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Immunization with Recombinantly Expressed LRP4 Induces Experimental Autoimmune Myasthenia Gravis in C57BL/6 Mice

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ABSTRACT

Background: Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction (NMJ), characterized with muscle weakness. While MG develops due to acetylcholine receptor (AChR) antibodies in most patients, antibodies to muscle-specific receptor tyrosine kinase (MuSK) or low-density lipoprotein receptor-related protein 4 (LRP4) may also be identified. Experimental autoimmune myasthenia gravis (EAMG) has been previously induced by both LRP4 immunization and passive transfer of LRP4 antibodies.

Objective: Our aim was to confirm previous results and to test the pathogenic effects of LRP4 immunization in a commonly used mouse strain C57BL/6 (B6) using a recombinantly expressed human LRP4 protein.

Methods: B6 mice were immunized with human LRP4 in CFA, Torpedo Californica AChR in CFA or only CFA. Clinical and pathogenic aspects of EAMG were compared among groups.

Results: LRP4- and AChR-immunized mice showed comparable EAMG clinical severity. LRP4-immunized mice displayed serum antibodies to LRP4 and NMJ IgG and complement factor C3 deposits. IgG2 was the dominant anti-LRP4 isotype. Cultured lymph node cells of LRP4- and AChR-immunized mice gave identical pro-inflammatory cytokine (IL-6, IFN-\textgamma and IL-17) responses to LRP4 and AChR stimulation, respectively.

Conclusion: Our results confirm the EAMG-inducing action of LRP4 immunization and identify B6 as a LRP4-EAMG-susceptible mouse strain. Demonstration of complement fixing anti-LRP4 antibodies in sera and complement/IgG deposits at the NMJ of LRP4-immunized mice indicates complement activation as a putative pathogenic mechanism. We have thus developed a practical LRP4-induced EAMG model using a non-conformational protein and a widely available mouse strain for future investigation of LRP4-related MG.

KEYWORDS: Antibody; autoimmunity; immunization; LRP4; myasthenia gravis

Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction (NMJ) characterized with fluctuating fatigue and muscle weakness (Binks et al., 2016). In around 80–90% of generalized MG patients, the symptoms are induced by antibodies against the...
postsynaptic nicotinic acetylcholine receptor (AChR), whereas in some of the remaining patients, muscle-specific receptor tyrosine kinase (MuSK) or low-density lipoprotein receptor-related protein 4 (LRP4) antibodies are detected (Higuchi et al., 2011; Pevzner et al., 2012; Zhang et al., 2012).

Different than AChR, MuSK and LRP4 are not directly involved in synaptic transmission in the NMJ but play crucial roles in synapse formation and AChR clustering. Agrin released by motor nerve fiber terminals binds LRP4-MuSK complex and thus, through a cascade of protein interactions, arranges the clustering of AChRs on the muscle cell surface and gives its characteristic appearance to the postsynaptic part of the NMJ. Moreover, MuSK-induced experimental autoimmune myasthenia gravis (EAMG) studies have shown that agrin/LRP4/MuSK signaling contributes to NMJ formation (Wu et al., 2012; Klooster et al., 2012; Otsuka et al., 2015). Additionally, while AChR antibodies that are mainly of complement fixing IgG1 and IgG3 isotypes disrupt NMJ transmission by way of complement mediated destruction, MuSK antibodies that are mainly of non-complement fixing IgG4 isotype block the interaction between LRP4 and MuSK (Klooster et al., 2012; Koneczny et al., 2013). There are likely other mechanisms as well including those induced by other anti-MuSK IgG subclasses (Koneczny et al., 2013).

LRP4 antibodies have been identified in different MG cohorts in widely ranging ratios (Higuchi et al., 2011; Pevzner et al., 2012; Zhang et al., 2012). Moreover, immunization (with rat AChR) and passive transfer studies (using IgGs purified from LRP4-immunized rabbits) in the A/J mouse strain have shown that LRP4 antibodies are pathogenic and are capable of inducing EAMG (Shen et al., 2013). However, still little is known about the immunological aspects of LRP4 autoimmunity. To investigate the exact pathogenic mechanisms of LRP4 antibody-associated MG, a practical EAMG method needs to be established using mouse strains with numerous available transgenic variants. For this purpose, we have immunized C57BL/6 (B6) mice with a recombinantly expressed LRP4 protein and compared clinical and immunological features of these mice with AChR-immunized mice.

