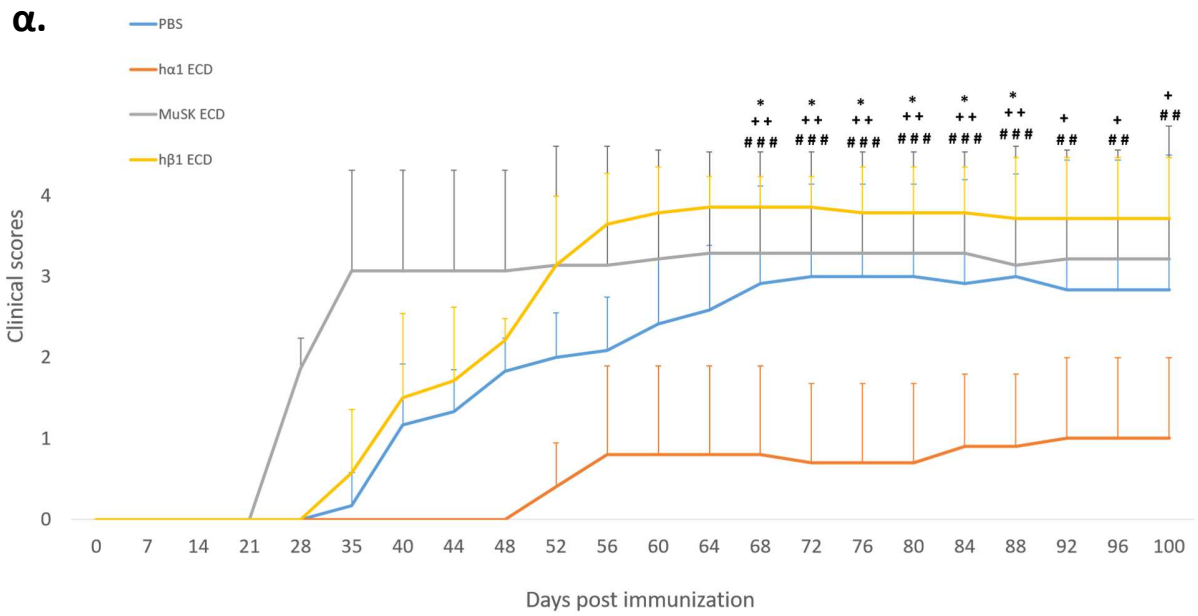
6–7 week old female Lewis rats were obtained from the animal breeding unit of the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute. They were maintained in the large rodent unit of the Department. All experiments described were conducted according to the regulations and guidelines for animal care. Before immunization, the rats were anaesthetized with 3% isoflurane supplemented with oxygen.

In previous studies of the laboratory, we have seen that administration of 100μg hα1 ECD 7 or 21 days after immunization resulted in reduction in the clinical score of the treated rats compared to mock-treated animals. Now we wanted to examine if the effect was specific for hα1 ECD, comparing it, i) to a different subunit ECD of the AChR (hβ1 ECD), ii) to another MG antigen, without homology to the AChR (MuSK) and iii) to an unrelated protein (OVA).

To this end, in a first experiment, the rats were immunized once in the hind footpads with hα1 ECD in CFA. Based on previous results, we administered intravenously different treatment proteins 7 days post immunization (p.i.) and repeating for 12 consecutive days. More specifically, 5 rats received 100μg hα1 ECD, 7 received 100μg MuSK ECD, 7 received 100μg hβ1 ECD and 6 received PBS (mock treatments). In a second experiment, 6 immunized rats received 100μg OVA, 6 received 100μg MuSK ECD and 7 received PBS, on the 21st day p.i. The treatment was administered intravenously at the animals' lateral tail veins.

Initially we evaluated the rats' clinical status by measuring their clinical scores. In the first experiment, the animals that received only PBS as treatment, were severely affected by EAMG, reaching mean clinical score of about 3. On the contrary, the animals that received hα1 ECD had delayed disease onset in comparison to the other three groups and were the least affected, showing signs of mild muscle weakness, with the average clinical score not exceeding 1. The animals treated with MuSK ECD and hβ1 ECD on day 7, which are proteins different to the one that they were immunized with, did not show any improvement, and in fact presented with more severe symptoms, reaching mean clinical scores of 3.29 and 3.86 respectively (**Fig. 1α**). Likewise, in the second experiment administration of MuSK ECD on day 21, or OVA on day 7, resulted in clinical manifestations similar to the PBS group (**Fig. 1β**).





