

Autoantibodies to Lipoprotein-Related Protein 4 in Patients With Double-Seronegative Myasthenia Gravis

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Objectives: To determine whether patients with myasthenia gravis (MG) have serum antibodies to lipoprotein-related protein 4 (LRP4), a newly identified receptor for agrin that is essential for neuromuscular junction formation, and to establish whether such antibodies contribute to MG pathogenesis.

Design: Serum samples from patients with MG with known status of serum antibodies to the acetylcholine receptor (AChR) and muscle-specific kinase (MuSK) and serum samples from control subjects (healthy individuals and individuals with other diseases) were tested for antibodies to LRP4. Serum samples with such antibodies were tested to determine whether they had the ability to inhibit 2 different functions of LRP4 at the neuromuscular junction.

Setting: Serum samples were collected at the Hellenic Pasteur Institute and Wayne State University. Samples were tested for LRP4 autoantibodies at Georgia Health Sciences University. Other immunoreactivities of the samples were tested at the Hellenic Pasteur Institute, Athens, Greece, or processed through University Laboratories of the Detroit Medical Center, Michigan.

Patients: The study included 217 patients with MG, 76 patients with other neurologic or psychiatric diseases, and 45 healthy control subjects.

Results: Anti-LRP4 antibodies were detected in 11 of 120 patients with MG without detectable anti-AChR or anti-MuSK antibodies (double seronegative) and in 1 of 36 patients without anti-AChR antibodies but with anti-MuSK antibodies, but they were not detected in any of the 61 patients with anti-AChR antibodies. No healthy control subjects and only 2 of the 76 control patients with neurologic disease had anti-LRP4 antibodies. Serum samples from patients with MG with anti-LRP4 antibodies were able to inhibit the LRP4-agrin interaction and/or alter AChR clustering in muscle cells.


Conclusions: Anti-LRP4 antibodies were detected in the serum of approximately 9.2% of patients with double-seronegative MG. This frequency is intermediate compared with 2 recent studies showing anti-LRP4 antibodies in 2% and 50% of patients with double-seronegative MG from different geographic locations. Together, these observations indicate that LRP4 is another autoantigen in patients with MG, and anti-LRP4 autoantibodies may be pathogenic through different immunopathogenic processes.

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MYASTHENIA GRAVIS (MG) affects about 20 per 100 000 people.¹ Patients with MG show characteristic fatiguable weakness of voluntary muscles including ocular, oral-facial, bulbar, and limb

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muscles and, in more severe cases, respiratory difficulty. In most patients with MG, the disease appears to stem from an autoimmune response against the muscle nicotinic acetylcholine receptor (AChR).

Autoantibodies against AChRs can be detected in approximately 85% of patients with generalized MG.² Evidence from classic experiments indicates that anti-AChR antibodies are pathogenic.³⁻⁵ About 40% of patients who are anti-AChR seronegative have antibodies against muscle-specific kinase (MuSK),^{6,7} a muscle tyrosine kinase critical for neuromuscular junction (NMJ)

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formation and agrin-induced AChR clustering.⁸ Also, MuSK antibodies have been shown to be pathogenic. They inhibit AChR clustering.⁶ Immunization with the extracellular domain of MuSK causes experi-

mental autoimmune MG in rodents.⁹⁻¹¹ Moreover, passive transfer of IgG from patients with anti-MuSK antibody-positive MG causes experimental autoimmune MG.^{12,13} The nature of the target antigen or antigens in double-seronegative MG (ie, without anti-AChR or anti-MuSK antibodies) is unclear, although the NMJ impairment appears to be involved. Recently, it has been reported that some of these individuals have anti-AChR antibodies of low avidity, which can be demonstrated *in vitro* by binding to AChR clusters.¹⁴

Lipoprotein-related protein 4 is a member of the low-density lipoprotein receptor family and contains a large extracellular N-terminal region that possesses multiple epidermal growth factor and low-density lipoprotein receptor repeats, a transmembrane domain, and a short C-terminal region without an identifiable catalytic motif.^{15,16} Recent studies indicate that LRP4 serves as a receptor of agrin^{17,18} and is required for agrin-induced activation of MuSK and AChR clustering and NMJ formation.¹⁹ Moreover, heterologous expression of LRP4 in non-muscle cells enables agrin-binding activity and reconstitutes agrin signaling including MuSK activation and Abelson murine leukemia viral oncogene homologue 1 phosphorylation.^{17,18} Evidence indicates that LRP4 interacts directly with agrin and MuSK.^{17,18} In a working model, agrin binds to LRP4 and increases its interaction with MuSK to activate the kinase and initiate downstream signaling cascades for AChR clustering.¹⁷