**Methods**

**Production of LRP4 and AChR**

AChR was affinity purified by the neurotoxin affinity column from the electric organ of Torpedo Californica (Wu et al., 2013). An expression clone (accession number: NM_002334.3) of human LRP4 (coding the entire LRP4 protein) was obtained from ImaGenes (Berlin, Germany). DNA was isolated for DNA sequencing (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Cloned cDNAs in the purified plasmid DNA were sequenced by Iontek Laboratory (Istanbul, Turkey). Nucleotide and translated amino acid sequences were compared with known sequences using BLAST algorithms (National Center for Biotechnology Information, Bethesda, MD). Following the confirmation of the selected clones, His-tagged proteins were recombinantly expressed in E. coli, purified by affinity chromatography (Preuss et al., 2009), and the purity of the protein was documented by SDS-PAGE analysis and immunoblotting using a commercial anti-human LRP4 antibody (Abcam, Cambridge, UK).
**Mice and EAMG**

Seven- to eight-week-old male B6 mice were purchased from the institutional animal facility of Istanbul University. All animals were housed in the viral antibody-free barrier facility in Istanbul University and maintained according to the Institutional Animal Care and Use Committee Guidelines.

Mice were anesthetized and immunized with 20 μg of AChR or LRP4 emulsified in complete Freund’s adjuvant (CFA, Difco, Detroit, MI) s.c. at four sites (two hind footpads and shoulders) on day 0 and were boosted with the same amount of AChR or LRP4 in CFA s.c. at four sites on the back on days 28 and 56. Control mice were immunized with only CFA. Mice were terminated 30 days after the 3rd immunization.

Mice were randomly allocated to different immunization groups, and clinical features were assessed by blinded researchers. For clinical examination, mice were left for 3 min on a flat platform and were observed for signs of EAMG. Clinical muscle weakness was graded as follows: grade 0, mouse with normal posture, muscle strength, and mobility; grade 1, normal at rest, with muscle weakness characteristically shown by a hunched posture, restricted mobility, and difficulty raising the head after exercise that consisted of 30 paw grips on a cage top grid; grade 2, grade 1 symptoms without exercise during the observation period on a flat platform; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead. For objective measurement of muscle strength, mice were first exercised with 40 paw grips on a cage top grid. Following exercise, mice were made to grasp a grid attached to a dynamometer (Chatillon Digital Force Gauge, DFIS 2, Columbus Instruments, Columbus, OH). The maximal force applied to the dynamometer while pulling the mouse by its tail until it lost its grip on the grid was recorded.

**ELISA for anti-LRP4 antibodies and isotypes**

Mice were bled from the tail vein 15 days after the last immunization. Sera were evaluated for anti-LRP4 IgG, IgG1, IgG2, IgG3 and IgM levels. Affinity-purified human LRP4 (1 μg/ml) or Torpedo AChR was coated onto 96-well microtiter plates in 0.1 M carbonate/bicarbonate buffer overnight at 4°C. Diluted 100 μl serum samples (1:100, 1:1000 or 1:10000 dilutions) were added, and plates were incubated at 37°C for 90 min. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, IgG1, IgG2, IgG3 or IgM antibodies (Abcam) (1:10000) were added, and plates were then incubated at 37°C for 90 min. A commercial mouse anti-human LRP4 (1:100, Abcam, Cambridge, UK) antibody was also used as a positive control. Subsequently, the peroxidase indicator substrate 2,2’-azinobis-(3-ethylbenzothiazoline 6-sulfonate) substrate (ABTS) solution in 0.1 M citric buffer (pH 4.35) was added in the presence of H₂O₂, and the mixture was allowed to develop color at room temperature in the dark. Plates were read at a wavelength of 405 nm. Normal mouse sera (collected from mice before immunization) were used for the background determination.

**Immunofluorescence for IgG and C3 deposits at NMJ**

Sections (10 μm thick) were obtained from forelimb muscle samples of mice, frozen in liquid nitrogen, and stored at −80°C. Slides were fixed in cold acetone and blocked in 10%
normal goat serum in PBS. After washing with PBS, the sections were incubated with
tetramethylrhodamine-conjugated bungarotoxin (BTx) (Molecular Probes, Eugene, OR)
(1:500 dilution) for 1 h at room temperature to label the NMJ. Sections were then
incubated for 1 h at room temperature with FITC-conjugated antibodies to mouse IgG
and complement C3 (Abcam) (diluted 1:1000) to colocalize IgG or complement deposits
at the NMJ. The sections were washed and viewed in a fluorescence microscope (Olympus
IX-70). The number of IgG isotype- and C3-positive BTx binding sites was counted in five
muscle sections from each mouse. The percentages of NMJs with deposits in each muscle
section were calculated by totaling the numbers of deposits divided by the numbers of BTx
labeled sites, times 100.