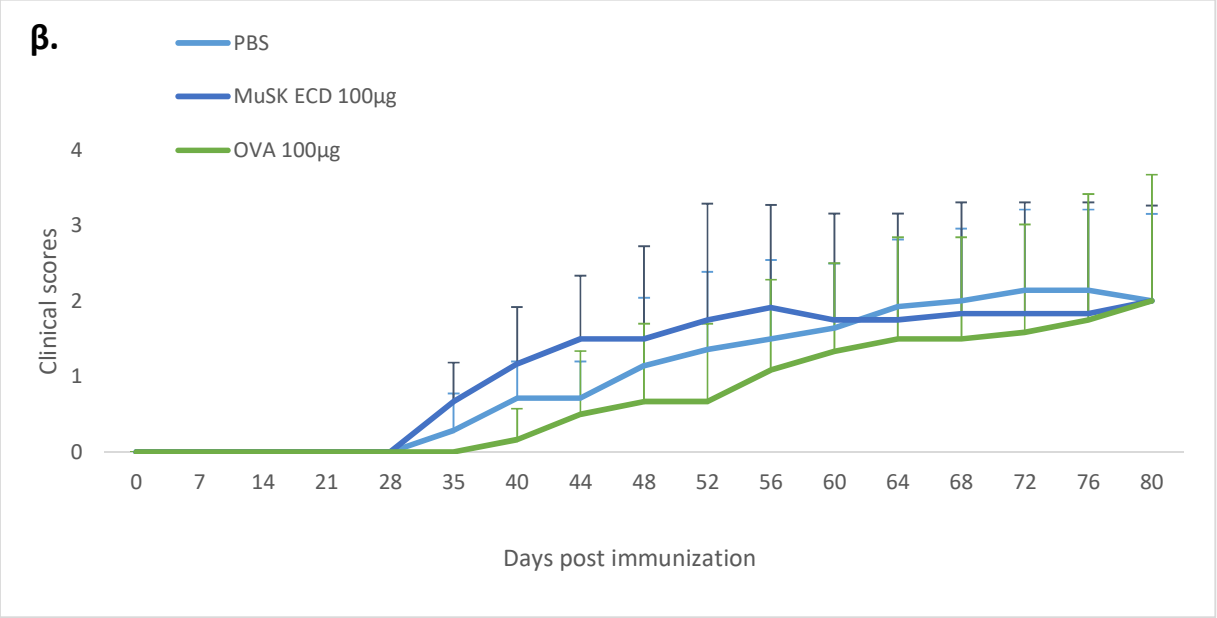


Figure 1: Clinical scores of hα1 ECD immunized rats treated with hα1 ECD, MuSK ECD, hβ1 ECD, or OVA. (a) EAMG scores of the animals treated on day 7 with PBS (blue, N=6), 100µg hα1 ECD (orange, N=5), 100µg MuSK ECD (grey, N=7) and 100µg hβ1 ECD (yellow, N=7). Administration of 100µg hα1 ECD lead to statistically significant lower clinical scores compared to controls and groups treated with 100µg MuSK ECD or 100µg hβ1 ECD. **(β)** EAMG scores of the animals treated with PBS (light blue, N=7), 100µg MuSK ECD on day 21 (blue, N=6) and 100µg OVA on day 7 (green, N=6). * compares 100µg hα1 ECD to PBS, + compares 100µg hα1 ECD to 100µg MuSK ECD, and # compares 100µg hα1 ECD to 100µg hβ1. Statistical significance: *=p<0.05 **=p< 0.005, ***=p<0.0005.

Next, we went on to measure the hα1 ECD serum antibody titers of the different treatment groups. For the first experiment, the serum samples tested were isolated from the treated animals on the 30th and 70th day after the immunization (i.e. 11 and 51 days after treatment termination respectively). When measuring the serum antibody titers from blood samples of the 30th day, we did not observe any difference between the various treatment groups. Similarly, on the 70th day, we did not see significant differences of hα1 ECD serum antibody titers, despite the seemingly elevated levels of the animals treated with 100μg hβ1 ECD (**Fig. 2**).

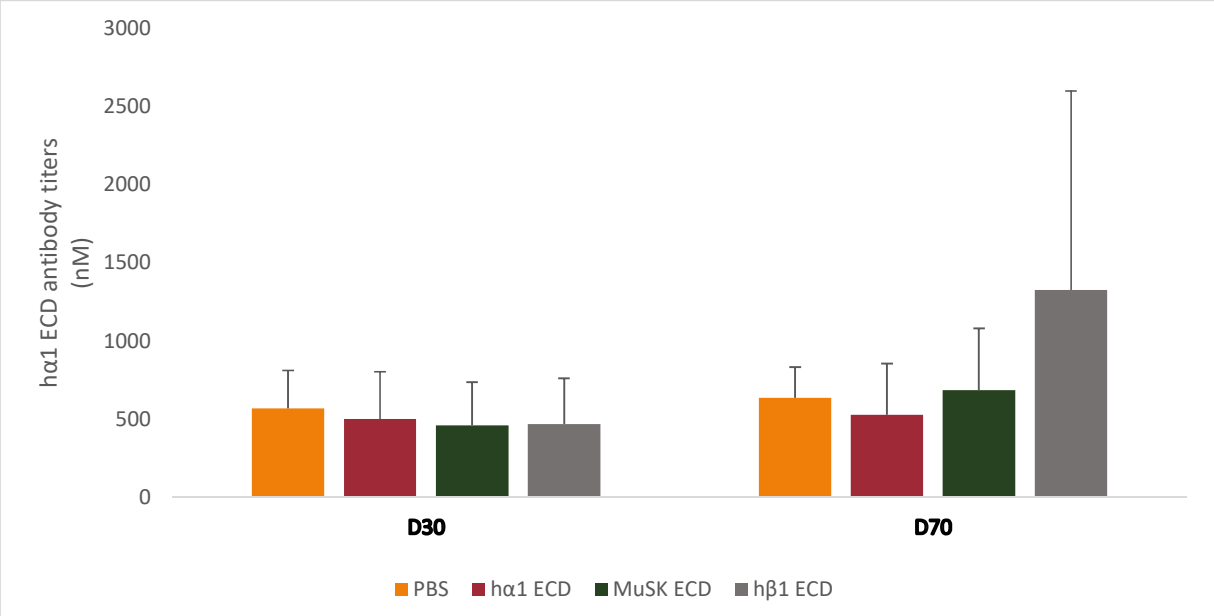


Figure 2: Serum hα1 ECD antibody titers. The mean hα1 ECD antibody titers of the treatment groups, as measured in serum samples taken at day 30 p.i. and day 70 p.i. Treatment groups: PBS treated animals (orange, N=6), animals treated with 100μg hα1 ECD (purple, N=5), animals treated with 100μg MuSK ECD on day 7 (green, N=7) and animals treated with 100μg hβ1 ECD (blue, N=7).

For the second experiment, the serum samples tested were isolated from the treated animals on the 43th and 70th day after the immunization, since treatment was initiated later than in the first group. We saw that all three groups did not have significant differences in hα1 ECD autoantibody levels in either of the two time points (**Fig. 3**).