Considering the critical role of LRP4 in NMJ formation and the fact that many agrin-signaling components have been implicated in muscular dystrophies, we hypothesized that LRP4 may be an autoantigen in patients with MG without antibodies to previously identified components of the NMJ. While our work was in progress, Higuchi et al²⁰ reported that 2% of Japanese patients with double-seronegative MG have anti-LRP4 antibodies and Pevzner et al²¹ reported that 6 of 13 patients with double seronegative MG tested positive for anti-LRP4 vs 0 of 4 healthy control subjects. We found that LRP4 autoantibodies were detected in 9.2% of patients with double-seronegative MG but not in those with anti-AChR or anti-MuSK autoantibodies. Furthermore, we found high specificity of anti-LRP4 autoantibodies for MG, exploring serum samples of patients with many neurologic and psychiatric diseases. We explored mechanisms by which LRP4 autoantibodies may alter the agrin-signaling pathway. Our results suggest pathophysiologic effects of LRP4 autoantibodies on AChR clustering and the agrin-LRP4 interaction. These results may provide insight into pathological mechanisms of double-seronegative MG.

METHODS

PATIENT SERUM SAMPLES

Serum samples from the Hellenic Pasteur Institute and Wayne State University were collected for diagnostic purposes or as part of approved research studies and had previously been tested for anti-AChR and anti-MuSK autoantibodies. Patients with MG and healthy volunteers gave their written informed consent. Anti-AChR and anti-MuSK antibody titers at the Hellenic Pasteur Institute were determined by anti-AChR and anti-MuSK anti-

Table. Non-Myasthenia Gravis Diseases in Patients

| Disease | Patients, No. |
|---|---------------|
| Amyotrophic lateral sclerosis | 9 |
| NMO | 16 |
| Schizophrenia | 10 |
| Other neurologic disorders | |
| Acute motor axonal neuropathy | 1 |
| Chronic inflammatory demyelinating polyneuropathy | 3 |
| CNS Lyme disease | 1 |
| CNS primary Sjögren syndrome | 2 |
| GBS with concomitant Isaac syndrome | 1 |
| GBS, acute inflammatory demyelinating polyneuropathy | 6 |
| Inflammatory myelopathies, not transverse myelitis or NMO | 3 |
| Multiple sclerosis | 18 |
| Neurosarcoidosis | 1 |
| Paraneoplastic neuropathies | 2 |
| Peripheral neuropathy of unknown etiology | 1 |
| Polychondritis with CNS vasculitis | 1 |
| Polymyositis with primary Sjögren syndrome | 1 |

Abbreviations: CNS, central nervous system; GBS, Guillain-Barré syndrome; NMO, neuromyelitis optica.

body radioimmunoprecipitation assay kits (RSR Ltd) according to the manufacturer's instructions with slight modifications as previously described.^{22,23} Anti-AChR titers below 0.2nM/L and above 0.5nM/L are considered negative and positive, respectively, whereas values between 0.2nM/L and 0.5nM/L are considered ambiguous. Similarly, anti-MuSK titers below 0.02nM/L and above 0.05nM/L are considered negative and positive, respectively, whereas values between 0.02nM/L and 0.05nM/L are considered ambiguous. Serum samples from Wayne State University were assayed for anti-AChR binding antibodies at ARUP Laboratories (positive, ≥ 5 nM/L) or at the Mayo Clinic (positive, >0.02 nM/L). Anti-MuSK testing was done by Athena Laboratories (MuSK antibody test or quantitative MuSK antibody titers) or by Angela Vincent, MD (Nuffield Department of Clinical Neurosciences, John Radcliffe Hospital, University of Oxford, UK) as part of a multi-institutional study of serum from patients with MG (positives were as defined by Hoch et al⁶). Seropositive MG was defined as being anti-AChR or anti-MuSK positive. Only definitely positive or definitely negative serum samples were examined for anti-LRP4 antibodies. Double-seronegative MG was defined by the documented MG symptoms, findings from neurologic examinations, pharmacologic response to anticholinesterase agents and/or clinical neurophysiological testing, and the concurrent absence of both types of antibodies. Normal control serum samples were obtained from age-matched volunteers serving as control subjects for other studies on MG. In addition, serum samples from patients with other diseases were examined (**Table**). Overall, we tested serum samples from 120 patients with double-seronegative MG (ie, without detectable anti-AChR and anti-MuSK antibodies) together with serum samples from 61 patients with anti-AChR antibodies, 36 patients without anti-AChR antibodies but with anti-MuSK antibodies, 45 healthy control subjects, and 76 control patients with other diseases.

