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Research Paper

Efgartigimod improves muscle weakness in a mouse model for muscle-specific kinase myasthenia gravis

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ABSTRACT

Myasthenia gravis is hallmarked by fatigable muscle weakness resulting from neuromuscular synapse dysfunction caused by IgG autoantibodies. The variant with muscle-specific kinase (MuSK) autoantibodies is characterized by prominent cranial and bulbar weakness and a high frequency of respiratory crises. The majority of MuSK MG patients requires long-term immunosuppressive treatment, but the result of these treatments is considered less satisfactory than in MG with acetylcholine receptor antibodies. Emergency treatments are more frequently needed, and many patients develop permanent facial weakness and nasal speech. Therefore, new treatment options would be welcome. The neonatal Fc receptor protects IgG from lysosomal breakdown, thus prolonging IgG serum half-life. Neonatal Fc receptor antagonism lowers serum IgG levels and thus may act therapeutically in autoantibody-mediated disorders. In MuSK MG, IgG4 anti-MuSK titres closely correlate with disease severity. We therefore tested efgartigimod (ARGX-113), a new neonatal Fc receptor blocker, in a mouse model for MuSK myasthenia gravis. This model involves 11 daily injections of purified IgG4 from MuSK myasthenia gravis patients, resulting in overt myasthenic muscle weakness and, consequently, body weight loss. Daily treatment with 0.5 mg efgartigimod, starting at the fifth passive transfer day, reduced the human IgG4 titres about 8-fold, despite continued daily injection. In muscle strength and fatigability tests, efgartigimod-treated myasthenic mice outperformed control myasthenic mice. Electromyography in calf muscles at endpoint demonstrated less myasthenic decrement of compound muscle action potentials in efgartigimod-treated mice. These substantial *in vivo* improvements of efgartigimod-treated MuSK MG mice following a limited drug exposure period were paralleled by a tendency of recovery at neuromuscular synaptic level (in various muscles), as demonstrated by *ex vivo* functional studies. These synaptic improvements may well become more explicit upon longer drug exposure. In conclusion, our study shows that efgartigimod has clear therapeutic potential in MuSK myasthenia gravis and forms an exciting candidate drug for many autoantibody-mediated neurological and other disorders.

1. Introduction

Myasthenia gravis (MG) is characterized by fatigable muscle weakness. It is caused by autoantibodies which target key neuromuscular junction (NMJ) proteins and thereby impair neuromuscular transmission (Gilhus and Verschuuren, 2015; Plomp et al., 2015). Autoantibodies against acetylcholine receptors (AChR), muscle-specific kinase (MuSK), low density lipoprotein receptor-related protein 4 (Lrp4) and agrin have been identified. Approximately 5–10% of MG patients has antibodies against MuSK, a postsynaptic membrane protein involved in embryonic

Abbreviations: α-BTx, α-Bungarotoxin; AChR, Acetylcholine receptor; CMAP, Compound muscle action potential; dTC, d-tubocurarine; ELISA, Enzyme-linked immunosorbent assay; EPP, Endplate potential; FDB, Flexor digitorum brevis; i.p., Intraperitoneal; i.v., Intravenous; IVIg, Intravenous immunoglobulin; LAL, Levator auris longus; Lrp4, Low density lipoprotein receptor-related protein 4; MEPP, Miniature endplate potential; MG, Myasthenia gravis; MuSK, Muscle-specific kinase; NMJ, Neuromuscular junction; n.s., Not statistically significant; OD, Optical density; PBS, Phosphate-buffered saline; SEM, Standard error of the mean

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clustering of AChRs at the NMJ, and their maintenance in later phases (Evoli et al., 2018; Hoch et al., 2001; Niks et al., 2007; Wu et al., 2010). MuSK MG autoantibodies are predominantly IgG4 isotype and within patients their titre correlates with disease severity (Bartoccioni et al., 2006; Huijbers et al., 2016; Klooster et al., 2012; McConville et al., 2004; Niks et al., 2008). IgG4 is unable to activate complement or to cause inflammation through Fc receptors on immune cells (Lighaam and Rispens, 2016). It undergoes half-molecule exchange with other IgG4 molecules so that human MuSK antibodies have only one MuSK-binding arm (Koneczny et al., 2017; van der Neut et al., 2007). MuSK autoantibodies are pathogenic by inhibiting agrin- and Lrp4-dependent MuSK activation through direct blockade of the interaction between Lrp4 and MuSK (Huijbers et al., 2013; Koneczny et al., 2013; Otsuka et al., 2015). This causes AChR declustering and synaptic transmission deficits resulting in fatigable muscle weakness, as demonstrated in passive transfer studies using patient IgG in mice (Cole et al., 2008; Klooster et al., 2012; Viegas et al., 2012).

Treatment of MuSK MG is challenging because symptomatic treatment with acetylcholinesterase inhibitors, the first-line and mostly effective drugs in AChR MG, is often not effective or even detrimental (Evoli et al., 2018). MuSK MG patients respond less well to immunosuppressive drugs than AChR MG patients do. High-dose immunosuppressant treatment does not prevent the frequent disease deteriorations requiring emergency treatment, and many patients develop irreversible facial weakness or nasal speech (Evoli et al., 2018; Evoli and Padua, 2013). Almost 40% of MuSK MG patients experiences a respiratory crisis at some point, which is mostly treated with plasmapheresis or intravenous immunoglobulin (IVIg) (Barth et al., 2011; Evoli et al., 2008). Rituximab, an anti-CD20 monoclonal antibody which eliminates B-cells, has been shown effective in case series (Diaz-Manera et al., 2012; Hehir et al., 2017; Tandan et al., 2017). These current treatments all have their inherent practical drawbacks or risks of side effects (Barth et al., 2011; Lee and Arepally, 2012; Lunemann et al., 2016; Okafor et al., 2010; Spath et al., 2015). After prolonged follow-up, complete remission was reached in only ~10% of the MuSK MG patients, and immunosuppressive treatment could be stopped in < 20% (Evoli et al., 2008). This prompts for more effective treatments with improved practical and safety features.

The neonatal Fc receptor (FcRn) IgG recycling pathway forms a major IgG level regulatory system (Martins et al., 2016). After IgG is taken up by pinocytotic vesicles of vascular endothelium and bone-marrow-derived cells, acidification of the vesicular lumen causes it to bind to FcRn (Roopenian and Akilesh, 2007). In this way IgG is spared from lysosomal degradation and recycled into the serum through exocytosis. FcRn-deficient mice have a reduced IgG half-life (Roopenian et al., 2003), and blocking the FcRn by either peptides or monoclonal antibodies has been shown to lower serum IgG levels in mice, monkeys and humans (Getman and Balthasar, 2005; Kiessling et al., 2017; Mezo et al., 2008; Nixon et al., 2015; Petkova et al., 2006; Ulrichts et al., 2018; Vaccaro et al., 2005). Thus, humoral immune responses can be regulated at the level of circulating IgG by manipulation of FcRn, suggesting this to be an interesting drug target for antibody-mediated autoimmune diseases (Challa et al., 2013; Liu et al., 2007; Patel et al., 2011; Roopenian and Akilesh, 2007; Sesarman et al., 2010). Efgartigimod (ARGX-113) is a monoclonal IgG1-Fc fragment modified with the so-called ABDEG™ mutations to increase its affinity for FcRn (Ulrichts et al., 2018; Vaccaro et al., 2005). Because the severity of MuSK MG is directly related to MuSK IgG4 antibody titre, we hypothesized that lowering IgG levels through inhibition of FcRn-mediated IgG recycling by efgartigimod could act therapeutically. Therefore, we studied the effect of efgartigimod in our patient IgG4 passive transfer mouse model for MuSK MG (Klooster et al., 2012), and report evidence for its therapeutic potential.

2. Materials and methods

2.1. Patient plasma and normal human serum

Therapeutic plasmapheresis waste material from five MuSK MG patients was obtained after informed consent at the Leiden University Medical Centre. All patients were positive for MuSK antibodies. Summary description of the clinical characteristics of the included patients is given in Supplementary Table. Normal human serum was obtained from Sanquin Blood Supply Foundation, Amsterdam, the Netherlands.