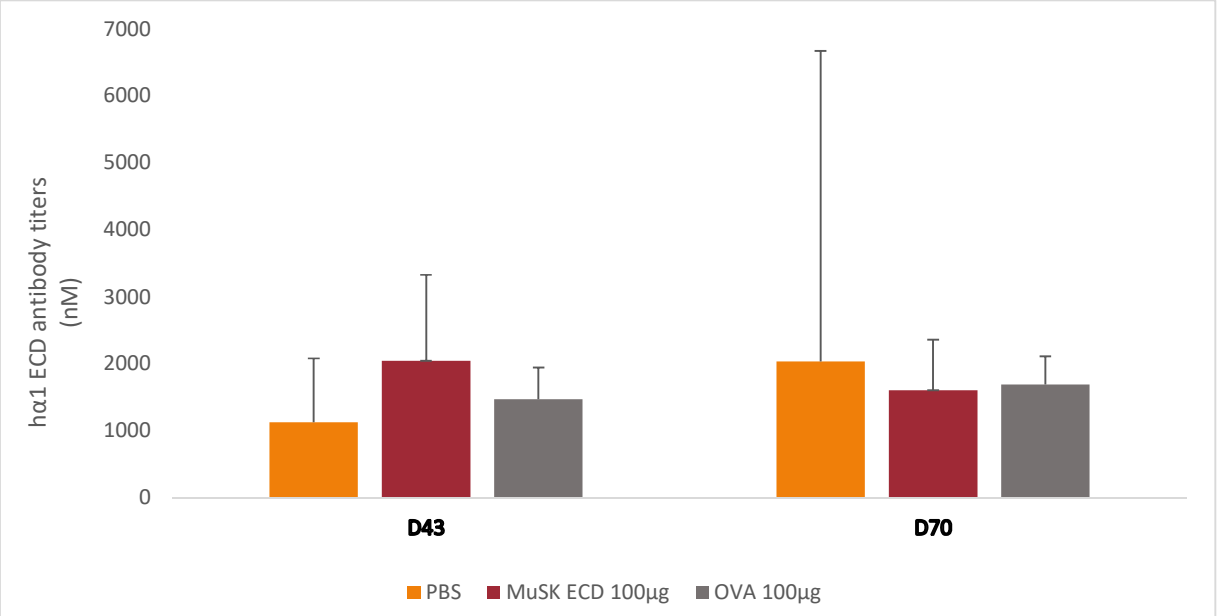


Figure 3: Serum hα1 ECD antibody titers. The mean hα1 ECD antibody titers of the treatment groups, as measured in serum samples taken at day 43 p.i. and day 70 p.i. Treatment groups: PBS treated animals (orange, N=7), animals treated with 100µg MuSK ECD on the 21st day p.i. (purple, N=6) and animals treated with 100µg OVA on the 7th day p.i. (grey, N=6).

Based on the results presented above, we decided to investigate the antibody responses against MuSK ECD and OVA, two proteins irrelevant to the immunization, since the rats that were treated with MuSK ECD on day 7, showed earlier symptom emergence than the control groups.

For the first experiment that consisted of 6 rats treated with PBS, hα1 ECD or MuSK ECD on day 7, we tested the serum samples that were isolated on the 30th day after immunization, using ELISA for the detection of antibodies against MuSK ECD. Interestingly, the rats that were administered with MuSK ECD 7 days after immunization, had developed significant titers of anti-MuSK antibodies (Fig. 4).

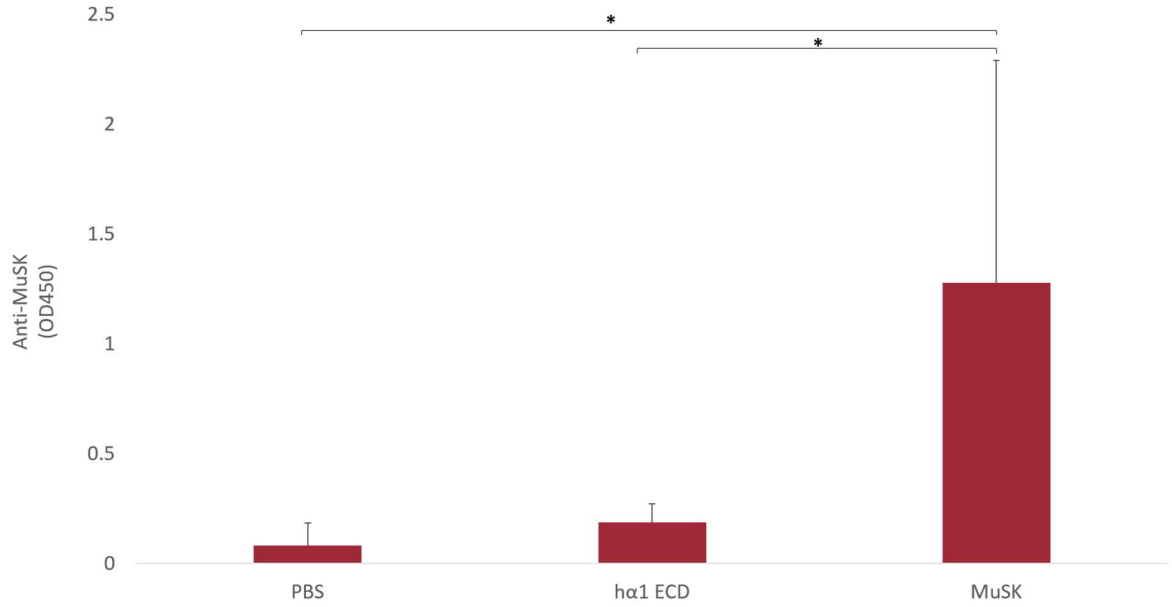


Figure 4: ELISA analysis of anti-MuSK titers in sera from rats. Sera from rats immunized with hα1 ECD and treated with PBS (N=6), 100μg hα1 ECD (N=5) or 100μg MuSK ECD (N=7), were tested for the presence of MuSK antibodies. Statistical significance: *=$p<0.05$.

Following, we used ELISA for the detection of antibodies against OVA in the serum samples of rats treated with PBS, MuSK ECD or OVA on day 7. The groups that were treated with PBS or hα1 showed no OVA serum antibody titers, in contrast to the animals that received OVA as treatment intravenously, that appear to have significant serum OVA antibody titers (**Fig. 5**).