RECOMBINANT PROTEIN PRODUCTION AND PURIFICATION

Constructs encoding full-length rat LRP4 and ecto-LRP4 in pcDNA3.1-Myc/His and alkaline phosphatase/Myc/His-tagged agrin in pAP5 were described previously.¹⁷

DETECTION OF ANTIBODIES TO LRP4

MaxiSorp Immuno 96-well plates (Nunc) were coated with 50 μ L of 1- μ g/mL ecto-LRP4 in the coating buffer containing 50mM carbonate (pH 9.6) at 4°C overnight, washed 6 times with TRIS-buffered saline with Tween 20 (TBST; 0.1% Tween 20 in 50mM TRIS buffer, 150mM sodium chloride, pH 7.6), and incubated with the blocking buffer containing 5% nonfat milk in TBST to block nonspecific binding. Serum samples were diluted 1:10 in the blocking buffer (100 μ L per well) and incubated for 1 hour at 37°C. After being washed with TBST, the wells were incubated with alkaline phosphatase–goat antihuman IgG + IgM + IgA secondary antibody (Abcam) diluted 1:30 000 in TBST at 37°C for 1 hour. Activity of immobilized alkaline phosphatase was measured using an optical density assay (at 405 nm) following incubation in the substrate buffer containing 0.5mM magnesium chloride, 3-mg/mL p-nitrophenyl phosphate, and 1M diethanolamine at room temperature for 30 minutes. Each sample was assayed in duplicate and repeated more than 3 times. Nonspecific signal was determined by optical density reading of wells coated with the coating buffer alone followed by incubation of secondary antibody and substrate. Intra-assay and interassay coefficients of variability were 8.3% and 12.4%, respectively. All samples were examined blindly without previous information of the patients' condition or diagnosis. The cutoff value was set as mean \pm 4 (SD) of control normal human serum samples, representing confidence of 99.99%.²⁴

IMMUNOPRECIPITATION OF LRP4 BY AUTOANTIBODIES

HEK293 cells were transfected by polyethylenimine with Myc-tagged full-length LRP4 as previously described.^{17,25} Lysates (500 μ L, 1 mg/mL protein, in radioimmunoprecipitation assay buffer) were incubated with 10 μ L of the serum sample (serum samples 21321, 22212, 23437, and 23473) at 4°C overnight with agitation followed by 2-hour incubation with 50 μ L of protein G beads at 4°C. Bead-immobilized proteins underwent sodium dodecyl sulfate – polyacrylamide gel electrophoresis and Western blotting with anti-Myc antibody.

EFFECTS OF LRP4 POSITIVE SERUM ON AGRIN/LRP4 INTERACTION

MaxiSorp Immuno plates were coated with ecto-LRP4 and incubated with 100 μ L of 0.5 μ M alkaline phosphatase–agrin, a fusion protein of alkaline phosphatase and agrin,¹⁷ together with 10 μ L of LRP4-positive serum (serum samples 21321, 22212, 23437, and 23473) or control normal human serum at 37°C for 1 hour. After being washed, activity of immobilized alkaline phosphatase was measured as previously described with p-nitrophenyl phosphate as the substrate.

EFFECTS OF LRP4 POSITIVE SERUM ON ACHR CLUSTERING

Clustering of AChR was assayed as previously described with minor modifications.^{17,25,26} The C2C12 myotubes were treated with neural agrin (10 ng/mL)¹⁷ together with LRP4-positive serum (1:150 dilution; serum samples 21321, 22212, 23437, and 23473) for 16 hours, fixed in 4% paraformaldehyde, and incubated with 50nM rhodamine-conjugated bungarotoxin (Invitrogen) to label AChR clusters. Myotubes were viewed under a Zeiss epifluorescence microscope and AChR clusters with diameters or an axis of 4 μ m or greater were scored. At least 10 views per dish and at least 2 dishes were scored in each of the 3 independent experiments.