2.2. IgG4 purification

Plasmapheresis fluid and normal human serum was stored at −20 °C or −80 °C until purification was initiated. IgG4 was purified as described previously (Klooster et al., 2012). In short, the fluid was diluted in citrate buffer and filtered before addition of the material to a phosphate buffered saline (PBS)-equilibrated Captureselect IgG4 affinity column (Thermo Fisher Scientific, Bleiswijk, The Netherlands). Next, the column was washed with PBS, and bound IgG4 was eluted with 0.1 M glycine pH 3.0 and neutralized with 1/10 volume of 1 M Tris pH 8.0. Purified IgG4 fractions were pooled, dialyzed against PBS, concentrated with a Vivaspin20 concentrator (Sartorius), filter sterilized (Millipore, Amsterdam, The Netherlands) and stored in aliquots at −20 °C until further use.

2.3. Efgartigimod and control Fc fragment production

Efgartigimod was produced and purified by Lonza Biologics (Slough, UK) using the CHOK1SV GS-KO system (Lonza Group Ltd., Basel, Switzerland) (Ulrichts et al., 2018). The wild-type control Fc fragment was transiently produced in CHO cells and purified using Mabselect™ Sure™ resin (Evitria, Schlieren, Switzerland), followed by rebuffering and concentration. Control Fc fragment solution matched efgartigimod solution in appearance, concentration and formulation, thereby keeping the investigators blinded.

2.4. MuSK ELISA and competition assays

To exclude that efgartigimod or the control Fc fragment binds MuSK and therefore interferes with the *in vivo* experiments we performed a MuSK enzyme-linked immunosorbent assay (ELISA) as described previously (Huijbers et al., 2016). As secondary antibody for efgartigimod or the control Fc fragment (both IgG1-derived) we used a mouse antibody against human IgG1 (m1325, Sanquin, Amsterdam, The Netherlands). Titration started at a concentration of 50 μg/ml. The same ELISA was used to investigate whether efgartigimod or the control Fc fragment could directly interfere with MuSK MG autoantibody binding to MuSK. To that end, MuSK was immobilized in Maxisorp Nunc plates (Thermo Fisher Scientific, Bleiswijk, The Netherlands) at 3 μg/ml. A fixed concentration of 15.625 μg/ml purified total IgG from one patient (#1, see Supplementary Table) and purified IgG4 from two other patients (#2 and #3, see Supplementary Table) was co-incubated with a concentration series of efgartigimod or the control Fc fragment, up to 50 μg/ml. A standard curve was included based on purified total IgG from another patient to serve as a positive control.

To determine the effect of efgartigimod or control Fc fragment on mouse serum levels of injected patient IgG4, passive transfer studies were performed during which serum was obtained (see below). This mouse serum was stored at −20 °C and assessed later for human MuSK reactivity in the MuSK ELISA described above. To that end sera were diluted starting at a dilution of 1:50 in block buffer. Serum MuSK reactivity at a concentration of 1:200 was in the linear range of the MuSK reactivity and was therefore plotted. Experiments were performed in duplicate. In a series of six efgartigimod and six control Fc fragment-treated mice, sera were obtained at 2 days before the start of the passive transfer, at 3 days after the start (*i.e.* the day before the start of the efgartigimod treatment) and at the endpoint. For logistic reasons, these sera were assayed in two separate (but methodologically identical) ELISAs on different days, with equal efgartigimod and control mice numbers included each time (*i.e.* one ELISA assayed sera of 4 *vs.* 4 mice

and the other assayed sera of 2 *vs.* 2 mice). Because the specific optical density (OD) values of the efgartigimod and control Fc fragment groups, as well as of the internal standards, were comparable between these two ELISAs, we pooled the data. From two more efgartigimod and two control Fc fragment-treated MuSK MG mice, endpoint sera were available and assayed in another ELISA. Because all experimental conditions were standardized, and group data was well within comparable ranges, we also pooled these endpoint data.

2.5. Normal human IgG level ELISA

Quantification of the levels of normal human IgG in mouse sera (see below) was performed using an ELISA (#88-50550, ThermoFisher Scientific, Bleiswijk, The Netherlands). In short, samples were initially diluted 200,000-fold, followed by a 2-fold dilution series and further processed according manufacturer's instructions. For all dilutions where a signal within the dynamic range of the assay was measured, IgG levels were determined using the standard curve. The averages of these values were plotted. Efgartigimod or control Fc fragment did not interfere with this ELISA assay (data not shown). Most likely (but not provided in the manufacturer's information), the capturing antibody of the kit is anti-Fab or anti-(Fab)2, *i.e.* IgG parts which are not present in efgartigimod and the control Fc fragment.

2.6. Mouse passive transfer studies

All experiments were carried out according to Dutch law and Leiden University guidelines, including approval by the National and Local Animal Experiments Committees. We used our passive transfer mouse model for MuSK MG in which purified MuSK MG patient IgG4 is injected daily, as described previously (Klooster et al., 2012). This model uses immunodeficient NOD.CB17-Prkdcscid/J (NOD/SCID) mice to prevent a mouse immune response to the injected human IgG4 (Shultz et al., 1995). Original breeders were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred and housed in sterile individually ventilated cages in the LUMC animal facilities. Sterilized food and drinking water were provided *ad libitum*.

First, we determined if efgartigimod is able to reduce the levels of injected human IgG in NOD/SCID mice. Four 2.5 months-old female littermate mice were loaded with normal human total IgG through daily i.p. injection of 35 mg for 7 days. On the last day, two mice were co-injected with 1 mg efgartigimod and two mice with control Fc fragment. Blood samples (50 μl, tail vein) were taken at days 5, 8, 11, 14, 17 and 20. They were allowed to clot at room temperature and were centrifuged thereafter. Serum was stored at −20 °C until IgG levels were determined in a subclass ELISA (see above).

To determine the appropriate daily MuSK MG IgG4 dose at which mice would develop phenotypical MG that could potentially be treated by therapeutic reduction of IgG4 level by efgartigimod we performed dose-finding experiments with the pooled purified IgG4 from two batches plasmapheresis fluid from patient #4 (see Supplementary Table). Ten-weeks-old female NOD/SCID mice were injected i.p. with different daily doses (0.025, 0.05, 0.1, 0.2, or 0.4 mg/g body weight). *In vivo* myasthenia was monitored by daily measurement of body weight, grip strength and inverted mesh hang time, as described in short below and in detail previously (Klooster et al., 2012).

Therapeutic experiments with efgartigimod and control Fc fragment were performed in 6–8 weeks-old female NOD/SCID mice (n = 16). On day 0, daily i.p. injection with purified IgG4 from patient #4 was started at the most likely suitable dose as determined above, *i.e.* 0.1 mg/g body weight. From day 4 on, half of the group (n = 8, randomly chosen) was co-injected with 0.5 mg/day efgartigimod and the other half (n = 8) with 0.5 mg/day control Fc fragment. One week later, on day 11, the experiment was ended and mice and dissected muscles were subjected to the endpoint analyses (see below). Neuromuscular performance (grip strength, inverted mesh hang test) and body weight of each mouse was measured daily, starting at 4 days before the start of the passive transfer to familiarize the mice with the handling and tests. To monitor serum levels of human MuSK MG IgG4, a blood sample was taken on experimental days −2, 3 and 11 and the serum was stored at −20 °C until analysis. Investigators were kept blinded for efgartigimod or control Fc fragment treatment. To this end, aliquots of efgartigimod and control Fc fragment stock solutions were coded by a lab member not performing the experiments. The coding was disclosed after completion of all *in vivo* studies and the analyses of the *ex-vivo* and morphological endpoint measurements. A group of five NOD/SCID mice was daily injected with PBS only and served as negative control.

2.7. In vivo assessment of neuromuscular function

The *in vivo* muscle strength and endurance of mice was assessed daily as described before (Klooster et al., 2012). With a grip strength meter (type 303,500, Technical and Scientific Equipment GmbH, Bad Homburg, Germany), a trial of 10 consecutive pulls with a few seconds pause in between was performed. The mean value of the peak force was calculated from these 10 pulls. An inverted mesh hang test was used to quantify fatigability of limb and abdominal muscles. The test ended upon completion of the maximum hanging time, set at 180 s, or falling off the mesh sooner (three attempts were allowed, duration of best attempt was taken for analysis).

