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IgG4 autoantibodies against muscle-specific kinase undergo Fab-arm exchange in myasthenia gravis patients

Inga Koneczny [a], Jo A.A. Stevens [a], Anna De Rosa [b], Saif Huda [c], Maartje G. Huijbers [d, h], Abhishek Saxena [a], Michelangelo Maestri [b], Konstantinos Lazaridis [e, f], Paraskevi Zisimopoulou [e, f], Socrates Tzartos [e, f], Jan Verschuuren [d], Silvère M. van der Maarel [h], Philip van Damme [f, g], Marc H. De Baets [a], Peter C. Molenaar [a], Angela Vincent [c], Roberta Ricciardi [b], Pilar Martinez-Martinez [a, **, 1], Mario Losen [a, *, 1]

[a] Department of Psychiatry and Neuropsychology, School for Mental Health and Neuroscience, Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands
[b] Department of Clinical and Experimental Medicine, Neurology Unit, University of Pisa, Via Paradisa 2, 56124 Pisa, Italy
[c] Neurology Department, Nuffield Department of Clinical Neurosciences, West Wing, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom
[d] Department of Neurology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands
[e] Hellenic Pasteur Institute, 127 Vasilissis Sofias Avenue 115 21, Ampelokipi, Athens, Greece
[f] Neurology Department, University Hospital, Herestraat 49, 3000 Leuven, Belgium
[g] KU Leuven - University of Leuven, Department of Neurosciences, VIB - Vesalius Research Center, Experimental Neurology - Laboratory of Neurobiology, Leuven, Belgium
[h] Department of Human Genetics, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands

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ABSTRACT

Autoimmunity mediated by IgG4 subclass autoantibodies is an expanding field of research. Due to their structural characteristics a key feature of IgG4 antibodies is the ability to exchange Fab-arms with other, unrelated, IgG4 molecules, making the IgG4 molecule potentially monovalent for the specific antigen. However, whether those disease-associated antigen-specific IgG4 are mono- or divalent for their antigens is unknown. Myasthenia gravis (MG) with antibodies to muscle specific kinase (MuSK-MG) is a well-recognized disease in which the predominant pathogenic IgG4 antibody binds to extracellular epitopes on MuSK at the neuromuscular junction; this inhibits a pathway that clusters the acetylcholine (neurotransmitter) receptors and leads to failure of neuromuscular transmission.

In vitro Fab-arm exchange-inducing conditions were applied to MuSK antibodies in sera, purified IgG4 and IgG1-3 sub-fractions. Solid-phase cross-linking assays were established to determine the extent of pre-existing and inducible Fab-arm exchange. Functional effects of the resulting populations of IgG4 antibodies were determined by measuring inhibition of agrin-induced AChR clustering in C2C12 cells. To confirm the results, κ/κ, λ/λ and hybrid κ/λ IgG4s were isolated and tested for MuSK antibodies.

At least fifty percent of patients had IgG4, but not IgG1-3, MuSK antibodies that could undergo Fab-arm exchange *in vitro* under reducing conditions. Also MuSK antibodies were found *in vivo* that were divalent (monospecific for MuSK). Fab-arm exchange with normal human IgG4 did not prevent the inhibitory effect of serum derived MuSK antibodies on AChR clustering in C2C12 mouse myotubes. The results suggest that a considerable proportion of MuSK IgG4 could already be Fab-arm exchanged *in vivo*. This was confirmed by isolating endogenous IgG4 MuSK antibodies containing both κ and λ light chains, i.e. hybrid IgG4 molecules. These new findings demonstrate that Fab-arm exchanged antibodies are pathogenic.

* Corresponding author.
** Corresponding author.
E-mail addresses: p.martinez@maastrichtuniversity.nl (P. Martinez-Martinez), m.losen@maastrichtuniversity.nl (M. Losen).
[1] These authors contributed equally.