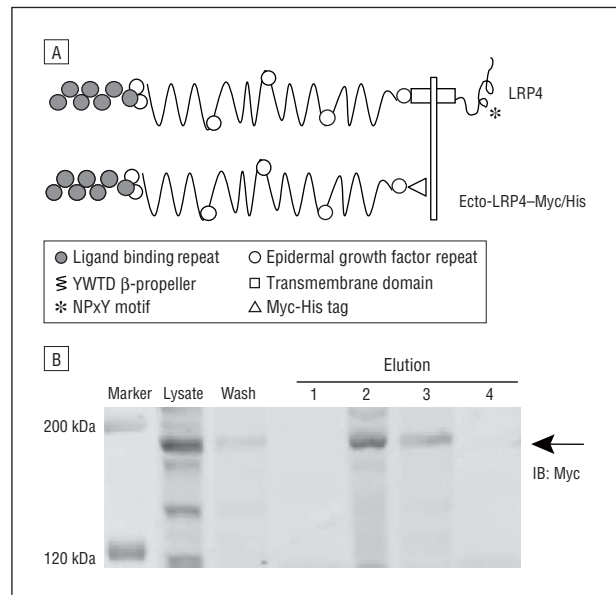


Figure 1. Preparation of ecto-lipoprotein-related protein 4 (LRP4). A, Structures of LRP4 and C-terminus-tagged ecto-LRP4. B, Preparation of ecto-LRP4 (arrow). Ecto-LRP4 was purified from transfected HEK293 cells by affinity chromatography and subjected to Western blotting by anti-Myc antibody.

RESULTS

DETECTION OF ANTI-LRP4 AUTOANTIBODIES IN SERUM SAMPLES OF PATIENTS WITH MG

To determine whether patients with seronegative MG produce anti-LRP4 autoantibodies, we generated Myc-tagged rat ecto-LRP4.¹⁷ The purified protein resolved around 200 kDa on sodium dodecyl sulfate – polyacrylamide gel electrophoresis in agreement with the predicted molecular weight (190 kDa). Moreover, it could be detected by a commercial antibody against the Myc epitope that is located at the C-terminus (**Figure 1B**), indicating that ecto-LRP4 contained the entire extracellular region of LRP4. The ecto-LRP4 protein was used in enzyme-linked immunosorbent assays for autoantibodies in serum samples from patients with double-seronegative MG as well as various groups of individuals. With the mean \pm 4 SD of normal serum samples as the cutoff, none of the normal serum samples tested positive for LRP4 autoantibodies. No positive was detected in serum samples from patients with psychiatric disorders or non-MG neurologic disorders as defined in “Methods,” with the exception of 2 of 16 serum samples of patients with neuromyelitis optica (NMO) (see “Comment”) (**Figure 2**). Of 217 patients with MG, 12 tested positive for LRP4 antibodies (Figure 2), 11 of whom were among 120 patients who were double seronegative and 1 of whom was among 36 patients without anti-AChR antibodies but with anti-MuSK antibodies. No patients with anti-AChR antibodies generated detectable LRP4 antibodies (**Figure 3**).

To confirm that the target antigen of these serum samples was full-length LRP4 rather than any contaminant in the ecto-LRP4 preparation, 4 LRP4-positive serum samples (3 from the group without anti-AChR antibodies and anti-MuSK antibodies [21321, 22212, and

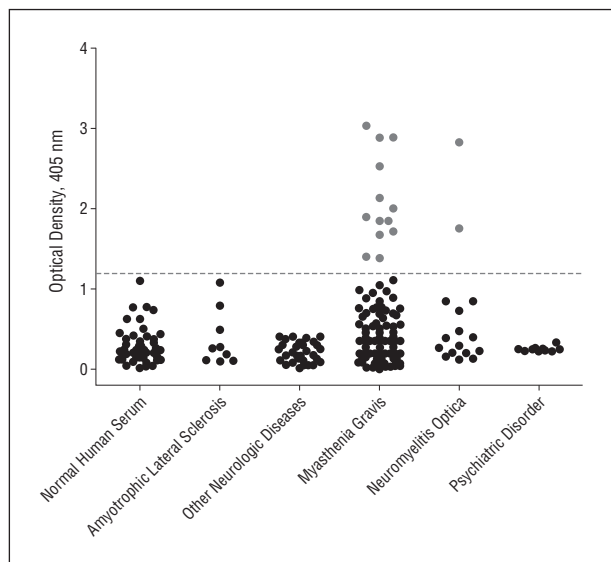


Figure 2. Enzyme-linked immunosorbent assays of anti-lipoprotein-related protein 4 autoantibodies. Mean (SD) optical density readings of normal human serum (control) were 0.31(0.22) (n=45). The dotted line indicates the cutoff.