2.8. Endpoint analyses

On day 11, mice were tested one last time for *in vivo* neuromuscular performance. Then, they underwent repetitive nerve stimulation electromyography of calf muscles under anaesthesia, as described before (Klooster et al., 2012). We used 40 Hz nerve stimulation, shown earlier to give prominent CMAP decrement in our model (Klooster et al., 2012), and being the approximate physiological firing frequency of rodent motor neurons (Eken, 1998). One myasthenic mouse from the control Fc fragment-treated group died soon after the anaesthesia had been installed, but muscles could be directly dissected for the *ex vivo* analyses described below. One mouse from the efgartigimod group did not respond and died overnight before the endpoint analyses day. Thus, endpoint EMG data was obtained from treatment groups of n = 7 each, and for all other endpoint analyses (see below) the efgartigimod and control Fc fragment group size was n = 7 and n = 8, respectively. After completing the electromyography, mice were killed by CO_2 inhalation without recovery from anaesthesia. Diaphragm, flexor digitorum brevis (FDB) and levator auris longus (LAL) muscles were quickly dissected for the analyses described below.

2.9. Ex vivo muscle contraction studies

Contraction force of the left phrenic nerve-hemidiaphragm was measured in Ringer's medium containing (in mM): NaCl 116, KCl 4.5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 23, glucose 11, pH 7.4) at room temperature (20–22 °C). A force transducer (type K30, Harvard Apparatus, Hugo Sachs Elektronik GmbH, March-Hugstetten, Germany), connected to an amplifier TAM-A 705/1 (Hugo Sachs Elektronik), was used. The output signal was digitized with a Digidata 1440 digitizer (Axon Instruments/Molecular Devices, Union City, CA), connected to a PC running Axoscope 10 (Axon Instruments). With a Vernier control, the optimal basic stretch tension was determined and maintained, *i.e.* the tension that gave maximal contraction upon 40 Hz supramaximal stimulation during 1 s. After equilibration and several Ringer medium washes, the phrenic nerve was stimulated supramaximally at several frequencies during 7 s, with 30 s pauses, to determine the stimulation frequency-contraction relationship. Area under the contraction curve was determined in later off-line analyses, using Clampfit 10 (Axon Instruments). Thereafter, the phrenic nerve was stimulated once every 5 min with 280 stimuli at 40 Hz, *i.e.* for 7 s. The safety factor of neuromuscular transmission was assessed by measuring

40 Hz nerve-stimulated contraction force in the presence of 125 nM) d-tubocurarine (Sigma-Aldrich, Zwijndrecht, The Netherlands). The amplitude of these recorded contractions was cursor-measured in Clampfit 10, at 2 s after the start.

2.10. Ex vivo neuromuscular junction electrophysiology

Intracellular recordings of miniature endplate potentials (MEPPs) and endplate potentials (EPPs) at the NMJ were made in Ringer's solution at 26–28 °C in right phrenic nerve-hemidiaphragm. At NMJs of FDB muscle preparations, MEPPs were recorded. For details, see (Klooster et al., 2012).

2.11. Fluorescence confocal laser-scanning microscopy of NMJs

Directly at the end of each *in vivo* experiment LAL muscles were dissected to visualize the AChRs at NMJs. LALs, together with a small strip of the right hemi-diaphragm were pinned out in Sylgard dishes and fixed in 1% paraformaldehyde in PBS for 1 h at room temperature. Next, the tissue was extensively washed in PBS. The tissue was subsequently incubated with 1 μg/ml α-bungarotoxin (α-BTX) conjugated with AlexaFluor488 (B13422, Molecular probes, Thermo Fisher Scientific, Bleiswijk, The Netherlands) for 30 min, followed by six 10 min washes in PBS. All incubations were performed at room temperature. Specimens were then mounted with Prolong Gold mounting medium (Thermo Fisher Scientific) and imaged under a SP8 confocal laser-scanning microscope using Las X software (Leica, Amsterdam, The Netherlands) or on a Zeiss Axioskop 2 FS with epifluorescence optics using Axiovision software (Carl Zeiss, Breda, The Netherlands).

2.12. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical significance of differences between the data from the efgartigimod group *versus* the control Fc fragment group was tested with Student's *t*-tests, with corrections for multiple testing wherever appropriate. Differences with P-values < 0.05 were considered statistically significant.

3. Results

3.1. Efgartigimod does not interfere in MuSK autoantibody binding to MuSK

We performed MuSK ELISAs to exclude the possibility that efgartigimod or control Fc fragment directly binds to MuSK or interferes with autoantibody binding to MuSK. Neither efgartigimod nor control Fc fragment bound recombinant MuSK, whereas purified MuSK MG patient IgG4 used as standard showed expected reactivity against MuSK (Fig. 1A). Possible competition of efgartigimod or control Fc fragment with autoantibody binding to MuSK was assessed by co-incubation at several concentrations with a fixed concentration of purified IgG or IgG4 from three MuSK MG patients (Fig. 1B). No effect on MuSK reactivity was observed.

3.2. Efgartigimod reduces levels of injected human IgG in serum of NOD/SCID mice

NOD/SCID mice are valuable for passive transfer studies with human patient-derived IgG as these mice cannot produce endogenous IgG and thus do not develop an immune response against the injected human IgG. This eliminates the need to add (toxic) immunosuppressants. However, injected IgG has a relatively short half-life in these mice (Pop et al., 2013). Although the FcRn gene sequence does not seem to be altered in NOD/SCID mice, human and chimeric IgGs bind more preferentially to Fc gamma receptor-expressing macrophages than in other mouse strains, possibly causing the reduced serum IgG half-life (Pop et al., 2013). We first aimed to establish that efgartigimod is capable of lowering injected human IgG serum levels in NOD/SCID mice. Four mice were loaded with seven daily injections of 35 mg normal human IgG (Fig. 2). In serum obtained on the fifth day, all four mice showed levels of 6–8 mg/ml human IgG. A single dose of 1 mg efgartigimod co-injected in two of the mice with the last IgG dose on the seventh day led to ~30–50% lower IgG levels as compared to the two Fc fragment controls, when assayed in serum obtained on the eighth day. At the eleventh day and onwards, serum IgG of all mice had returned to very low levels due to the short serum half-life of IgG in NOD/SCID mice. These results suggest that efgartigimod is capable of acutely lowering serum IgG levels in NOD/SCID mice.

3.3. Efgartigimod improves in vivo muscle weakness in myasthenic NOD/SCID mice

NOD/SCID mice (n = 16) were injected with purified IgG4 from one MuSK MG patient to generate a passive transfer MuSK MG model, essentially as described previously (Klooster et al., 2012). The daily dose of purified MuSK MG IgG4 injected was set to 0.1 mg/day per g of the body weight on the day before the first injection. This dose was determined in initial dose-ranging experiments for the used batch of purified IgG4, aiming to establish the minimal dosing that would give clear *in vivo* myasthenic features (Supplementary Fig. 1).

Passive transfer mice were tested daily for neuromuscular performance and body weight was noted, starting at 4 days before the first IgG4 injection to familiarize the mice with handling and tests. On the day of the fifth MuSK MG IgG4 injection, mice were co-injected with either 0.5 mg efgartigimod (n = 8) or control Fc fragment (n = 8). The efgartigimod treatment prevented or reduced the progressive body weight loss and myasthenic muscle weakness that was observed in control Fc fragment-treated mice after ~5–7 days of MuSK MG IgG4 injection (Fig. 3A and see below). The mean reduction of body weight in the control Fc fragment group on the endpoint day, as compared to the first day of MuSK MG IgG4 injection, was 22.6 ± 2.9% (from 18.4 ± 0.5 g to 14.2 ± 0.6 g, $P < 0.001$, paired *t*-test), whereas that of the efgartigimod group was 7.0 ± 4.6% (from 18.3 ± 0.5 g to 17.1 ± 1.0 g, $P = 0.16$, paired t-test) (Fig. 3B). One of the eight efgartigimod-treated mice had an early onset of the myasthenia and apparently did not respond. It showed accelerated body weight loss as compared to the control Fc fragment-treated mice (Supplementary Fig. 2, Fig. 3B) and died in the night before the endpoint analyses were scheduled.

Efgartigimod-treated mice performed better in muscle weakness tests. In the grip strength measurements, control Fc fragment injected MuSK MG mice developed progressive loss of muscle strength starting at the fifth passive transfer day (Fig. 3C). The mean strength loss at endpoint as compared to the start was 79.9 ± 10.5% (from 80.2 ± 4.7 g to 16.9 ± 9.0 g, $P < 0.001$, paired t-test). The efgartigimod group lost much less strength, 27.3 ± 12.3% (from 73.4 ± 3.7 g to 53.4 ± 8.9 g, $P < 0.05$, paired t-test). This difference in strength reductions between the groups was statistically significant ($P < 0.01$, Fig. 3D). The one non-responding efgartigimod-treated MuSK MG mouse lost all its strength in this test. PBS-only treated mice (*i.e.* which did not receive patient IgG4) showed a slight increase of force during the experiment, presumably due to a training effect.