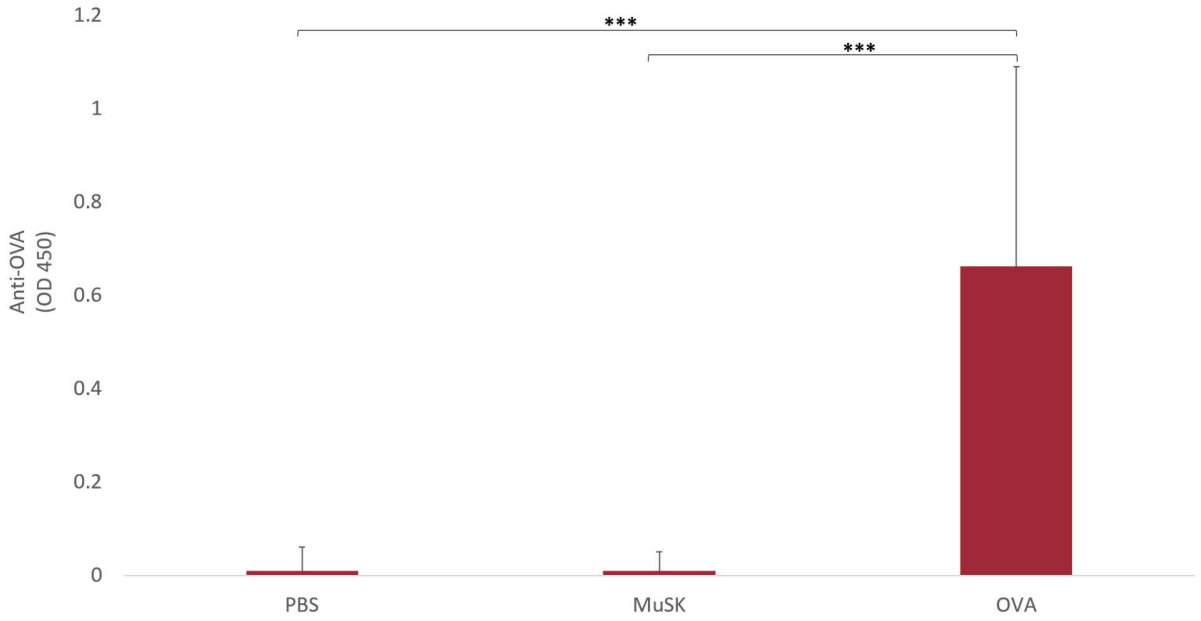


Figure 5: ELISA analysis of anti-OVA antibody titers in sera from rats. Sera from rats immunized with hα1 ECD and treated with PBS (N=7), 100μg MuSK ECD (N=6) or 100μg OVA (N=6), were tested for the presence of OVA antibodies. Statistical significance: ***=p<0.0005.

4.2 Assessment of the contribution of epitope spreading

For the next set of experiments, we wanted to determine the potential effect of epitope spreading on the therapeutic effect. In a first experiment rats were immunized once in the hind footpads with a mixture of AChR subunit ECDs (80μg of each hα1, hβ1, hγ and hε) in CFA. We then administered intravenously different treatments, 7, 21 and 40 days p.i. for 12 consecutive days. In more detail, 6 rats received only PBS as treatment, 6 received 100μg hα1 ECD on the 7th day p.i., 7 received 100μg hα1 ECD on the 21st day p.i., 7 received 500μg hα1 ECD on the 40th day p.i and finally, 6 received ECD mix (100μg of each hα1, hβ1, hγ and hε) on the 21st day p.i. Their monitoring and sample gathering was done at the appointed time points as previously described.

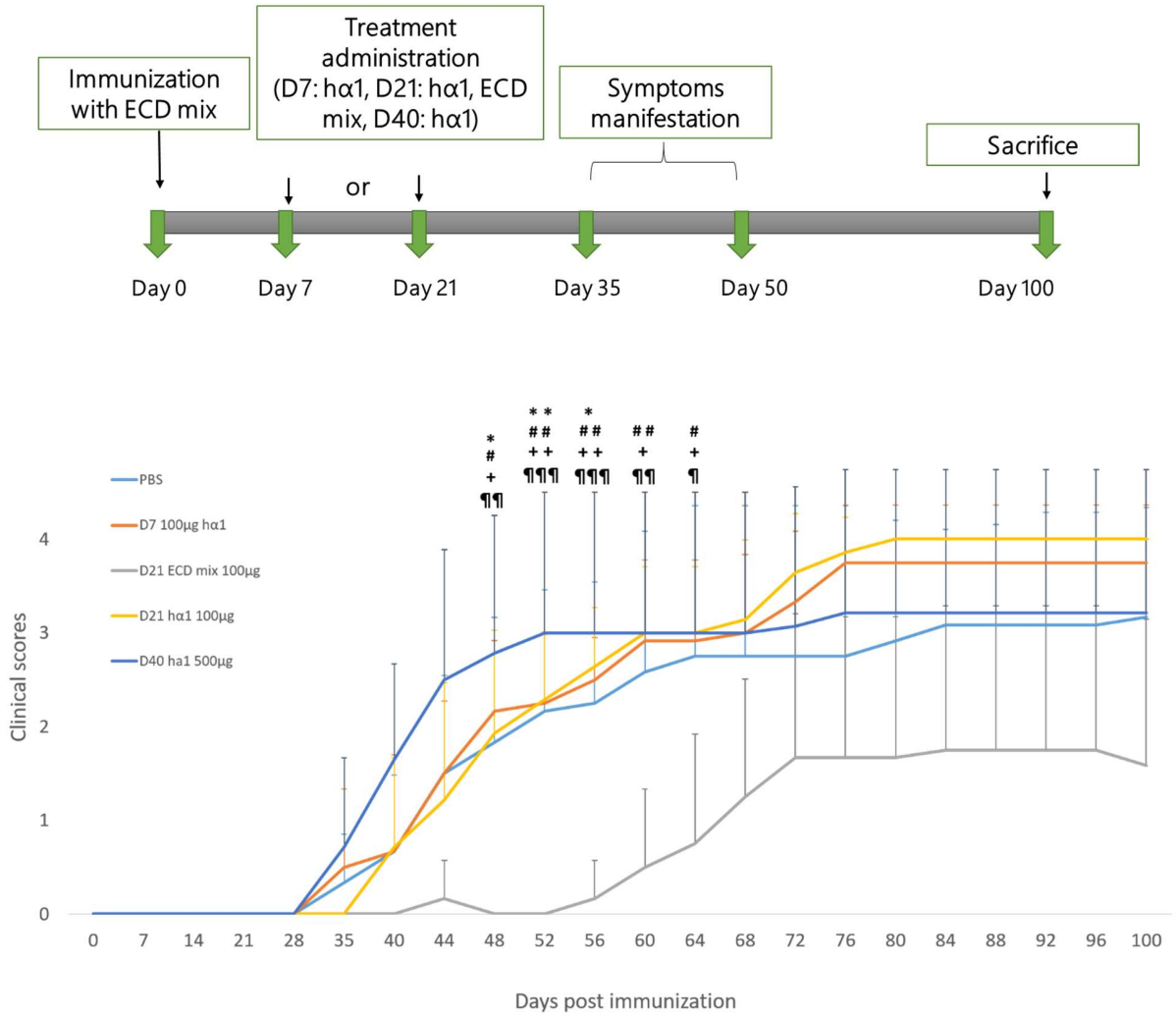


Figure 6: Clinical scores of ECD mix immunized rats treated intravenously with PBS, hα1 ECD or ECD mix. The lines represent the EAMG scores of the animals treated only with PBS (light blue, N=6), 100µg hα1 ECD on the 7th day p.i (orange, N=6), 100µg each ECD mix on the 21st day p.i (grey, N=6), 100µg hα1 ECD on the 21st day p.i (yellow, N=7) and 500µg hα1 ECD on the 40th day p.i. (blue, N=7). * compares ECD mix to PBS, +compares ECD mix to D7 hα1 ECD, # compares ECD mix to D21 hα1 ECD, ¶ compares ECD mix to D42 hα1 ECD. *=p<0.05, **=p<0.005, ***=p<0.0005.