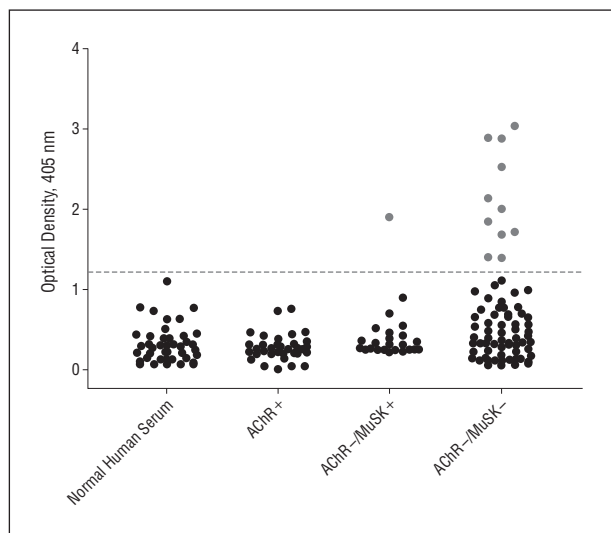


Figure 3. Distribution of anti-lipoprotein-related protein 4 autoantibodies among patients with myasthenia gravis. Of 217 myasthenia gravis samples, 61 had anti-acetylcholine receptor (AChR) antibodies (AChR+), 36 had no anti-AChR antibodies (AChR-) but did have anti-muscle-specific kinase (MuSK) antibodies (MuSK+), and 120 were AChR- and had no anti-MuSK antibodies (MuSK-). The cutoff (the dotted line) was set as mean \pm 4 SD.

23437] and 1 from the group without AChR but with anti-MuSK antibodies [23473]) were incubated with lysates of HEK293 cells expressing Myc-tagged full-length LRP4. The immunocomplex was purified by protein G immobilized on beads, was resolved by sodium dodecyl sulfate – polyacrylamide gel electrophoresis, and underwent Western blot analysis with anti-Myc antibody. As expected, full-length LRP4 was not detectable in the immunocomplex by normal human serum. However, Myc-tagged LRP4 was detected in the precipitates by 4 LRP4-positive serum samples, indicating that LRP4 autoantibodies were able to recognize full-length LRP4 expressed in transfected cells (**Figure 4**).

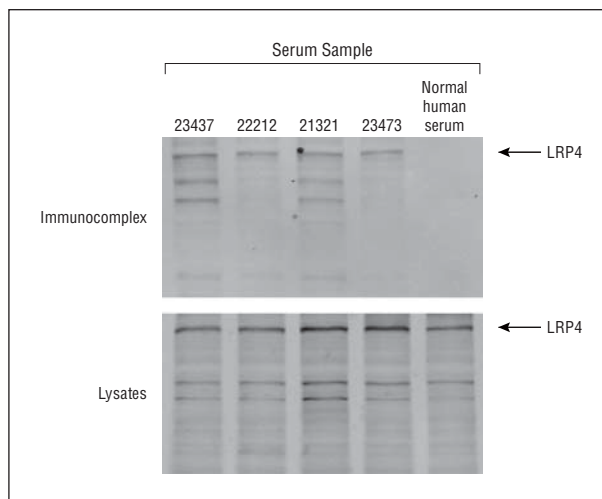


Figure 4. Recognition of full-length lipoprotein-related protein 4 (LRP4) by serum samples with anti-LRP4 antibodies. Lysates of LRP4-transfected HEK293 cells were incubated with serum samples with anti-LRP4 antibodies or normal human serum samples. Resulting immunocomplex and lysates (to indicate equal amounts of input) underwent Western blotting with anti-Myc antibody.

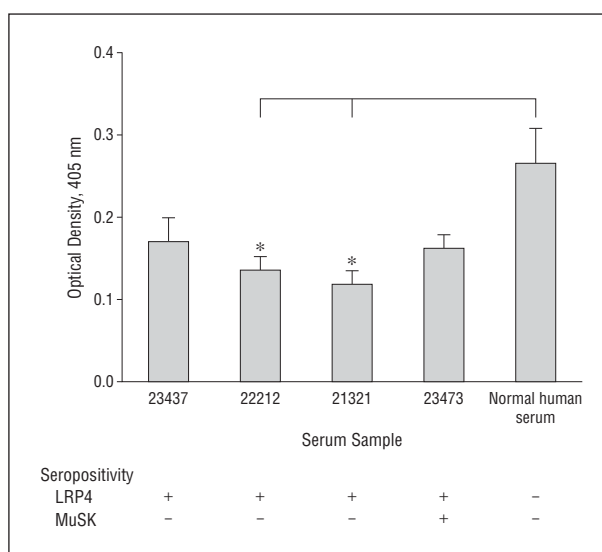


Figure 5. Inhibition of agrin-lipoprotein-related protein 4 (LRP4) interaction by serum samples with anti-LRP4 antibodies. The interaction was tested by enzyme-linked immunosorbent assay in the presence of control serum samples or serum samples with anti-LRP4 antibodies. Data are shown as mean \pm SD (n=3). * P < .05 compared with normal human serum samples. MuSK indicates muscle-specific kinase.