Similarly, efgartigimod-treated MuSK MG mice performed better than control Fc fragment-treated MuSK MG mice in the inverted mesh hang test, which tests for muscle fatigue (Fig. 3E). The mean hang time on the endpoint day of control Fc fragment-treated mice was 40.1 ± 22.1 s, whereas efgartigimod-treated mice hung for 143.0 ± 18.2 s ($P < 0.001$). All PBS-only treated mice completed the 180 s hanging time.

At the end of the experiment, mice were anaesthetized and repetitive nerve stimulation EMG was performed at the left calf muscles to assess a possible myasthenic decrement of compound muscle action potentials (CMAPs). The absolute value of the recorded first CMAP in the control Fc fragment-treated group was ~25% lower than in the efgartigimod-

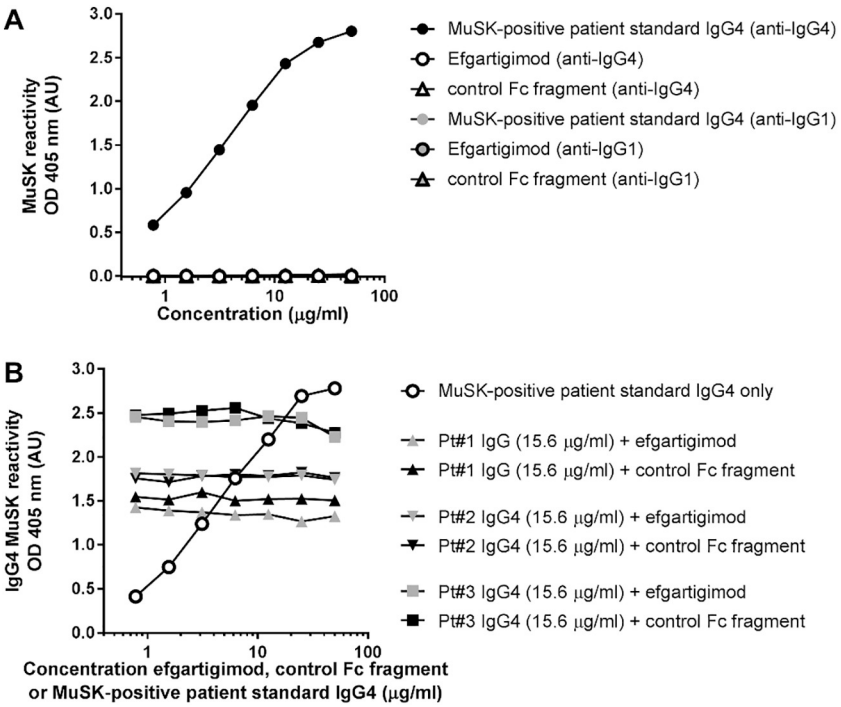

Fig. 1. Efgartigimod and control Fc fragment do not bind recombinant extracellular MuSK in ELISA. (A) The positive control standard, a purified patient IgG sample, showed clear IgG4 reactivity against MuSK, while efgartigimod or the control Fc fragment showed no MuSK reactivity at all (symbols overlap near OD zero for all concentrations). Furthermore, no IgG1 anti-MuSK reactivity was detected in the control standard nor with efgartigimod or the control Fc fragment (symbols overlap near OD zero for all concentrations). (B) Co-incubation of a fixed concentration of 15.6 μg/ml MuSK MG patient IgG(4) with increasing concentrations of efgartigimod or control Fc did not inhibit the ability of the patient material to bind to MuSK. A concentration range of a MuSK-positive patient standard alone was included in the experiment to demonstrate the efficacy of the ELISA.

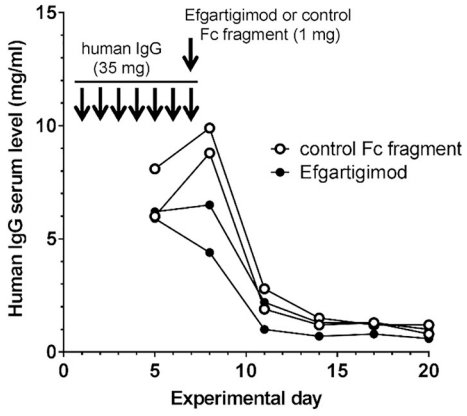

Fig. 2. Efgartigimod is capable of lowering serum levels of injected human IgG levels in NOD/SCID mice. Four NOD/SCID mice were loaded for 7 days with 35 mg normal human IgG per day. Human IgG levels in the serum of the individual mice are shown, as determined from blood samples taken at day 5, 8, 11, 14, 17 and 20. Two mice co-injected with 1 mg efgartigimod on the seventh experimental day revealed ~30–50% lower IgG level on the next day, as compared to the two control Fc fragment co-injected mice. At the eleventh day and onwards, serum IgG levels of all mice returned to low levels due to the short serum half-life of IgG in NOD/SCID mice.

treated group ($P < 0.05$, Fig. 3F). CMAP decrement upon 40 Hz nerve stimulation was more pronounced in most control Fc fragment-treated MuSK MG mice, as compared to the efgartigimod-treated group (mean decrements of 29.4 ± 9.6% and 5.2 ± 1.7%, respectively, $P < 0.05$, Fig. 3G and H). As expected, untreated PBS-only mice showed no CMAP decrement. Thus, efgartigimod improved CMAP decrement, an important clinical neurophysiological feature of MG.

3.4. Efgartigimod causes a prominent reduction of the level of human IgG4 autoantibodies in MuSK MG passive transfer mice

To monitor the effect of efgartigimod on the levels of human MuSK antibodies in the serum of the MuSK MG passive transfer mice, blood samples were obtained and tested in a MuSK IgG4 ELISA (Fig. 4). IgG4 MuSK antibody levels were undetectable at the start of the experiment in all mice and rose to a similar level on day 3 (*i.e.* one day before start of the efgartigimod treatment) in the mice assigned to either the efgartigimod group or control Fc fragment group (Fig. 4A). At endpoint, it appeared that efgartigimod clearly had reduced the serum IgG4 MuSK antibody level, as compared to control Fc fragment treatment ($P < 0.001$). At experimental day 3, the maximum serum levels of MuSK IgG4 were apparently already reached, as the titres in the control Fc fragment group hardly increased over the next 8 days in spite of continued daily MuSK MG IgG4 injections. The mean OD value of endpoint sera was ~80% lower in the efgartigimod-treated group ($P < 0.001$, $n = 7$ efgartigimod mice *vs.* $n = 8$ control Fc fragment mice, Fig. 4B). From the titration curves that were made in these ELISAs (Fig. 4C and D), it can be deduced that efgartigimod caused an about eight-fold reduction of MuSK IgG4 levels at the endpoint.

3.5. Ex vivo analyses of diaphragm muscle

Nerve stimulation-evoked contraction of dissected diaphragm muscles was measured. Stimulation frequencies higher than 20 Hz caused tetanically fused contractions with, in the MuSK MG mouse diaphragms, a clear fatiguing component after a few seconds. Area under the contraction curve analysis showed a modest improvement of the myasthenic fatigue by efgartigimod, as compared to the control Fc fragment group, statistically significant at frequencies of 60 Hz and higher ($P < 0.05$, Fig. 5A and B). The peak force delivered upon 40 Hz nerve stimulation (*i.e.* the physiological motor neuron firing frequency) of the efgartigimod group showed a non-statistically significant tendency of increase (~11%), as compared to the control Fc fragment group ($P = 0.07$, Fig. 5C). The safety factor of neuromuscular transmission was assessed by measuring the 40 Hz tetanic contraction in 125 nM d-tubocurarine, a reversible blocker of AChRs. This too revealed a non-statistically significant tendency of improvement in the efgartigimod group, as compared to the control Fc fragment group ($P = 0.11$, Fig. 5D). Still, the safety factor remained compromised, as compared to the PBS-only group of which the diaphragm contraction was almost unaffected by 125 nM d-tubocurarine.