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Abbreviations

AChR	Acetylcholine-receptor
ELISA	enzyme-linked immunosorbent assay
Fab	antigen binding fragment
GSH	reduced glutathione
IgG1-3	Immunoglobulin G1, G2 and G3
IgG4	Immunoglobulin G4
Lrp4	low-density lipoprotein receptor-related protein 4
MG	myasthenia gravis
MuSK	muscle-specific (tyrosine) kinase
NMJ	neuromuscular junction
PBS	phosphate buffered saline
κ/κ	IgG4 with two κ light chains
λ/λ	IgG4 with two λ light chains
κ/λ	bispecific IgG4 with one κ and one λ light chain

1. Introduction

Various antibody-mediated autoimmune diseases are caused by the IgG1 and IgG3 subclasses [1–6], through cross-linking-induced endocytosis of cell surface antigens, and activation of complement that attacks the cell membrane resulting in cell damage and loss of antigen function [7–15]. This relies upon the ability of IgG1 and IgG3 subclasses to bind divalently (i.e. two antigen molecules cross-linked by the antibody's two identical antigen-binding fragments, Fab) to their cognate antigen on the cell surface and to activate the complement cascade via C1q ([16–19], reviewed by Ref. [20]).

The case with IgG4 antibodies (around 5% of total IgG) is different [21,22]. Plasma cells produce cross-linking/monospecific IgG and each B-cell clone produces IgG with either κ light chains (κ/κ) or λ light chains (λ/λ) in an overall ratio of κ/λ = 2:1 [23], and a ratio of 3:1 in IgG4 [24]. After secretion into the plasma, IgG4 has the ability to exchange half-molecules (one heavy-chain with one attached light chain), with other IgG4 half-molecules, a process that has been coined "Fab-arm exchange" [25–28]. This process can be induced *in vitro* under reducing conditions [25,29]. Spontaneous Fab-arm exchange *in vivo* leads to bispecific IgG4s made up of κ/κ, κ/λ and λ/λ IgGs in serum of healthy human individuals [24,30]; elevated levels of these Fab-arm exchanged antibodies were measured in both rheumatoid arthritis [31] and autoimmune pancreatitis [32]. Overall, steady state calculations (supplementary data) suggest that we could expect that a large proportion of normal serum IgG4 is already Fab-arm exchanged *in vivo*.

Therapeutic monoclonal antibodies of the IgG4 subclass (with the natural IgG4 hinge, e.g. natalizumab) were found to exchange half-molecules with plasma IgG4 in patients [33,34], which may take up to 24 hours [25,35]. Also in IgG4 antibody diseases, the IgG4 molecules are likely to contain two different antigen-binding fragments (Fab) and cannot cross-link membrane-bound antigens to cause endocytosis [25,36,37], or form immune complexes on antigen expressing cells to activate the complement cascade [25,38,39]. Furthermore IgG4 antibodies to a specific antigen can inhibit immune precipitation by IgG1 antibodies [40,41]. Indeed several studies indicate that IgG4 dampens hypersensitivity reactions caused by antibodies with the same specificity but a different isotype or subclass that remains cross-linking [41–51] (summarized in schematic Fig. 1A).

There are now well recognized autoimmune diseases that are mediated by autoantibodies of the IgG4 subclass such as pemphigus [52–56], limbic encephalitis, neuromyotonia, Morvan's disease [57–62], Goodpasture syndrome [63], thrombotic thrombocytopenic purpura [64–67] and muscle specific kinase myasthenia gravis (MuSK MG) [36,37,68–72].

MuSK-MG is caused by IgG antibodies, mainly IgG4, that bind to MuSK [36,68–70,73] which is a key orchestrator of development and maintenance of the high density of acetylcholine receptors (AChRs) at the neuromuscular junction [74]. Agrin, a heparan-sulfate proteoglycan, is released from the motoneuron [75] and binds its co-receptor, Lrp4 [76,77], which subsequently activates MuSK leading to an intracellular pathway that results in AChR clustering [74,78]. The pathogenic role of MuSK IgG4 antibodies is strongly supported by *in vitro* and *in vivo* models; IgG4 from MuSK MG patients is pathogenic by passive transfer into (complement deficient) mice [70] and purified IgG and IgG4 fractions block binding between Lrp4 and MuSK [36,37,79] and reduce agrin induced AChR clustering in myotubes [36].