We evaluated the rats' clinical status by measuring their clinical scores. The rats that received PBS or hα1 ECD at 7, 21 or 40 day p.i. as treatment, were severely affected by EAMG, in contrast with the animals that were treated with the ECD mix (Fig. 6). More specifically, the rats that were treated only with PBS reached a mean clinical score of 3.3, while the animals treated with hα1 ECD on the 7th, 21st or 40th day p.i. did not show significant

improvement, with mean scores for these groups 3.75, 4 and 3.2 respectively. On the other hand, the animals treated with 100μg ECD mix on the 21st day p.i. had delayed disease onset in comparison to the other four groups, and were the least affected from EAMG, showing signs of moderate muscle weakness, with the average clinical score not exceeding 1.75.

As previously, we measured the serum hα1 ECD antibody titers of the different treatment groups. The serum samples tested were isolated from the treated animals in the 38th and 70th day after immunization. All treatment groups showed similar hα1 ECD serum antibody titers on day 38 after immunization. However, on the 70th day p.i., the sera isolated from animals that received PBS and 500μg hα1 ECD on day 40 p.i., showed an increase of hα1 ECD serum antibody titers (**Fig. 7**). The groups that received 100μg hα1 ECD on the 7th day p.i., 100μg hα1 ECD on the 21st day p.i. and 100μg ECD mix on the 21st day p.i., had similar antibody titers.

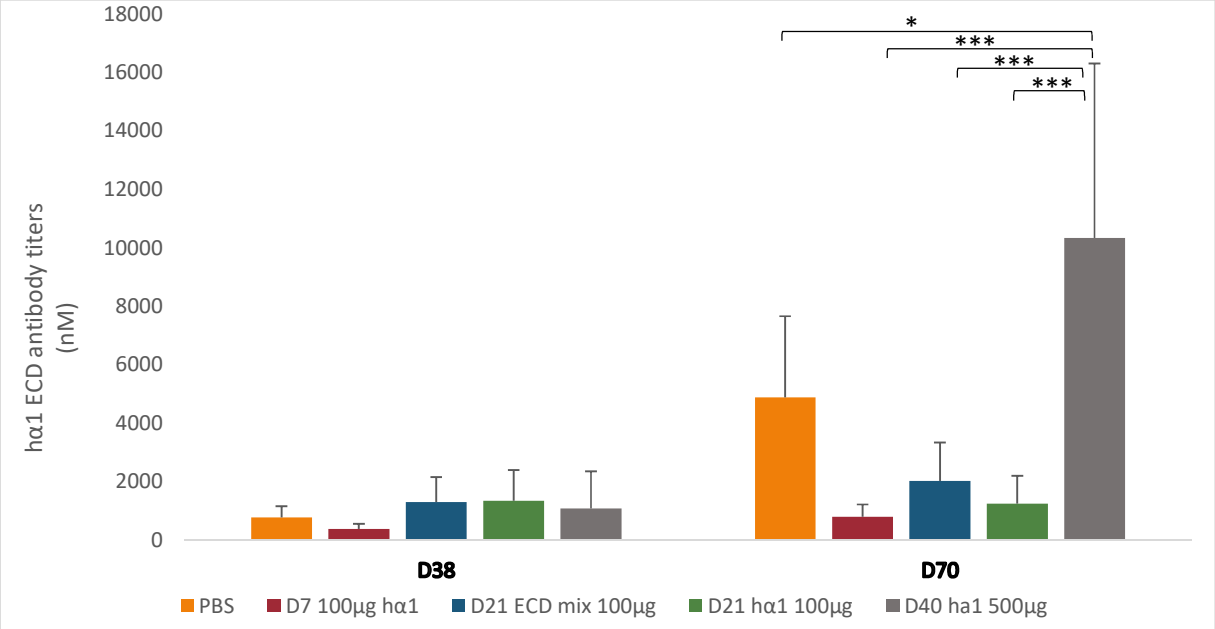
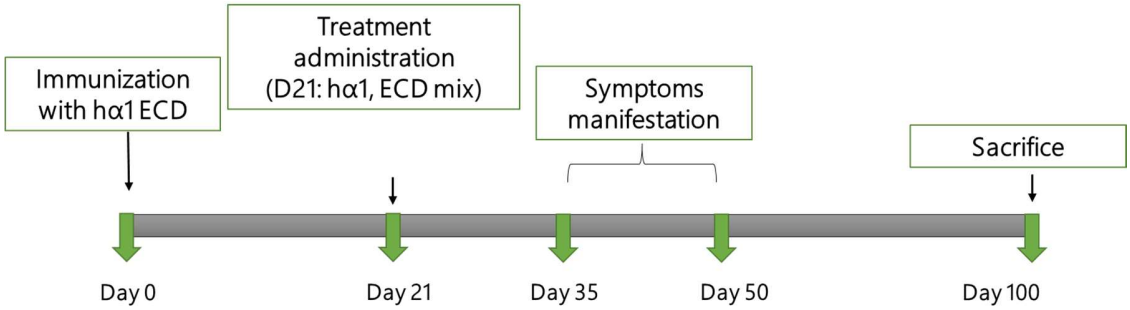


Figure 7: Serum hα1 ECD antibody titers. The mean hα1 ECD antibody titers of the treatment groups, as measured in serum samples taken at day 38 p.i. and day 70 p.i. Treatment groups: PBS treated animals (orange, N=6), animals treated with 100μg hα1 ECD on the 7th day p.i (purple, N=6), animals treated with 100μg ECD mix on the 21st day p.i (blue, N=6), animals treated with 100μg hα1 ECD on the 21st day p.i (green, N=7) and animals treated with 500μg hα1 ECD on the 40th day p.i. (grey, N=7). Statistical significance: *=$p<0.05$, ***=$p<0.0005$.

In a next experiment the rats were immunized with hα1 ECD in CFA. We then administered intravenously at the lateral tail veins different treatment proteins 21 days p.i. for 12 consecutive days. More specifically, 7 rats received PBS, 7 received 100μg hα1 ECD, 7 received 100μg ECD mix (20μg each: hα1, hβ1, hγ, hδ, hε) and 7 rats received 500μg ECD mix (100μg each ECD). Their monitoring and sample gathering was done at the appointed time points as previously described.



The rats that were treated with PBS showed EAMG symptoms with the mean clinical score reaching 2.24. On the other hand, the rats that received 100μg hα1 ECD, 100μg ECD mix and 500μg ECD mix showed similar behavior, with slightly delayed disease manifestation, and mean clinical scores 1.5, 1.4 and 1.6 respectively.