Cytokine measurements in culture supernatants

Lymph node cells (2 × 10^5 cells/well) obtained at termination were seeded in triplicate into
96-well, round-bottomed microtiter plates in 0.2 ml of culture medium with or without 15
μg of LRP4 or AChR. The cells were cultured for 48 h at 37°C in humidified 5% CO2-
enriched air. Supernatants were collected and stored at −80°C until analyzed. The
supernatant levels of IL-6, IFN-γ, and IL-17 were measured by ELISA kits (Invitrogen,
Carlsbad, CA, USA), according to the manufacturer’s instructions and expressed in pg/ml.

Statistical analysis

Clinical EAMG incidences were compared using chi-square test. Clinical scores were
compared by Kruskal–Wallis test and Dunn’s post hoc test. All other parameters were
compared using ANOVA (and Tukey’s post hoc test). Correlation studies were done with
Pearson’s test. p values less than 0.05 were considered statistically significant.

Results

Immunization of B6 mice with LRP4 induces EAMG

To confirm the previously reported pathogenic effect of LRP4 immunity and to
demonstrate its pathogenicity in a different mouse strain, 10 B6 mice were immunized
with recombinantly expressed human LRP4 emulsified in CFA. Control groups
included B6 mice immunized with Torpedo AChR in CFA (n=10 mice) and B6 mice
immunized with only CFA (n=10 mice). All mice received two more immunizations.
Shortly after the second immunization, LRP4- and AChR-immunized mice started
developing muscle weakness as demonstrated by clinical scoring and grip strength
measurements, whereas CFA-immunized mice did not display any muscle weakness.
At termination, 9/10 of AChR-immunized and 7/10 of LRP4-immunized mice had
clinical muscle weakness (p < 0.001 for AChR- and LRP4-immunized vs CFA-immu-
nized groups). EAMG prevalence did not significantly differ between AChR- and
LRP4-immunized mice (p = 0.531 at termination). Grip strengths and clinical scores
were significantly different between AChR-immunized and CFA-immunized mice
starting from the 6th week, whereas significant differences between LRP4- and CFA-
immunized mice began on the 7th week. Although AChR-immunized mice showed
higher muscle weakness severity than LRP4-immunized mice, there were no significant differences between AChR- and LRP4-immunized mice by means of clinical scores and grip strengths at any time point (Figure 1).

**LRP4-immunized mice show characteristic features of EAMG**

Antibody responses to the affinity-purified human LRP4 were measured with ELISA. Anti-LRP4 IgG, IgG1, IgG2, IgG3 and IgM responses were detected in LRP4-immunized mice in all tested dilutions but not in AChR- or CFA-immunized mice. The dominant isotype of LRP4 antibodies was IgG2 in LRP4-immunized B6 mice (Figure 2). The commercial anti-LRP4 antibody also showed strong reactivity with the coated human LRP4 (OD values between 0.8 and 1.0 in different experiments). By contrast, LRP4-immunized mice did not display antibodies against Torpedo AChR (not shown). All AChR-immunized and LRP4-immunized mice showed

![Figure 1](image.png)

**Figure 1.** Kinetics of EAMG clinical scores and grip strengths of LRP4 + CFA (close squares), AChR + CFA (open squares) or CFA-immunized (close circles) mice after first immunization. Vertical bars indicate standard errors. Asterisks denote significant differences between LRP4 + CFA or AChR + CFA-immunized vs CFA-immunized mice; *p < 0.05, **p < 0.01, ***p < 0.001. One representation of two independent experiments.
an abundancy of NMJ IgG and C3 deposits (~15–20% of all NMJs per muscle section), whereas no IgG or C3 deposits were observed in CFA-immunized mice. Thus, both LRP4- and AChR-immunized mice had significantly higher NMJ deposit numbers than CFA-immunized mice, whereas LRP4- and AChR-immunized mice showed identical numbers of both types of deposits (Figure 3). Percentages of C3 and IgG deposits were significantly correlated in LRP4- and AChR-immunized mice (p = 0.003, R = 0.823 and p = 0.015, R = 0.737 for LRP4- and AChR-immunized mice respectively by Pearson’s test).

**LRP4- and AChR-immunized mice show identical cytokine production patterns**

Inflammatory cytokines are important participants of inflammation and autoimmunity (Ebrahimnezhad et al., 2016; Ramroodi et al., 2015). Cytokine responses of immunogen-stimulated lymph node cells were measured and compared among study groups. Both LRP4- and AChR-immunized mice showed significantly increased IL-6, IFN-γ and IL-17 responses to stimulation by their respective immunogens,
whereas CFA-immunized mice did not show an appreciable cytokine production in response to LRP4 or AChR stimulation (Figure 4).