If MuSK antibodies undergo Fab-arm exchange *in vivo,* they would be bi-specific and only monovalent for MuSK. Interestingly, MuSK Fabs were sufficient for pathogenicity *in vitro* [36,80], suggesting this might be the case, but that observation left open the question of whether the MuSK antibodies were Fab-arm exchanged *in vivo*. Indeed, as MuSK antibodies may not be representative of the normal IgG4 population, it was possible that some difference in IgG4 itself or in its processing pathway might have impeded Fab arm exchange. Therefore, we felt it important to test whether the MuSK IgG4 antibodies could undergo exchange *in vitro*, whether they were exchanged *in vivo*, and whether exchange *in vitro* with normal healthy IgG4 antibodies affected their pathogenicity. To this end we used IgG subclass and light chain purification methods and functional assays to explore the evidence of Fab arm exchange in MuSK autoantibodies. We demonstrate for the first time that MuSK autoantibodies do undergo Fab-arm exchange *in vitro* and *in vivo*, but without affecting pathogenicity in *in vitro* tests.

2. Materials & methods

2.1. Patients

All patients' material was obtained with approval from the relevant ethical boards and after informed consent of the patients. Serum samples or plasmas derived from plasmapheresis from 51 MuSK MG patients (44 from Pisa, 4 from Leiden, 2 from Leuven and 1 from Maastricht) were used. The patients (44 female, 7 male) had MGFA (Myasthenia Gravis Foundation of America) scores between 0 and V. Most patients were treated with immunosuppressive medication. The AChR-Ab negativity was confirmed by radioimmunoassay (RIA, RSR, Cardiff). Sera from thirteen healthy individuals were used as controls. Clinical data of MuSK MG patients whose plasma or serum was investigated in detail, and are individually referred to in the results is summarized in Table 1, clinical information on the remaining MuSK MG patients used in this study is summarized in Supplemental Table 1.

2.2. MuSK titer measurement

MuSK autoantibodies were assessed by RIA (RSR, Cardiff, UK) according to the manufacturer's instructions. Alternatively, to verify binding of antibodies to immobilized MuSK, an ELISA was used. ELISA plates (Greiner, Microlon, catalogue number 655092) were coated with 1 μg/mL MuSK extracellular domain (ECD). The MuSK ECD was produced in mammalian cells (a kind gift of Dr Bernard Rees-Smith, RSR) or in yeast [81]. Samples were added together with a standard dilution series of MuSK MG patient serum of patient P19 with predetermined MuSK titer by RIA to calculate the MuSK antibody titer of each sample or purified fraction (Supplementary Tables 3 and 4) in the plasma. Bound MuSK