LRP4 AUTOANTIBODY-MEDIATED DISRUPTION OF THE AGRIN-LRP4 INTERACTION

Lipoprotein-related protein 4 interacts directly via its extracellular domain with agrin. Knowing that LRP4 autoantibodies interact with full-length LRP4, we wondered whether they interfere with the agrin-LRP4 interaction. The interaction was tested using enzyme-linked immunosorbent assays by coating plates with ecto-LRP4 followed by incubation with alkaline phosphatase – agrin in the presence of normal or LRP4-positive serum samples (1:10 dilution). As shown in **Figure 5**, the optical density readings in the enzyme-linked immuno-

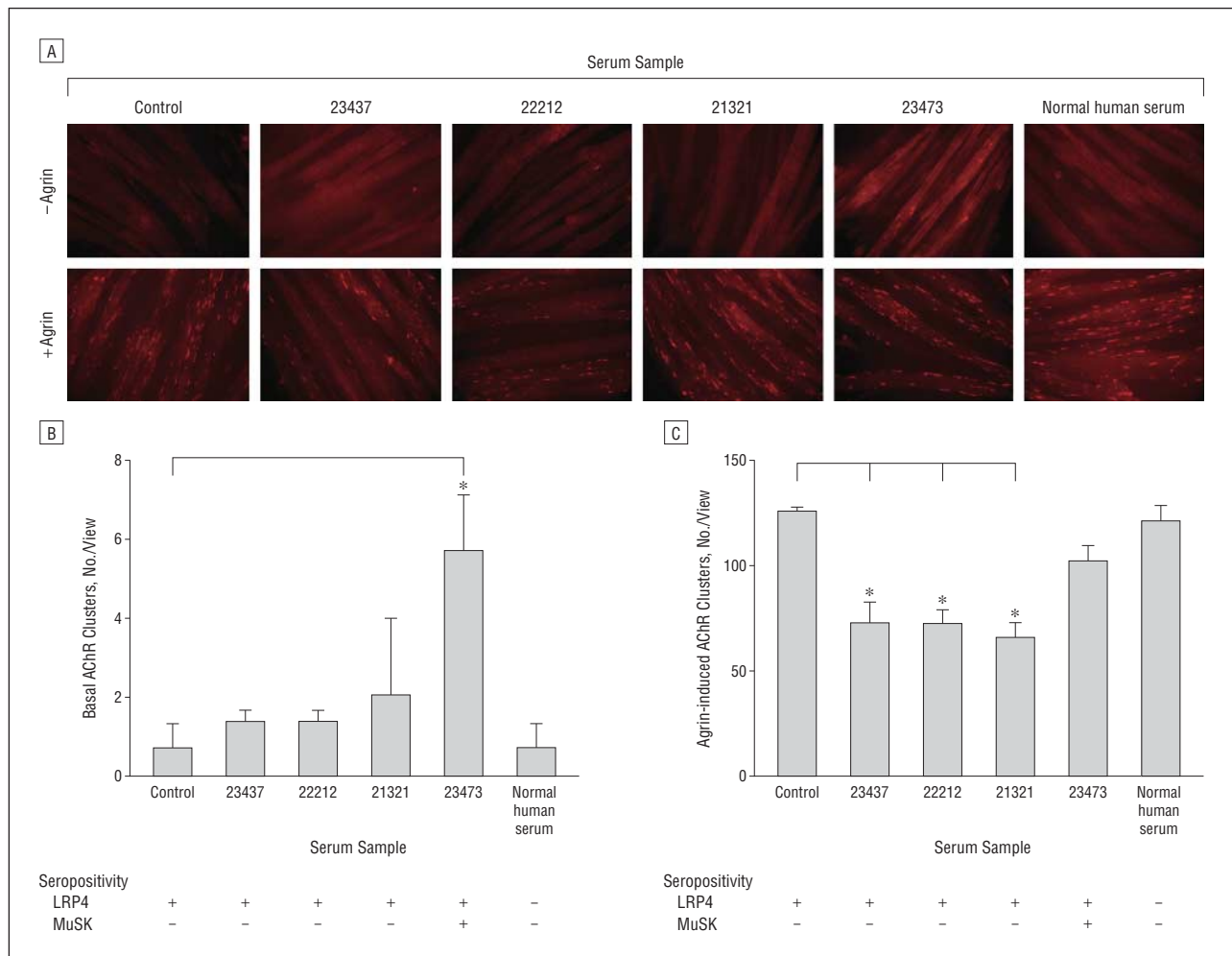


Figure 6. Serum samples with anti-lipoprotein-related protein 4 (LRP4) antibodies alter acetylcholine receptor (AChR) clustering in myotubes. A, Representative images. B, Quantitative data of basal AChR clusters. C, Quantitative data of induced AChR clusters. Data are shown as mean (SD). * $P < .05$ compared with control. MuSK indicates muscle-specific kinase.

sorbent assays were reduced at least in the presence of serum samples 21321 and 22212, compared with readings in the presence of normal human serum, suggesting that LRP4 autoantibodies may inhibit the agrin-LRP4 interaction.

ALTERATION OF BASAL AND AGRIN-INDUCED AChR CLUSTERING BY PATIENT LRP4 AUTOANTIBODIES

Lipoprotein-related protein 4 is a component of the agrin receptor complex and is critical for NMJ formation and agrin-induced AChR clustering. With the ability to recognize full-length LRP4 (Figure 4) and interfere with agrin-LRP4 interaction (Figure 5), the autoantibodies may change agrin-induced AChR clustering. To test this hypothesis, C2C12 myotubes were treated with neural agrin alone or together with control or LRP4-positive serum samples and examined for AChR clusters. As shown in **Figure 6**, induced AChR clusters were not altered by normal human serum samples, but they were inhibited by serum samples 21321, 22212, and 23437. Serum sample 23473 had no significant effect on agrin-induced AChR clustering. These results suggest that LRP4

autoantibodies may have a differential effect on AChR clustering induced by agrin.

Antibodies interacting with a transmembrane protein may cause its dimerization or oligomerization, which may result in its activation.²⁷ Antibodies against the extracellular domain of MuSK were shown to activate MuSK, leading to AChR clustering in cultured myotubes in the absence of agrin.²⁸ Moreover, MuSK autoantibodies from patients with MG also induced AChR clustering.⁶ We previously showed that overexpressed LRP4 enhances MuSK activity in the absence of agrin.¹⁷ Thus, we wondered whether LRP4 autoantibodies were able to induce AChR clustering in the absence of agrin because aggregated LRP4 may promote MuSK dimerization and/or activation. To test this hypothesis, C2C12 myotubes were treated without LRP4-positive serum samples (control) or with LRP4-positive serum samples and assayed for spontaneous AChR clusters. No apparent effect was observed with serum samples 21321, 22212, and 23437 or the normal human serum sample NHS2. However, the serum sample 23473 with anti-MuSK antibodies and anti-LRP4 antibodies, which did not inhibit agrin-induced AChR clustering (Figure 6B), was able to increase the number of spontaneous AChR clusters.