Subsequently, we went on to examine the potential effect of the different therapeutic approaches on the serum antibody titers. The serum samples tested were isolated from the treated animals on the 43rd and 70th day after the immunization. The groups that were treated with 100μg hα1 ECD, 100μg ECD mix and 500μg ECD mix, showed high hα1 ECD serum antibody titers on the 43rd day, which is decreased on the 70th day, to levels similar to the PBS group (Fig. 9), but none of the groups differences was statistically significant.

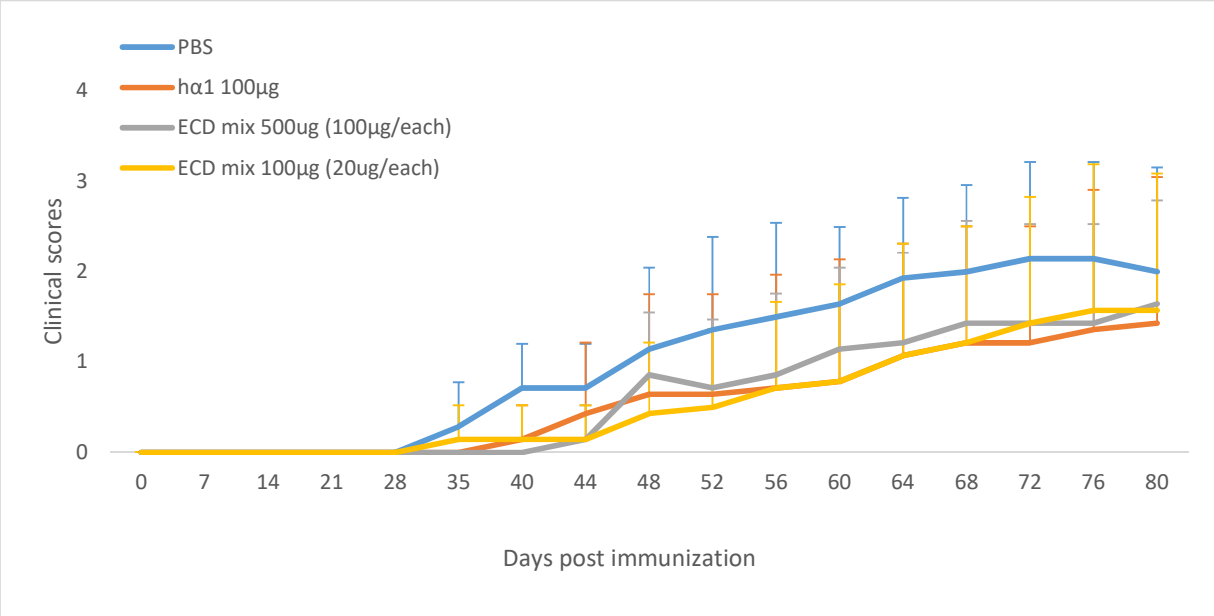


Figure 8: Clinical scores of rats treated intravenously with PBS, hα1 ECD or ECD mix. EAMG scores of the animals treated only with PBS (light blue, N=7), 100µg hα1 ECD on the 21st day p.i (orange, N=7), 500µg ECD mix on the 21st day p.i. (grey, N=7), 100µg ECD mix on the 21st day p.i. (yellow, N=7).

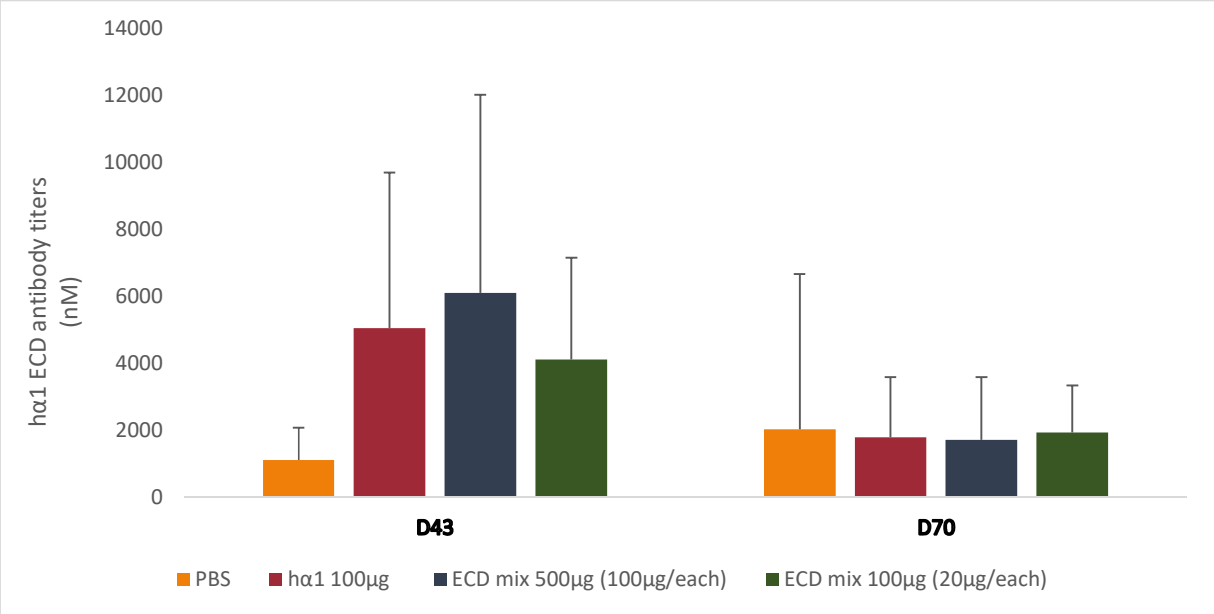


Figure 9: Serum hα1 ECD antibody titers. The mean hα1 ECD antibody titers of the treatment groups, as measured in serum samples taken at day 43 p.i. and day 70 p.i. Treatment groups: PBS treated animals (orange, N=7), animals treated with 100µg hα1 ECD on the 21st day p.i (purple, N=7), animals treated with 500µg ECD mix on the 21st day p.i. (blue, N=7) and animals treated with 100µg ECD mix on the 21st day p.i. (green, N=7).

5. Discussion

MG is a prototypical antibody mediated, T-cell dependent autoimmune disease caused by antibodies targeting components of the neuromuscular junction. In the majority of patients (about 85%), the antibodies are directed against the acetylcholine receptor (AChR) in the post-synaptic membrane. This process leads to damage of the postsynaptic muscle membrane, impaired signal transduction and, consequently, to muscle weakness and fatigability. The exact cause that leads to the initiation of the autoimmune response to nAChR is still unknown. Various therapeutic strategies have been used over the years to alleviate MG symptoms. These strategies aim at improving the transmission of the nerve impulse to muscle or at lowering the immune system activity with corticosteroids or other immunosuppressant drugs. Since the current treatments for MG are not specific and can cause adverse side effects, it would be very beneficial to develop an antigen-specific therapy that would target only the pathogenic components of the immune system.