Similarly, the electrophysiological parameters determined with microelectrode measurements at single diaphragm NMJs showed only slight

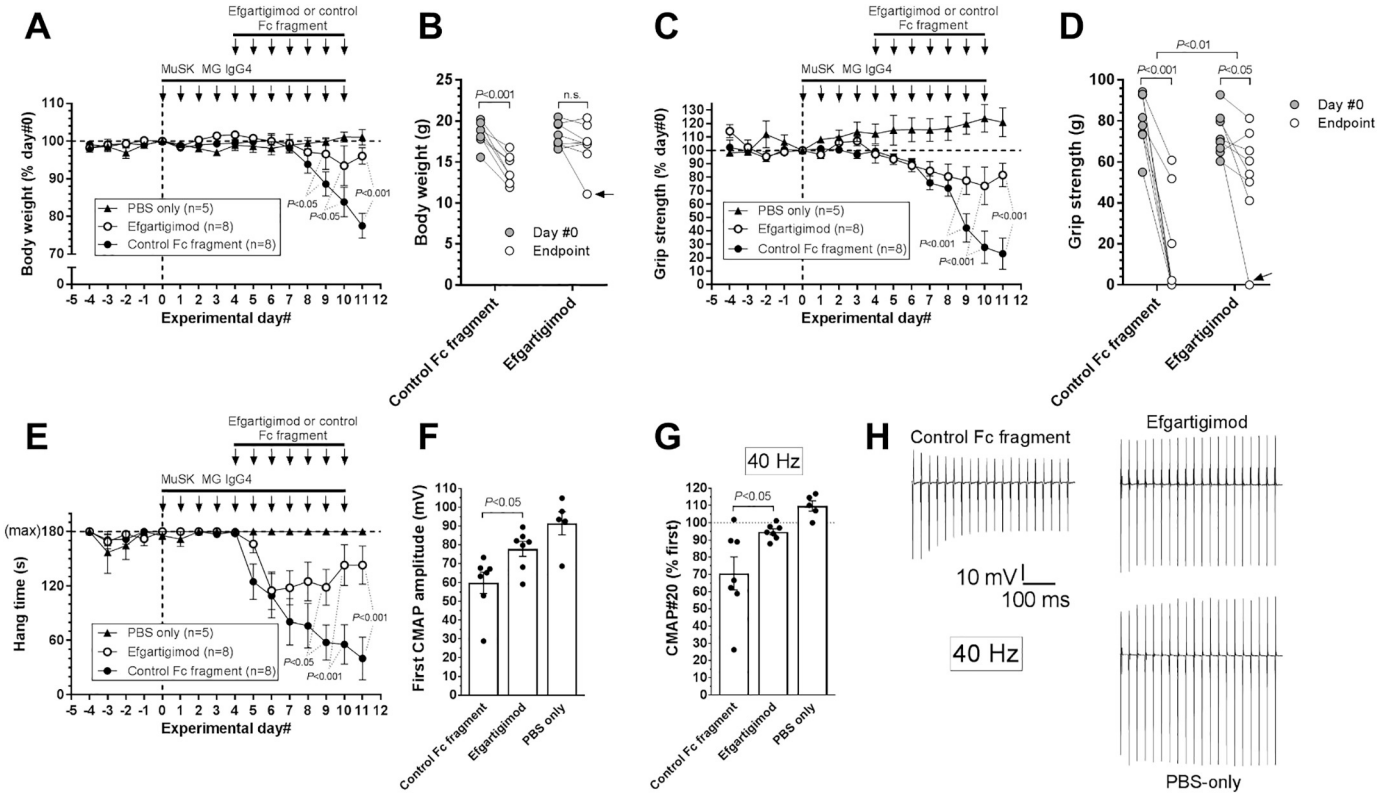

Fig. 3. Efgartigimod improves *in vivo* neuromuscular performance in myasthenic MuSK MG passive transfer mice. NOD/SCID mice were passively transferred with 11 daily injections with 0.1 mg/g purified MuSK MG IgG4, and 0.5 mg efgartigimod (n = 8) or control Fc fragment (n = 8) was co-injected daily, starting at the fifth MuSK MG IgG4 injection day. (A) Efgartigimod prevented for the greatest part the progressive body weight loss associated with the myasthenia in the model, as observed in the control Fc fragment group starting after ~7 days. For comparison, stable body weight of a negative control group of five mice injected with PBS only (*i.e.* no MuSK MG IgG4, efgartigimod or control Fc fragment) has been included in the graph. The difference between the efgartigimod and control Fc fragment group means was statistically significant on experimental day 9–11, as indicated. (B) All eight mice in the control groups showed clear body weight loss (mean 22.6 ± 2.9%, P < 0.001) at the endpoint day, as compared to the starting day of the MuSK MG IgG4 injections. In the efgartigimod group this was only the case in one of the eight treated mice (indicated with black arrow). n.s. = not statistically significant. (C) Control Fc fragment-treated MuSK MG mice progressively lost grip strength, starting at the fifth MuSK MG IgG4 injection day, while this was less outspoken in the efgartigimod-treated group (P < 0.001). (D) Mice in the control group (n = 8) showed severe loss of force at the endpoint day, as compared to the start (mean 79.9 ± 10.5%, P < 0.01), while this was less outspoken in the efgartigimod group (27.3 ± 12.3%, n = 8, P < 0.05), except for the one non-responding mouse (black arrow), which progressively lost all force in this test during the course experiment. (E) Efgartigimod induced partial recovery from the progressive fatigable muscle weakness occurring in the MuSK MG passive transfer mice from the fifth MuSK MG IgG4 injection day onwards in the inverted mesh test. (F) The sciatic nerve of the left hind leg was electrically stimulated repetitively at supramaximal amplitude with subcutaneous needle electrodes. Compound muscle action potentials (CMAPs) were measured with a second set of subcutaneous needle electrodes. Mean amplitude of the first CMAP was ~25% lower in the control Fc fragment-treated group than in the efgartigimod-treated group (P < 0.05). (G) About 25% less CMAP decrement in the efgartigimod-treated MuSK MG mice than in the control Fc fragment-treated group (P < 0.05), indicating improved neuromuscular synaptic function. (H) Representative examples of 40 Hz nerve stimulation-evoked CMAPs.

(non-statistically significant) tendencies of improvements in the efgartigimod group, as compared to the control Fc fragment group (Fig. 6). The amplitudes and frequencies of MEPPs and amplitudes of 0.3 Hz nerve stimulation-evoked EPPs tended to be ~10–20% larger in the efgartigimod group than in the control Fc fragment group (P = 0.11, 0.31 and 0.09, respectively), but were still largely reduced compared to those of the PBS-only group (Fig. 6A–C). The quantal content of EPPs (Fig. 6D) and rundown of high-rate (40 Hz) nerve stimulation-evoked EPP amplitudes (Fig. 6E and F) were unchanged in the efgartigimod group, as compared to the control Fc fragment group. MEPP amplitudes and frequencies measured at NMJs of FDB muscles of efgartigimod-treated mice, were also not or only marginally improved, respectively, as compared to control Fc fragment-treated mice (Supplementary Fig. 3).

3.6. No clear normalization of the MuSK MG neuromuscular junction morphological aberrations by efgartigimod

NMJs of MuSK MG passive transfer mice have severely fragmented AChR clusters (Ghazanfari et al., 2014; Klooster et al., 2012). We studied AChRs cluster appearance at NMJs of diaphragm and LAL muscles of the efgartigimod-treated and control Fc fragment-treated MuSK MG IgG4 mice. This revealed that NMJs of efgartigimod-treated mice, even those with improved muscle strength, still had fragmented AChR clusters, indiscernible from the situation in NMJs of control Fc fragment-treated mice (Fig. 7). Since these appearances were very similar in both treatment groups, as judged upon visual observations by an experienced investigator blinded for the treatment, no further quantifications were made because we presumed that these (tedious) analyses would at best reveal only very subtle differences.