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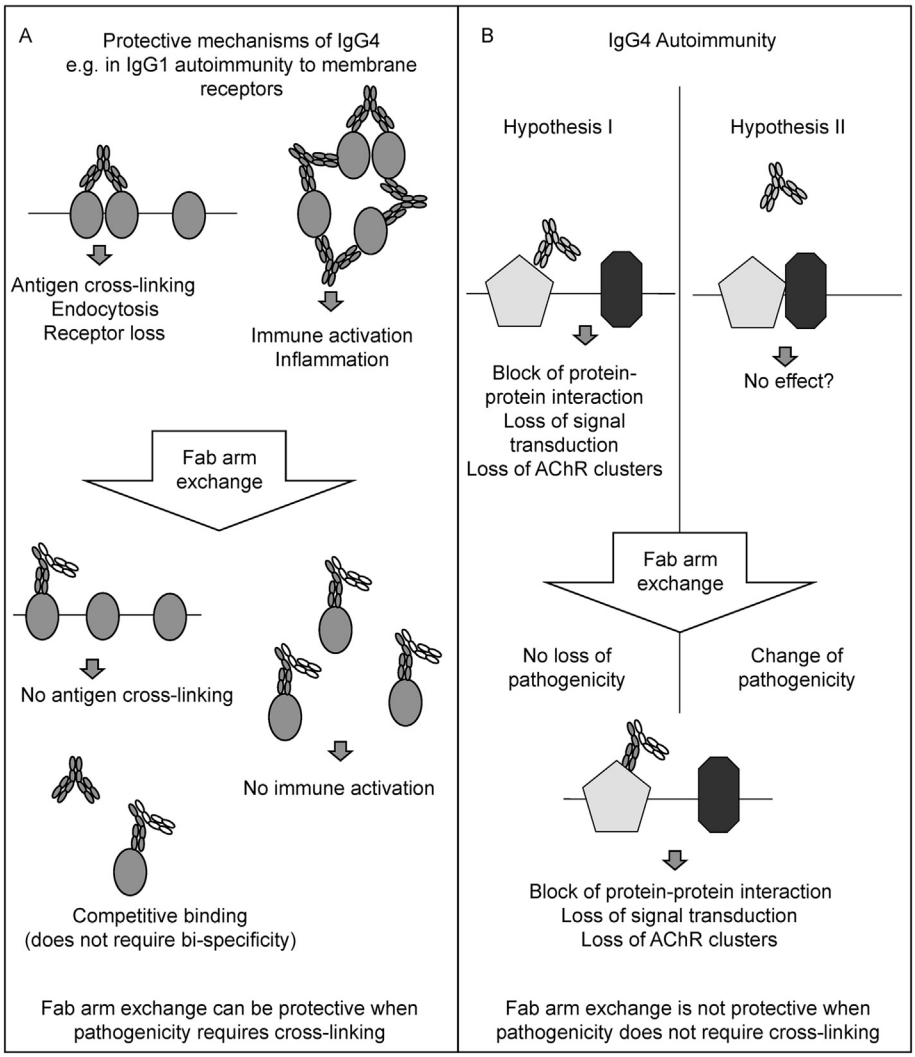

Fig. 1. Hypothesis for the role of Fab-arm exchange in IgG4 mediated autoimmunity. A) IgG4 can be protective by loss of cross-linking ability [25,36,37], preventing endocytosis as observed in IgG1 autoimmunity [12,13,50,95–98]. Bispecificity also prevents the formation of large antigen-antibody complexes, also containing complement molecules that facilitate uptake by antigen-presenting cells and subsequent activation of antigen-specific immune cells [25,38–41]. Competitive binding and the immunological inertness of the IgG4 Fc region also convey protection against hypersensitivity reactions induced by IgG1-3 [41–50]. B) Hypothesis I: IgG4 may not be protective in IgG4 autoimmunity. The main pathogenic mechanism in IgG4 autoimmune diseases is the blockade of protein-protein interactions, which does not require cross-linking. Thus Fab-arm exchange may not reduce the pathogenicity. Hypothesis II: While no supporting evidence was found in this study, it may be possible that Fab-arm exchange changes pathogenicity. To study this possibility, monoclonal MuSK IgG4 would be required.

antibodies were detected with goat $F(ab)_2$ anti-human IgG Fcγ conjugated to horseradish peroxidase (1:20,000, Jackson ImmunoResearch, USA, catalogue number 109-036-008) or alternatively with mouse anti-human IgG4:HRP (1:3500, AbD Serotec, Germany, catalogue number MCA2098P). Samples were incubated with substrate containing 3,3′, 5, 5'-tetramethylbenzidine, and absorbance at 450 nm was measured using a VictorX3 plate reader (PerkinElmer, USA).