About 85% of patients with MG have detectable serum antibodies against AChRs, with 20% to 40% of the remaining patients being positive for anti-MuSK antibodies.^{6,29} This would leave about 10% of patients with double-seronegative MG (ie, without detectable antibodies against any known autoantigen). This study presents evidence that anti-LRP4 autoantibodies exist in serum samples of patients with double-seronegative MG. In our cohort of 120 patients without anti-AChR and anti-MuSK antibodies, 11 were found to be positive for anti-LRP4 antibodies, accounting for 9.2%. While our work was in progress, 2 studies identified LRP4 autoantibodies in patients with double-seronegative MG.^{20,21} Together with our work, the research suggests that LRP4 may be a novel antigen in many patients with double-seronegative MG. It is worth noting that in agreement with the study by Higuchi et al,²⁰ we failed to detect LRP4 autoantibodies in the cohort of 61 patients with AChR antibodies. We found 1 LRP4-positive serum sample in the cohort of 36 patients with MuSK antibodies, whereas 3 of 28 patients with MuSK antibodies in the cohort from the study by Higuchi et al were positive for LRP4. In the rare patients with MG who test positive for both anti-LRP4 and anti-MuSK antibodies, the relative role of these 2 different antibodies in disease pathogenesis is unknown. Interestingly, Higuchi et al found that of a cohort of 272 patients with double-seronegative MG, only 6 patients were positive for LRP4 antibodies; this accounted for about 2% of the double-seronegative serum samples differing with the 9.2% reported in our study. Pevzner et al²¹ reported that about 50% of the tested patients with double-seronegative MG (6 of 13) had anti-LRP4 antibodies. The reason for the difference among the 3 studies is unclear. It may result from the difference of patient ethnicity and countries of origin. Indeed, a similar geographic difference was also observed in patients with MG with MuSK autoantibodies; the reported percentage of patients with anti-MuSK antibodies among all patients without anti-AChR antibodies varies from 0% to 50%.³⁰ Intriguingly, LRP4 autoantibodies were detected in 2 of 16 patients with NMO. It is known that several patients have both anti-AChR and NMO antibodies (anti-aquaporin-4),³¹ while many patients with NMO often have other autoantibodies such as antinuclear antibodies and anti-extractable nuclear antigen antibodies without having systemic lupus erythematosus or Sjögren syndrome.^{32,33} In addition, NMO and/or transverse myelitis have been reported in the same individuals, and the onset of the 2 diseases may occur years apart (Gotkine et al³⁴ and personal observation by R.P.L.).