So far, past studies have achieved tolerance in EAMG, when the nAChR or its fragments were administered nasally or orally. In more detail, it has been demonstrated that nasal administration of recombinant fragments of the hα1 ECD in female Lewis rats, prior to or after immunization with nAChR isolated from the electric organ of Torpedo californica (TAChR), results in amelioration of EAMG symptoms in the treated animals (lower mean clinical scores, decreased serum antibody titers) [162]. The same team has also attempted to delineate the underlying mechanisms of the therapeutic effect observed. Their observations suggest, that the mechanism responsible for the induction of nasal tolerance, was based on active suppression involving a shift from Th1 (IL-2, IL-12, IFN-γ) to a Th2 (IL-10) and Th3 (TGF-β) regulated nAChR-specific responses through the downregulation of co-stimulatory factors [169]. The therapeutic potency of recombinant fragments of the hα1 ECD when administered orally has also been investigated. The data from this series of experiments were similar to previous observations, as active suppression seems to be implicated also in the

induction of oral tolerance. In line with these results, it has been shown that the nasal administration of TAChR diminished the incidence and severity of clinical muscular weakness, but also significantly suppressed AChR-specific B and T cell responses in both peripheral blood and lymphoid organs [158]. Additionally, a switch in nAChR specific IgG antibody subclasses from IgG2 to IgG1 was reported [169]. However, the majority of these studies have been performed on animals that were immunized with the TAChR.

In addition, there is evidence to suggest that nasal administration of synthetic peptides failed to induce nasal tolerance to EAMG [167]. In more detail, peptides of TAChR that were selected based on specific considerations were used: 1. α 61–76, being the main immunogenic region (MIR) for anti-AChR antibodies, was selected to study if the unresponseness can be established directly by focusing on the antibody response, the final pathway in the development of MG; 2. α 100–116, which is considered as an important epitope for T cell responses in EAMG rats and provides help in driving the disease-causing anti-AChR antibody response; 3. α 146–162, which is a predominant T cell epitope in mice and important for induction of tolerance to EAMG in neonatal mice, to define if it is effective in tolerance induction in rats, and to study the cross-reactivity between different animal species; 4. α 354–367, which is effective in tolerance induction in rabbits, to examine the effect in rats, and potential cross-reactivity between species; 5. α 261–277, which is an unbiased peptide to be used as control; 6. the mixture of all these epitopes, to study if it is more useful than the single peptides. However, it was shown that these peptides did not induce tolerance after administration by the nasal route, irrespective if the dose was lower, the same or higher than that of whole AChR that is effective in preventing EAMG. The possible reasons suggested to be behind these results were that the suppression induced by one epitope is not strong enough to overcome the responses induced by the many other epitopes by 'spreading' mechanism, and removal of one set of T cells, regardless of their 'dominance' in the response, may allow other less prominent T cell reactivities, initially lower in the hierarchy of activities in the T cell repertoire, to move forward in predominance.

As EAMG is an animal model for a human disease, the efficiency of antigen specific therapies evaluated on animal models that are induced by non-human sequences could be underestimated [132]. Until now one research team has managed to ameliorate EAMG symptoms manifestations by administering intravenously solubilized MHC Class II and TAChR α1 subunit complexes [165]. Therefore, it is essential to elucidate the immunological mechanisms involved following i.v. antigen administration.

Our experimental design was based on past studies of the laboratory, which have achieved treatment in EAMG, when the nAChR hα1 ECD was administered intravenously to rats immunized with hα1 ECD. Briefly, it was found that intravenous antigen injection ameliorated EAMG symptoms in a dose dependent manner, and that the effect was stronger when treatment was initiated earlier post immunization.

Initially, we conducted experiments in order to determine the specificity of the therapeutic protein. To this end, we administered intravenously hα1 ECD, hβ1 ECD, MuSK ECD and OVA to female Lewis rats, 7 days p.i. with hα1 ECD. At day 7 after the immunization, the immune response is established but at its initial stages. In our EAMG model the symptoms manifest between 35 and 50 days after the immunization. We observed that the animals that were treated with hα1 ECD had delayed disease manifestations and milder symptoms, confirming the previous results of the laboratory. The animals that received hβ1 ECD showed no improvement of EAMG symptoms, although hβ1 is a component of AChR, and could potentially contribute to a positive therapeutic effect, due to epitope spreading. Epitope spreading takes place when the targets of immune response do not remain fixed and they are directed against different epitopes within a protein. Epitope spreading plays a significant role in the development of autoimmune diseases when it is initiated as a result of tissue damage but on the other hand, it could be crucial for protective immune responses since it would enhance their efficiency [170], [171]. This is in contrast with the positive results we have when rats are immunized and treated with hα1 ECD since treatment with hα1 ECD can be suppressive for hα1 ECD-induced disease. We assume that the administration of an

AChR peptide or domain different than the one that the animals are immunized with, does not have a significant therapeutic effect, since we don't observe epitope spreading between the different subunits of the AChR.

Even though MuSK takes part on the formation and maintenance of NMJs, it's not a component of the AChR, so it's not involved in AChR MG. The animals that were treated with MuSK ECD, showed somewhat earlier disease onset and slightly more severe symptoms than the control group that were treated with PBS. The treatment affected negatively the animals, since they produced antibodies against MuSK ECD following i.v. administration, possibly owing to the inflammatory environment created due to prior immunization with CFA. This hypothesis was also confirmed by ELISA, which showed that the animals that received this treatment, had high MuSK antibody titers, although they were not immunized with MuSK but with hα1 ECD. The animals that were treated with OVA, an irrelevant protein for the disease and different from the one that the animals were immunized with, as expected showed no improvement of EAMG symptoms. With ELISA, we confirmed that these animals too had high OVA antibodies, further supporting the assumption that the inflammatory environment created from the immunization with CFA, is conducive for the production of antibodies against antigens administered intravenously in the days following immunization.