2.3. Induction of IgG4 Fab-arm exchange reaction

Fab-arm exchange was induced based on previous studies [25,29]. The exchange was conducted in 1.5 mL microcentrifuge tubes using soluble IgG4, to avoid confounding Fc-Fc interactions that can occur with immobilized IgG4 [82]. Twenty-five μg of monoclonal antibody against AChR [83] of IgG1 (IgG1-637) or IgG4 (IgG4-637) [25] subclass were added to 25–125 μg of purified MuSK-MG IgG4 or healthy control IgG4 or IgG1-3 in the presence or absence of 0.5 mM reduced glutathione (GSH, Merck, Germany, catalogue number 1.04090) and incubated 48 h at 37 °C. Alternatively, 75 μg of IgG4-637 was incubated with 100 μL serum from MuSK-MG patients. This volume would contain approximately 1 mg IgG, and therefore approximately 50–80 μg IgG4, resulting in a 1:1 ratio between serum IgG4 and monoclonal IgG4-637. As further controls, the same amounts of IgG4-637, IgG1-637, purified IgG4, purified IgG1-3 or serum in the absence of the reaction partner were used. Notably, both in patient serum and in purified IgG4 from patient plasma, normal IgG4 molecules with specificities other than MuSK are present and participate in the Fab-arm exchange.

2.4. Bispecificity solid phase radioimmunoassay

After *in vitro* Fab-arm exchange in solution, as above (2.3), the samples were tested for newly formed MuSK-AChR bi-specific antibodies by a solid phase radioimmunoassay (SPRIA). Nunc-

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Table 1
Clinical information on selected MuSK MG patients used in detail this study (na = data not available).

Patient code	Treating center (original code)	Sex	Age at sample	Age of onset	MGFA at onset	MGFA at sample	Symptoms at onset	Symptoms at sample	MuSK titer (nM, 0.05 cut-off)	AChR MG titer at diagnosis (Pisa RIA, nM, 0.05 cut-off)	AChR MG titer at sample (Oxford RIA, nM, 0.5 cut-off)	Therapy at sample
P19	Pisa (M 132)	F	52	18	IIIB	IIA	Diplopia, mild dysartria, episodic dysphagia, limb and neck muscles weakness	Limb muscles weakness	13.6	0	0.45	Prednisone, cyclosporine, IVIG every month
P33	Pisa (M 183)	F	60	56	IIB	IIA	Ptosis, diplopia then dysphagia	Mild limb muscles weakness	11.2	0	0.24	Dexamethasone
P41	Pisa (M 116b)	F	40	32	IIIB	0	Mild dysphagia, then dysarthria and limb muscles weakness		1.2	na	na	
P43	Pisa (M 211)	F	69	53	IIB	0		No symptoms	4.1	0	na	Prednisone, Azathioprine
P46	Leiden	M	54	52	IIIB	IIIB	Oculobulbar	Ocular bulbar with proximal arm weakness	na	0	0	Pyridostigmine, prednisone 80 mg dd, azathioprine 150 mg dd was just started before admission
P47	Leiden	F	19	6	IIB	IIIB	Ocular bulbar with proximal arm weakness	Ocular bulbar with proximal arm weakness	na	0	0	Mycophenolate mofetil
P48	Leiden	M	55	na	na	na	na	Ptosis, ophthalmoparesis, dysarthria and weakness of facial muscles and both neck flexors and extensors	na	na	na	Azathioprine, Prednisone
P49	Leiden	F	25	2	I	IVB	Ocular	severe facial, bulbar and respiratory weakness	na	0	0	none
P50	Leuven	M	36		I	IIA	Ocular	Generalized weakness	11.3	Negative	na	Azathioprine
P51	Leuven	F	50		IIB	IVB	Dysarthria	Generalized weakness	4.9	Negative	na	Pyridostigmine
P52	Maastricht	F	17		V	V	Respiratory crisis, neck, dysarthria	Respiratory crisis, neck, dysarthria	6.6	na	na	Prednisone, plasmapheresis

Immuno maxisorp breakable module wells (VWR, USA, catalogue number 62409-293) were used to enable radioactive counting. The plates were coated and treated as above and incubated for 60 min with 50 μL undiluted samples. Next, the wells were washed and incubated for 60 min with 50 μL of a 1:4 dilution of AChR containing extract from CN21 cells (in 0.5% triton X-100 in PBS) that express the adult form of the AChR, with shaking at 1000 rpm. After washing, bound AChR was detected by incubation with 50 μL excess ^{125}I-alpha-bungarotoxin (^{125}I-BuTx, 370–740 kBq specific activity, Perkin Elmer, USA, catalogue number NEX126050UC) followed by washing and measuring cpm using a 2470 Wizard2 gamma counter (Perkin Elmer, USA). The results were presented as pmol/L.