4. Discussion

Inhibition of the serum IgG recycling system through FcRn blockade is a method by which circulating IgG levels can be rapidly reduced (Sesarman et al., 2010; Wang et al., 2014). This can be used as therapeutic strategy in antibody-mediated autoimmune disorders such as MG. In the present study we provide preclinical evidence of a therapeutic effect of FcRn inhibition in MuSK MG. In a passive transfer mouse model, based on daily purified MuSK MG patient IgG4 injections, we demonstrate that administration of efgartigimod, an engineered Fc

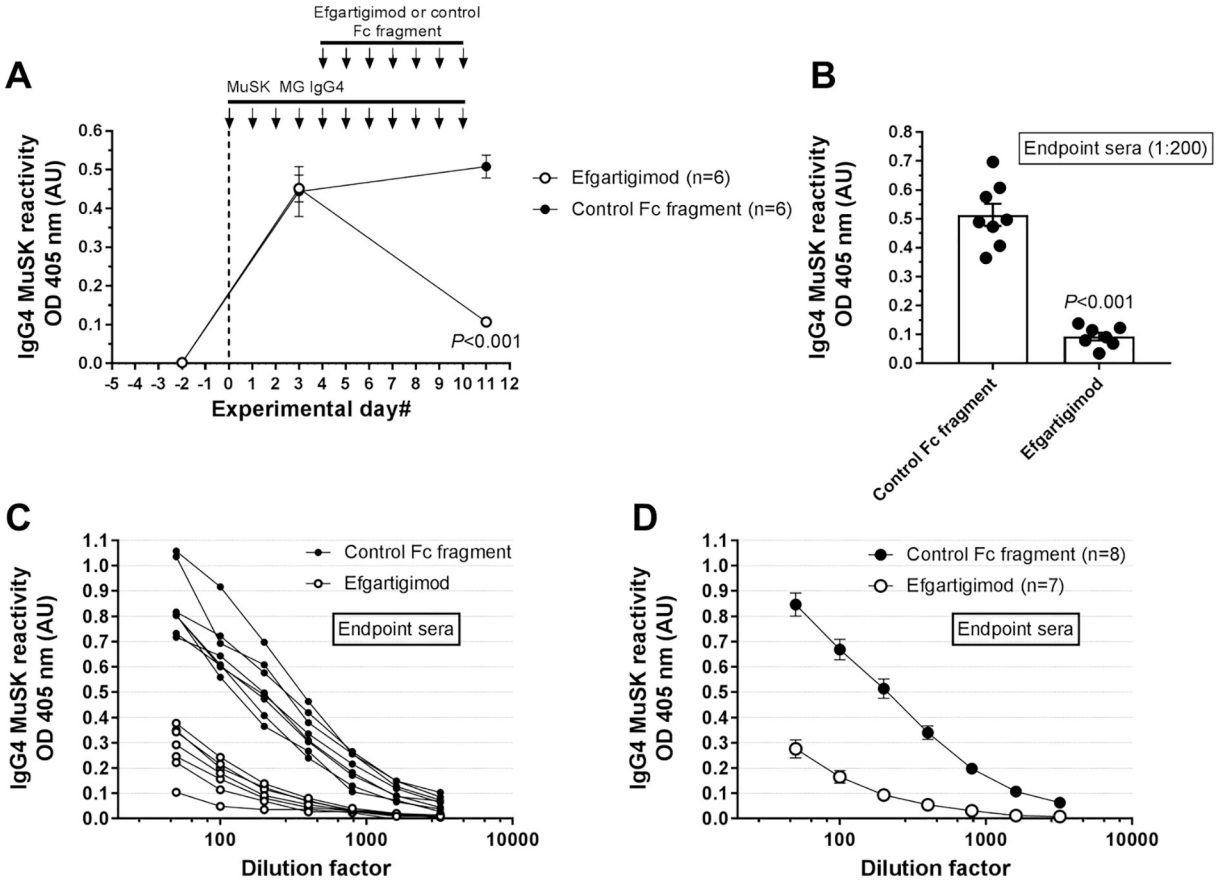

Fig. 4. Efgartigimod greatly reduces IgG4 MuSK antibody titres in MuSK MG passive transfer mice. Human IgG4 MuSK reactivity in mouse sera was determined in ELISAs. (A) Pooled data from two ELISAs under identical conditions (n = 5–6 mice per group per time-point). At 2 days before the start of the passive transfer, MuSK reactivity was absent. After 3 days of injections with purified MuSK MG IgG4, *i.e.* one day before the start of the efgartigimod or control Fc fragment treatment, mice of both groups had comparable levels of MuSK reactivity. At endpoint, efgartigimod-treated mice had clearly lower levels of MuSK reactivity ($P < 0.001$), in spite of continued MuSK MG IgG4 injections. (B) Pooled data from three ELISAs under identical conditions of endpoint serum (1:200; n = 7–8 mice per group), showing greatly reduced MuSK IgG4 level by efgartigimod ($P < 0.001$). (C) Titration curves of endpoint sera of individual mice. (D) Group mean values of titration curves indicating that efgartigimod causes an about eight-fold reduction of MuSK MG IgG4 in the endpoint sera.

fragment which binds and thereby blocks FcRn, considerably lowers the serum levels of patient autoantibodies and improves the myasthenic muscle weakness and fatigability, thereby largely preventing the secondary occurrence of body weight loss.

Daily efgartigimod treatment was started at the fifth day of MuSK MG IgG4 injection in our passive transfer mouse model. As of day 7 of the experiment, this treatment improved the *in vivo* myasthenic features as compared to the control myasthenic group, which was co-injected with a wild-type control Fc fragment that lacks the optimized FcRn binding properties of efgartigimod. Indeed, grip strength and hang time in the inverted mesh test stabilized as of day 7 in the efgartigimod group and increased towards the end of the experiment whilst the control group showed progressive loss of muscle strength and increased fatigability. Similarly, the weight loss associated with the myasthenic features in this model was reverted in the efgartigimod-treated group.

Not surprisingly, in view of the relatively short treatment duration, some degree of myasthenia remained present in the *in vivo* tests up to the endpoint. At least three explanations for this incomplete recovery can be theorized: 1) The lowered MuSK MG IgG4 titres might still suffice to induce NMJ pathology. However, our ELISAs showed an about eight-fold reduction of MuSK MG IgG4 at endpoint, and the MuSK MG IgG4 dose-range finding experiments (see Supplementary material) showed that the chosen daily dose of 0.1 mg/g body weight was already at the threshold of inducing overt myasthenic muscle weakness. It is thus not to be expected that eight-fold lowered serum levels by itself would still cause overt myasthenia. 2) The efgartigimod treatment might have been started too late. Passive transfer of high doses MuSK MG IgG has been shown to induce acute NMJ pathology by eliminating 3–5% of the AChRs per day, directly from the start (Morsch et al., 2012). Thus, 12–20% of AChR density must already have been lost at the start of the efgartigimod treatment on the fifth passive transfer day, and this loss will likely have progressed for a number of days, in spite of lowered MuSK IgG4 levels. 3) Recovery from this loss depends on insertion of new AChRs, a process that takes a few days (Bruneau et al., 2005). Furthermore, AChR density is at dynamic equilibrium with degradation compensated for by insertion of newly synthesized *plus* recycling of existing AChRs (Bruneau et al., 2005). Both these mechanisms may be disturbed at the MuSK MG NMJ, hampering recovery. In line with this is the observation that recovery to full muscle strength in our passive transfer model after cessation of the MuSK MG IgG4 injections takes one week (JJ Plomp, unpublished data). Thus, the remaining degree of myasthenia in efgartigimod-treated mice is most likely due to a lag in the degradation of AChR density after lowering the IgG levels, in combination with slow and possibly disturbed recovery of AChR density within the time-frame of our model. Further studies with longer passive transfer duration and/or earlier start of efgartigimod treatment will reveal if, due to the currently used paradigm, the therapeutic potency of efgartigimod was underestimated and whether complete remission is possible.