Pathogenic mechanisms of anti-AChR antibodies have been well studied. In rabbit, mouse, and rat models of experimental autoimmune MG, anti-AChR antibodies blocked the activity of the AChR,³⁵⁻³⁷ accelerated the internalization and degradation of AChRs,³⁸⁻⁴⁰ and fixed complement, which could mediate NMJ destruction and AChR loss.^{37,41,42} The AChR deficiency decreases the amplitude of miniature end plate potentials and hence that of end plate potentials, which consequently reduces the safety margin of neuromuscular transmission.^{43,44} On the other hand, anti-MuSK antibodies seem to inhibit the activity of MuSK, lead-

ing to attenuation of agrin-induced AChR clustering and thus reducing AChR levels at the junctional folds.^{10,11,13,45,46} In addition, NMJs and AChR scaffolds are disrupted in anti-MuSK-induced experimental autoimmune MG. However, anti-MuSK antibodies in patients with MG are predominantly of the IgG4 subclass,^{47,48} which do not bind and activate complement. Thus, it seems that anti-MuSK antibody-associated MG may have different etiologic and pathologic mechanisms from those of the anti-AChR-associated MG. In addition, patients with anti-MuSK do not appear to have thymic hyperplasia or thymoma.⁴⁹⁻⁵³

Whether and how LRP4 autoantibodies are pathogenic requires further study. We have demonstrated that some serum samples with anti-LRP4 antibodies but without anti-AChR antibodies and anti-MuSK antibodies were able to disrupt the agrin-LRP4 interaction and inhibit agrin-induced AChR clustering. Serum sample 23473, which also had anti-MuSK antibodies, had no effect on the agrin-LRP4 interaction, nor did it inhibit agrin-induced AChR clustering. It increased basal AChR clusters, which may be due to anti-MuSK antibodies instead of those directed against LRP4. Considering the large size of the extracellular domain of LRP4, it is likely that the pathogenic mechanisms of LRP4 antibodies could be complex. For example, LRP4 also interacts with MuSK in addition to agrin.^{17,18} Therefore, the anti-LRP4 antibodies might prevent LRP4 from interacting with MuSK. They may also cause its internalization and subsequent degradation. Finally, most LRP4 autoantibodies appeared to be IgG1,²⁰ similar to those against AChR, which are able to activate complement.⁵⁴ Therefore, it is possible that complement may be involved in the pathogenesis of MG in some patients with LRP4 autoantibodies. If so, this would differ from the presumed mechanism of action of anti-MuSK autoantibodies.

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Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Zhang, Xiong, Lisak, S.J. Tzartos, and Mei. **Acquisition of data:** Zhang, J. S. Tzartos, Belimezi, Ragheb, Bealmear, Lewis, Lisak, and S. J. Tzartos. **Analysis and interpretation of data:** Zhang, J. S. Tzartos, Xiong, Lisak, S. J. Tzartos, and Mei. **Drafting of the manuscript:** Zhang, Ragheb, Xiong, Lisak, S.J. Tzartos, and Mei. **Critical revision of the manuscript for important intellectual content:** J. S. Tzartos, Belimezi, Ragheb, Bealmear, Lewis, Lisak, S. J. Tzartos, and Mei. **Statistical analysis:** Zhang. **Obtained funding:** Xiong, Lisak, S. J. Tzartos, and Mei. **Administrative, technical, and material support:** J. S. Tzartos, Belimezi, Ragheb, Lisak, S. J. Tzartos, and Mei. **Study supervision:** Lisak, S. J. Tzartos, and Mei.

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