2.5. Purification of IgG4, IgG1-3, Igκ and Igλ

Purified IgG fractions were isolated as described previously [70]. In short, IgG4, IgG1-3, Igκ and Igλ were isolated from plasmapheresis material from MuSK MG patients and healthy controls using one of the following affinity columns installed in an ÄKTA laboratory scale chromatography system (GE Healthcare Lifesciences): 1) IgG4 (Hu) affinity matrix (life technologies, catalogue number 290005) to isolate IgG4 subclass antibodies, 2) HiTrap Protein G HP (GE Healthcare Lifesciences, catalogue number 17-0404-01) to isolate IgG1-3 from IgG4 depleted plasma, 3) HiTrap KappaSelect (based on a single chain (scFv)) antibody, GE Healthcare, catalogue number 17-5458-11) to isolate Igκ or 4) CaptureSelect LC-lambda (Hu) Affinity Matrix (based on single domain V_HH antibody Thermo Fisher Scientific, USA, catalog number 084905) to isolate Igλ. The samples were run multiple times over the relevant affinity columns to ensure complete depletion (Supplementary Table 2). The purified IgG4, κ/λ, κ/κ and λ/λ as well as the κ and λ depleted flow-through (−/−) were then analyzed for protein concentration (Supplementary Tables 3 and 4) and the presence of MuSK antibodies using MuSK ELISA. After elution, the buffer was exchanged using gel filtration using G-25 Sephadex gel filtration medium (GE, catalogue number 17-0033-01). Eluted antibody was concentrated up to 50-times using Vivaspin 6 spin columns with 30 kDa MWCO (GE Healthcare, catalogue number 28-9323), achieving protein concentrations of 0.25–12.81 mg/mL (Supplementary Table 3) that were determined using the DC Protein Assay Kit I (BioRad, USA, catalogue number 5000111). The MuSK antibody titers of the concentrated samples were determined by MuSK ELISA and the yield of MuSK IgG4 in nmol/L was calculated (Supplemental Table 4).

2.6. Monospecific antigen cross-linking assay to determine non-Fab exchanged IgG antibodies

The monospecific cross-linking assay was a modification of the MuSK ELISA described above. In a first step, the *in vitro* Fab-arm exchange was conducted in 1.5 mL microcentrifuge tubes with purified IgG4 or IgG1-3 from MuSK MG patients as described in 2.3. As controls, non-reducing conditions were used. After the Fab-arm exchange was conducted in solution, the next step was to measure the amount of monospecific MuSK IgG4. For this purpose, the samples were applied to the MuSK-ELISA plate, and after washing and blocking, mono-specific (i.e. cross-linking) MuSK antibodies that bound only by one arm to the MuSK coated plates, were detected by binding of biotinylated MuSK ECD produced in mammalian cells, followed by washing and incubation with horseradish-peroxidase coupled streptavidin (Jackson ImmunoResearch, USA, catalogue number 016-030-084, 1:5000 in PBS, 60 min at 37 °C). The wells were washed, incubated with substrate and OD450 values were assessed as described above.