Interestingly, while efgartigimod clearly improved all studied *in vivo* parameters of our MuSK MG IgG model, only subtle improvements were seen on the tested *ex vivo* parameters of the diaphragm muscle, *i.e.* muscle contraction force and single NMJ electrophysiology. Firstly, the

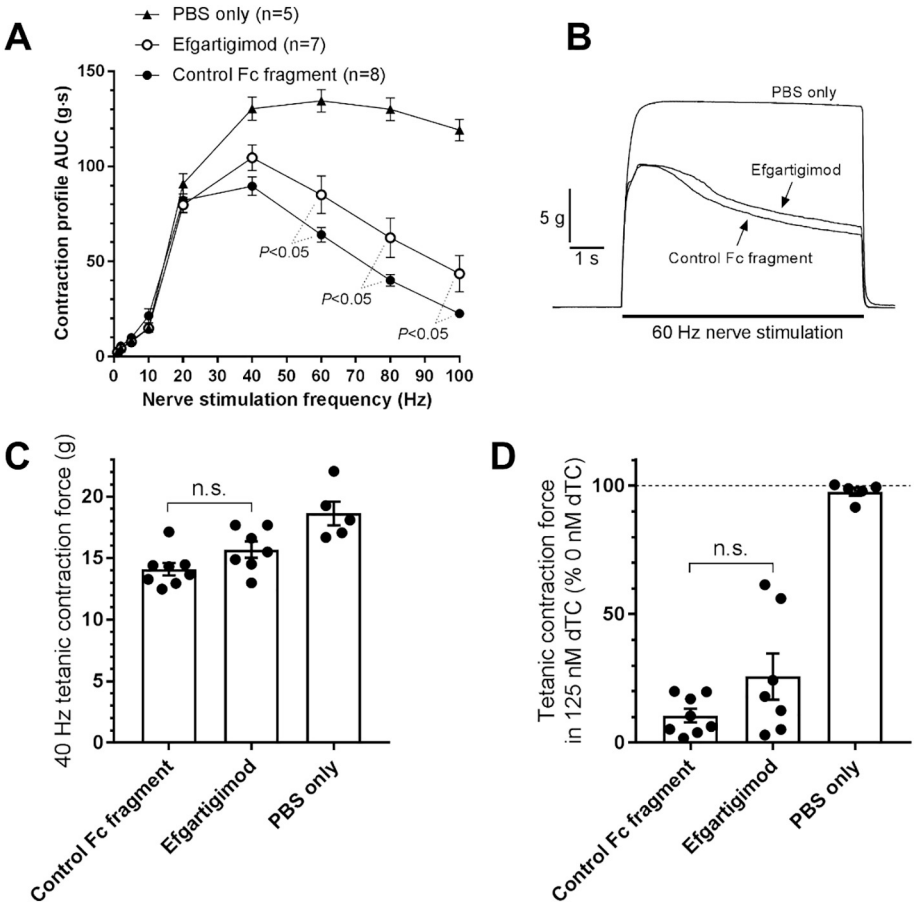

Fig. 5. Slightly improved *ex vivo* muscle contraction in diaphragm of Efgartigimod-treated MuSK MG IgG4 passive transfer mice. Supramaximal nerve stimulations of the phrenic nerve. (A) Area under the curve measurement of the diaphragm contraction profiles obtained at several stimulation frequencies for 7 s. (B) Representative contraction profiles, recorded at 60 Hz nerve stimulation for 7 s, showing slightly better, but still myasthenic, contraction. (C) Non-statistically significant tendency of improvement of the force determined at 2 s after start of the 40 Hz nerve stimulation of diaphragm of efgartigimod-treated mice. (D) Similarly, there was a tendency for improvement of safety factor of neuromuscular transmission as assessed by measuring the 40 Hz tetanic contraction in the presence of 125 nM d-tubocurarine (dTC), which was more outspoken in two of the efgartigimod-treated mice.

diaphragm muscle may be disproportionally exposed and affected by the injected MuSK MG IgG4 due to the intraperitoneal administration route. This causes direct exposure, on every injection day, before the IgG is taken up from the peritoneal cavity into the circulation. The observation that calf muscles showed clear improvement of the CMAP decrement in EMG might indicate that muscles more distant from the injection site (and only indirectly exposed to MuSK IgG4 *via* the circulation), are improving better than the diaphragm from the serum IgG lowering by efgartigimod. Indeed, in lower leg muscles (extensor digitorum longus and soleus) from a separate set of passive transfer MuSK MG mice, we found a clear tendency of improved MEPP amplitudes and AChR staining intensity at NMJs by efgartigimod (Supplementary Fig. 4). On the other hand, AChR morphology of LAL muscle and NMJ electrophysiology of FDB muscle, both muscles at distance from the diaphragm, did not show major improvements, either. Differences in muscle MuSK levels and muscle-specific sensitivities to MuSK autoantibodies may play a role here (Punga et al., 2011). Secondly, the all-or none nature of neuromuscular transmission at NMJs, in combination with the reduced safety factor following from the reduced AChR density in the MuSK MG model makes transmission extremely critical (Klooster et al., 2012; Plomp et al., 2015). In this critical state, minor recovery of AChR density following MuSK IgG4 lowering by efgartigimod might be just sufficient to restore successful neuromuscular transmission at a proportion of the NMJs and this could result in the considerable improvement of the *in vivo* fatigable muscle weakness. However, this functional recovery may not be clearly visible yet as major improvements in the AChR cluster morphology and electrophysiology at NMJs. In fact, this apparent disbalance between recovery at NMJ level and improvement of muscle weakness is similar to acetylcholinesterase inhibitor treatment of myasthenia. This can cause substantial muscle strength improvements from only minor elevation of ACh concentration at NMJs (restoring the transmission by slightly increasing EPPs to just-suprathreshold).

One of the efgartigimod-treated myasthenic mice was not responding, and developed progressive myasthenia. This mouse had the most early onset of the myasthenia of all, suggesting that the therapeutic intervention by efgartigimod was started too late. Alternatively, this mouse may have had unrelated underlying pathology, exacerbating the consequences of myasthenia. Unfortunately, no post-mortem pathology could be done.

Aside from MuSK MG in our present study, the therapeutic potential of FcRn blockade is supported by observations in other neuroimmunological disease models. In experimental autoimmune encephalomyelitis mice, modelling multiple sclerosis, an FcRn blocker based on the same ABDEG™ mutations as incorporated in efgartigimod, reduced disease symptoms (Challa et al., 2013). Furthermore, in an acute passive transfer rat MG model, involving a single anti-AChR monoclonal antibody injection, prophylactic administration of an FcRn blocking monoclonal antibody reduced myasthenic symptoms (Liu et al., 2007). It was not reported whether FcRn inhibition in a more therapeutic setting after disease induction, such as used in our current study, was equally effective in this passive transfer model. In a more chronic, active immunization AChR MG rat model in the same study, therapeutically applied FcRn monoclonal antibody reduced myasthenic symptoms, comparable to immunosuppression with dexamethasone.

It will be exciting to test FcRn therapeutics in patients with antibody-mediated (neuro)immunological diseases like MuSK-MG. First evidence for clinical efficacy of this approach has recently emerged as