**Discussion**

Immunization of A/J mice with the rat LRP4 protein has been shown, in a single study, to induce the clinical and immunopathological features of EAMG. In this previous study,
LRP4 protein used for immunization was purified from lysates of transfected HEK293 cells by affinity chromatography (Shen et al., 2013). In our study, we have confirmed that LRP4 immunization induces EAMG with clinical and immunological features that are highly comparable to those of AChR-induced EAMG. However, different from the previous study, we have immunized B6 mice with a human LRP4 protein recombinantly expressed by E. coli thus displaying that LRP4-related EAMG induction does not require immunization with conformational LRP4 epitopes. Similarly, EAMG can be induced by both native AChR pentamer and recombinantly expressed subunits of the AChR (Yang et al., 2007).

It is well known that the major histocompatibility complex (MHC) molecules are significant contributors of autoimmunity (Liu et al., 2016). Not all mouse strains are susceptible to EAMG, and EAMG susceptibility is at least partially controlled by the MHC H-2 haplotypes. Strains of H-2^b haplotype (e.g., B6 and C57BL10/J) are susceptible to AChR-induced EAMG, whereas strains of H-2^k, p haplotypes are relatively resistant (Christadoss, 1989). Our results indicate that, in addition to A/J mice (carrying H-2^d/k haplotype), B6 (carrying H-2^b haplotype) is a susceptible mouse strain to LRP4-induced EAMG. As indicated in the previous study (Shen et al., 2013), at least two LRP4 immunizations are required to generate muscle weakness. In our study, we have obtained a 70% EAMG incidence in B6 mice with three immunizations, as opposed to 56% incidence with three immunizations and 66% incidence with four immunizations in A/J mice. Higher EAMG incidence in our study might suggest that B6 mice are slightly more susceptible to LRP4-induced EAMG. It may also be associated with use of CFA in all immunizations in our study, as opposed to use of CFA in the first immunization and incomplete Freund’s adjuvant in subsequent immunizations in the other study. The B6 mouse strain has numerous genetic variants (e.g., IL-6 knockout or C3 knockout mice on the B6 background) thus facilitating future experimental studies that will be performed to dissect the key immunological factors involved in LRP4-related MG such as specific cytokines, costimulatory molecules, and complement factors.

Immunization with the human LRP4 does not only generate muscle weakness but also mimics major hallmark immunopathological features of MG, namely serum antibodies to a NMJ protein and IgG and complement accumulation at the NMJ (Tüzün and Christadoss, 2013). The identification of LRP4 antibodies only in LRP4-immunized but not AChR- or CFA-immunized mice indicates specificity of these antibodies and proves that LRP4 antibody production is not merely a bystander effect of NMJ destruction or immune system activation. Major complement fixing human IgG isotype IgG1 is the dominant isotype in LRP4 antibody positive MG patients (Higuchi et al., 2011). Similarly, the chief complement fixing mouse isotype IgG2 was the dominant anti-LRP4 isotype in EAMG induced by LRP4 immunization. This finding and abundancy of complement deposits at the NMJ of LRP4-immunized mice suggest that complement activation is an important contributor of NMJ destruction in LRP4-induced EAMG. In line with our results, anti-LRP4 antibodies of EAMG induced mice were found to be capable of activating the complement cascade (Shen et al., 2013).

A limitation of our study was that murine LRP4 antibodies could not be identified in sera of LRP4-immunized mice. Since these mice did not show AChR antibodies and yet displayed NMJ IgG deposits, there is a good likelihood that LRP4 immunization resulted in anti-mouse LRP4 antibodies. Nevertheless, for better characterization of the disease
model, we recommend direct demonstration of murine LRP4 antibodies in future experiments.

The immunological aspects of LRP4 immunity have not been studied at all. Here, we show for the first time that lymph node cells obtained from LRP4-immunized mice show enhanced production of pro-inflammatory cytokines IL-6, IFN-γ and IL-17, all of which have been shown to display seminal roles in AChR-related EAMG (Deng et al., 2002; Schaffert et al., 2015; Zhang et al., 1999). By contrast, lymph node cells of LRP4-immunized mice did not give an enhanced cytokine response to AChR stimulation indicating the specificity of the cytokine results. Our EAMG model thus constitutes a potentially useful method to study the immunopathological aspects of LRP4 immunity. Investigation of a broader panel of cytokines and induction of LRP4-related EAMG in specific cytokine knockout B6 mice will shed further light on dominant Th-types and pathogenic aspects of LRP4 immunity.

**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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