2.7. AChR clustering assay

This was performed as described elsewhere [36,68]. A soluble c-terminal splice form of neural agrin (short rat agrin-pBC12—CMV, a kind gift of David Beeson and Judith Cossins [84]) was produced in HEK293 cells as described [36]. Three healthy control sera (C1, C2, C3) were randomly selected; three MuSK-MG patient sera (P33, 41, 43) were selected for their high capacity to exchange Fab-arms with AChR mAb IgG4-637. To induce Fab-arm exchange, 100 μL serum was incubated with 75 μg purified IgG4 derived from healthy control serum under reducing or non-reducing conditions. As controls, PBS only or PBS with a total concentration of 0.5 mM GSH were used. The samples were diluted in DMEM (Gibco, USA, catalogue number 10938-025) to a final serum dilution of 1:20 (healthy controls) or 1:40 (MuSK-MG patients). The samples were dialyzed against DMEM, blinded and used on C2C12 myotubes in the presence of agrin as described [36]. AChRs were visualized with AlexaFluor 488-labeled α-bungarotoxin, images of 20 randomly selected microscopic fields per well at 20× magnification were acquired and analyzed using MicroManager software [85]. The numbers of AChR clusters per field ≥5 μm were divided by the area of myotubes per field to obtain AChR cluster number per μm^2. Mean numbers of AChR clusters per myotube area of three experiments were calculated.

2.8. Statistics

One-way ANOVA with Bonferroni post-test was used to analyze the data of the bispecific cross-linking assays and 2-way ANOVA with Bonferroni post-test was used to analyze whether Fab-arm exchange affected AChR clustering. Distribution of samples was analyzed with D'Agostino Pearson test. Linear regression and correlation analysis with Pearson was performed with normal distributed samples; the Spearman correlation coefficient was used in case of non-Gaussian distribution. All statistical tests were performed with GraphPad prism software version 4.0. All error bars in the figures indicate standard deviation; the used tests are mentioned in the corresponding legends.

3. Results

3.1. MuSK autoantibodies from MuSK MG patient sera and IgG4 fractions exchanged Fab-arms in vitro

MuSK IgG4 from each patient serum (n = 32) bound to the MuSK-coated ELISA plates (Supplementary Fig. 1A, each data-point corresponding to a patient serum) correlated broadly with the MuSK RIA titer ($r^2 = 0.28$; $p = 0.001$) although, of note, two sera were negative in the MuSK IgG4 ELISA while they were strongly positive by RIA. To determine whether Fab-arm exchange of patient-derived autoantibodies could be induced *in vitro*, sera from MuSK MG patient or healthy controls were first incubated for 48 h at 37 °C with an AChR monoclonal antibody of IgG4 subclass (AChR mAb IgG4-637 [25,83]), under either normal (PBS) or reducing conditions (0.5 mM GSH, Fig. 2A). Thereafter, the samples were transferred to the ELISA plates and tested for the presence of newly-exchanged MuSK-AChR antibodies using a bispecific solid-phase radioimmunoassay (SPRIA; illustrated in Fig. 2B) based on the ability of hybrid IgG4 antibodies to cross-link soluble AChR antigen to the immobilized MuSK; the AChR was then identified by binding of ^{125}I-BuTx (Fig. 2C). Subtraction of the results in the absence of GSH from those in GSH for each of the sera showed that 16/31 of the MuSK-MG samples contained a detectable amount of MuSK IgG4 that could exchange with the AChR IgG4 (Supplementary Fig. 1B). Notably, as the content of IgG4 in normal serum is between 5 and 8% [21,22] both patient sera and the purified IgG4 from patient