Previous studies have shown a weak correlation of hα1 ECD antibodies and a moderate of rat-AChR antibodies with disease severity [172]. However, more recent data support the lack of correlation between either hα1ECD or rat-AChR autoantibody titers and disease severity following intravenous treatment with antigen (unpublished data). Therefore, only the hα1 ECD antibodies were measured for assessment of the immunization. Indeed, the results from the hα1 ECD serum antibody titers, did not correlate with the clinical scores following treatment, so they were not indicative of disease severity, and they did not provide significant insight to the immunological changes taking place due to treatment. This is in contrast to the MuSK-EAMG, since it seems to be a strong correlation between anti-MuSK

antibody and disease severity [159]. Further studies aimed at dissecting antibody subclasses, epitope specificities, binding affinity or antibody activity may prove more informative.

Next, we wanted to investigate the specificity of a subunit of the AChR as a therapeutic approach, for animals that were immunized with a mix of ECDs. This is important since many MG patients have antibodies against more than one AChR subunit [105] and it would be advantageous for treatment if a single subunit was sufficient to induce tolerance for the whole AChR. We thus administered intravenously hα1 ECD and ECD mix on different treatment initiation dates, 7, 21 and 40 days p.i.. We chose to use hα1 ECD subunit as treatment, since it contains the MIR area, so it is a major MG autoantigen. From our results, we observed that the animals treated with hα1 ECD were severely affected from EAMG, regardless of the treatment initiation day. Even the administration of higher amount of hα1 ECD did not lead to a more robust effect on the treated animals. Since EAMG in this experiment was caused by immunization with all the AChR subunits, administration of hα1 ECD alone was not sufficient as a therapeutic approach. On the other hand, the ECD mix treatment was beneficial, even when it was administered 21 days p.i. with the animals showing milder symptoms than PBS treated controls.

Our goal with this experimental approach, was to determine whether treatment with hα1 ECD would cover additional AChR subunits by epitope spreading. Our data suggest that the phenomenon of epitope spreading does not contribute to a significant degree, thus it appears that treatment requires all the pathogenic epitopes for a robust effect, further corroborating the previous results from hβ1 ECD treatment of hα1 ECD immunized animals.

Another important aspect we wanted to investigate was the efficacy of all subunits of the AChR ECDs administered together, on animals that were immunized with only one of them. We had observed from previous results, that the treatment with hα1 ECD on a later time point (day 21 and 40 p.i.) on animals immunized with hα1 ECD, does not have optimal therapeutic results (unpublished laboratory data). The reduced therapeutic efficacy of hα1 ECD at later time points could be the result of epitope spreading following immunization,

so administration of hα1 ECD alone is no longer sufficient, since it does not have therapeutic effect on different subunits. To investigate this, we administered intravenously hα1 ECD or an ECD mix of the hα1, hβ1, hγ and hε subunits 21 days p.i., in different doses. The rats that received 100μg hα1 and ECD and 500μg ECD mix showed similar behavior, with delayed disease manifestation and milder symptoms than the PBS group. These two therapeutic approaches have in common the presence of hα1 ECD, with the difference that the ECD mix contains all the other AChR subunits. From these results, since the ECD mix had a similar clinical outcome with the hα1 ECD, we assume that even if epitope spreading is observed as a phenomenon, it does not play a major role towards pathology in our model. It is thus likely that other mechanisms are responsible for the reduced treatment efficiency of hα1 ECD observed at later time points. More specifically, the accumulation of damage on muscles could be so severe that it cannot be easily recovered. In addition, long-lived immune memory cells could be produced during disease progression, decreasing the efficacy of the treatment. Data from multiple studies on mucosal tolerance support that low doses of a tolerogen favor active suppression, while high antigen doses favor clonal deletion and clonal anergy. It remains to be seen if such mechanisms are also relevant in i.v. treatment.

It has been shown that the administration of a mixture of extracellular and cytoplasmic domains of AChR (α1, β1, γ, δ and ε subunits) orally prevents the induction of EAMG and reduces the severity of ongoing EAMG, whilst nasally is highly efficient in preventing EAMG but ineffective at treating ongoing EAMG [157] The suppression of ongoing EAMG, is more difficult and requires a nasal administration of ten-fold higher amounts of TAChR compared to the amounts required for prevention of EAMG. These experiments were conducted in rats immunized with Torpedo AChR and the administration route of the therapeutic approach was nasal or oral, in contrast with the intravenous route that was used in our experiments. It is important to evaluate the selection of peptides that are used for treatment, because an antigen can exacerbate, rather than suppress, the pathogenesis of autoimmune diseases when the antigen is exposed to compartments containing more helper than regulatory

lymphocytes. The heterogeneity of T- and B-cell epitopes of AChR could challenge the treatment of the disease by using synthetic peptides that depend on one or two particular epitopes. This is especially a problem if the therapeutic antigen also contains pathogenic B cell epitopes that could provoke the production of pathological autoantibodies [157]. It was shown that removal of two major B-cell epitopes from the thioredoxin- fused human a1 1–210 turns it from an exacerbator of EAMG into an effective tolerogen via oral administration. It was suggested that the usage of cytoplasmic domains of human AchR could have therapeutic effect if the autoimmune response is diverted to the cytoplasmic surface and generate regulatory T cells. The therapy using only cytoplasmic domains would completely avoid the liability of introducing potentially pathogenic B cell epitopes [157]. However, it seems that intravenous administration alleviates those problems, since the protein used in our experiments was produced in yeast, and has been shown to have near native conformation, but despite this, it has been found an efficient therapeutic in EAMG. Taking into consideration all of the above and the results obtained from our experiments so far, we suggest that the therapeutic effect of a peptide administered intravenously is antigen specific. Thus the presentation of adverse effects are unlikely, but on the other hand administration of a protein different than the one the animals are immunized with, does not have a therapeutic effect. It is important that the therapeutic antigen is relevant with the disease-inducing autoantigen since as it was shown, irrelevant antigens are ineffective in treating EAMG. Therefore, it is important to define the autoantigen(s) responsible for the pathology of individual MG patients and administer the relevant antigen as a therapeutic approach. The robust effect of hα1 ECD when administered at an early stage after the induction of the disease, leading to the amelioration of EAMG paves the way for its further characterization as a novel therapy for MG. The results obtained from our experiments so far, even though at a preliminary stage, strongly support the development of a treatment that has the capacity to reprogram the immune system and eliminate the pathogenic factors that result in the manifestation of MG symptoms. It is important to understand in depth and

characterize the underlying molecular mechanisms that are involved in MG in order to develop an antigen-specific therapy that will target only the pathogenic and disease-related immune cells. Finally, further studies are required in order to fully characterize the clinical potential and the safety of these therapeutic approaches, such as long-term monitoring of treated animals and comparison of therapeutic efficacy to current mainstay treatments for MG. Importantly, any findings and advances made for MG could be applied to other related antibody-mediated diseases, increasing the impact of these studies.

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