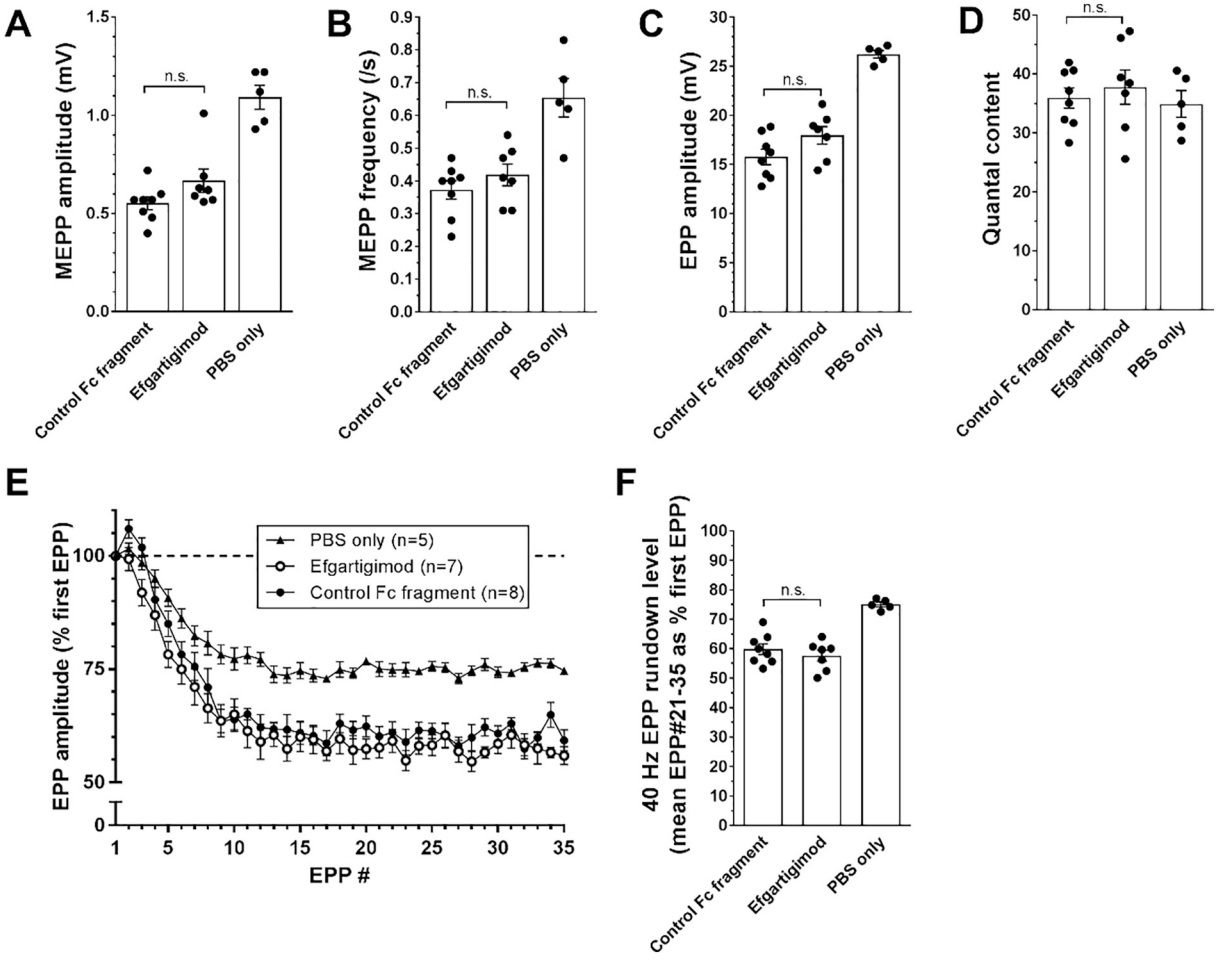

Fig. 6. Slight tendencies of improvement of synaptic signals at NMJs of efgartigimod-treated MuSK MG IgG4 passive transfer mice. Electrophysiological parameters were determined with micro-electrode measurements at single diaphragm NMJs. (A) MEPP amplitude and (B) frequency showed only slight (non-statistically significant) tendencies of improvements in the efgartigimod group. (C) Similar effect on EPPs evoked by low-rate (0.3 Hz) nerve stimulation, (D) Similar quantal contents in all groups. (E) The myasthenic extra rundown of EPPs during high-rate (40 Hz) nerve stimulation was not affected by efgartigimod treatment. (F) Further quantification showed that mean myasthenic rundown level of EPPs#21–35, as compared to the first EPP in the train, was unchanged by efgartigimod.

efgartigimod treatment was shown to induce a marked disease improvement paralleled with a substantial (auto-)antibody reduction in a double-blind, placebo-controlled phase 2 study in anti-AChR-positive MG patients (ClinicalTrials.gov NCT02965573; JF Howard, Jr., et al., manuscript submitted). Interim data from studies investigating other FcRn antagonists, in the autoimmune skin blistering disease pemphigus (ClinicalTrials.gov NCT03075904) (Werth et al., 2018) or primary immune thrombocytopenia (ClinicalTrials.gov NCT02718716) (Robak et al., 2017) further underscore the therapeutic potential of FcRn blockade.

As yet, it is not completely clear which extent of autoantibody reduction is sufficient to result in meaningful decrease of MG disease symptoms, without compromising general protective immunity. A recent study prospectively followed serum levels of pathogenic AChR antibodies as well as protective antibodies against varicella zoster, Epstein-Barr virus, diphtheria toxin, and tetanus toxoid during and after a therapeutic plasmapheresis course in ten exacerbating AChR MG patients (Guptill et al., 2016). A maximal reduction of ~60–70% of serum total IgG and similar reductions in AChR antibodies at the end of the 2 weeks plasmapheresis treatment was shown. Epstein-Barr virus, diphtheria toxin, and tetanus toxoid antibody titres remained above protective level, suggesting that a general lowering of circulating IgG levels of this magnitude confers no major extra risk for most infections. Similarly, efgartigimod was shown to be safe and well-tolerated in AChR MG patients and induced IgG reductions comparable to those achieved with therapeutic plasmapheresis (JF Howard, Jr., et al., manuscript submitted).

For MuSK MG, a correlation between disease severity and MuSK antibody concentration in the serum has been demonstrated (Bartoccioni et al., 2006). Reduction of 5 nM in MuSK antibody serum concentration roughly corresponded with a decrease of ~10 points in the quantitative MG (QMG) score, or a one-grade drop in the MG Foundation of America (MGFA) classification. In view of the range of MuSK autoantibodies of 0.1 - ~40 nM determined in the cohort of patients in that study, both the > 80% reduction of serum anti-MuSK IgG levels in our current mouse model as well as the ~75% IgG lowering in humans achieved after about two weeks and maintained for several more weeks following efgartigimod treatment (Ulrichts et al., 2018) would predict substantial improvement in many MuSK MG patients.

In conclusion, we have provided preclinical evidence of therapeutic potency of efgartigimod for MuSK MG. This was associated with only slight tendencies of functional NMJ improvements. Future experiments with longer treatment duration will show whether this was due to a lag in NMJ recovery or, much less likely, to some unexpected direct positive effect of efgartigimod on muscle function. Together with the encouraging preliminary results of preclinical and clinical studies on other FcRn inhibitors, this holds great promise for a new, easy-to-use and safe therapy in MuSK MG and other autoantibody-mediated disorders.

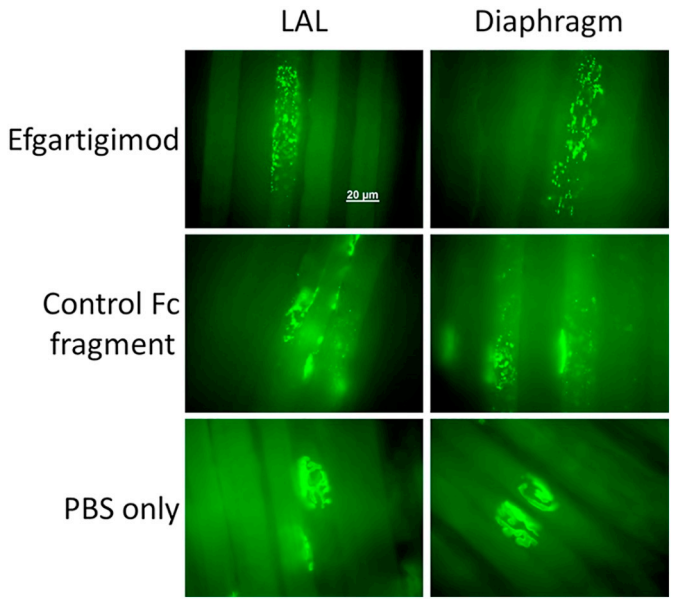

Fig. 7. Preserved AChR fragmentation at NMJs of efgartigimod-treated MuSK MG IgG4 passive transfer mice. Levator auris longus (LAL) and diaphragm NMJs were stained with AlexaFluor488-αBTx to visualize acetylcholine receptor (AChR) clusters. Upon visual inspection, no clear improvement by the efgartigimod treatment of the AChR cluster fragmentation at NMJs of the MuSK MG IgG4 passive transfer mice were apparent.

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Conflicts of interest

LUMC receives royalties for a MuSK ELISA.
JJV has been involved in MG research sponsored by the Prinses Beatrix Fonds, NIH, FP7 European grant (#602420), consultancies for argenx bvba, Alexion and Rapharma. All reimbursements were received by the LUMC.
PU, HdH and EH are full-time employees of argenx bvba.
MGH, JJP, SMvdM and JJV are co-inventors on two patent applications related to MuSK MG pathogenesis.

Declaration of authorship

MGH designed and performed experiments, analysed data and wrote the manuscript.
JJP designed and performed experiments, analysed data and wrote the manuscript.
IEvE performed experiments, purified IgG and supported *in vivo* experiments.
YEFG purified IgG, supported *in vivo* experiments.
SK performed experiments and analysed data.
PU supplied efgartigimod and control Fc fragment, performed *in vitro* experiments, analysed data and wrote the manuscript.
HdH analysed data and wrote the manuscript.
EH performed *in vitro* experiments and analysed data.
JJV designed experiments and wrote the manuscript.
SMvdM designed experiments and wrote the manuscript.

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Appendix A. Supplementary data

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