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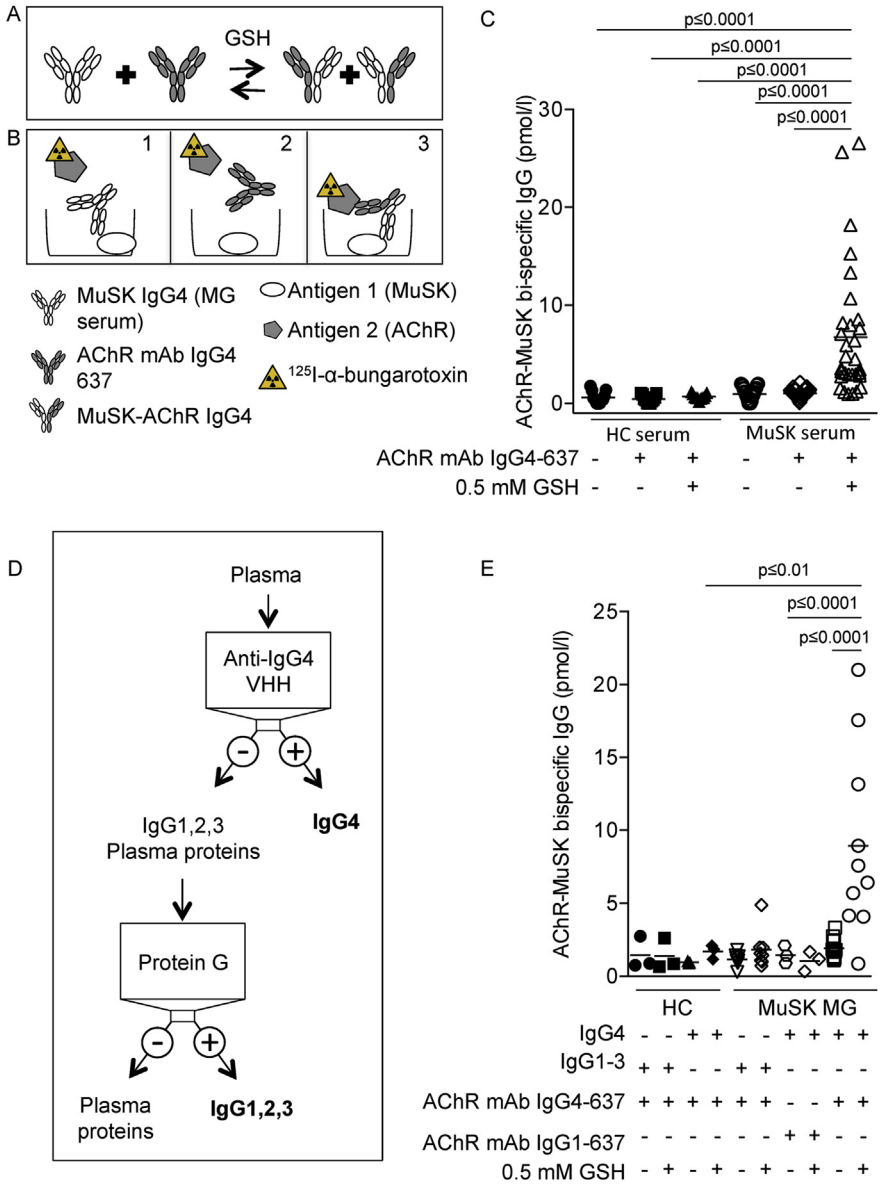

Fig. 2. MuSK IgG4 from patient serum exchanges half-molecules with monoclonal AChR mAb IgG4 antibody. A) The principle of Fab-arm exchange. Two IgG4 with different specificity (MuSK = white, AChR = grey) can undergo Fab-arm exchange to form bispecific antibodies (white/grey). B) The principle of the bispecific cross-linking assay. MuSK-AChR bispecific IgG4 (B3) can cross-link immobilized MuSK and soluble radiolabeled AChR. Monospecific antibodies against AChR (B2) or MuSK (B1) are unable to cross-link both antigens. C) Sera from 31 MuSK-MG patients or eleven healthy controls were incubated with human AChR mAb IgG4-637 under reducing (0.5 mM GSH) or non-reducing (PBS) conditions. In addition, serum samples were incubated in PBS only. Each serum was measured once; data were pooled from five individual experiments and each experiment included patient and healthy control samples of all experimental conditions. Black shapes = healthy control samples, white shapes = MuSK MG samples. Circles = serum only, rectangles = serum and AChR mAb IgG4-637, triangles = serum, AChR mAb IgG4-637 and 0.5 mM GSH. One-way ANOVA with Bonferroni post-test. **: p = 0.01,****: p = 0.0001. D, E) Fab-arm exchange of MuSK antibodies is limited to the IgG4 subclass. D) IgG4 and IgG1-3 were isolated from patient or healthy control plasma by affinity chromatography using immobilized IgG4-specific antibody and protein G. E) *In vitro* Fab-arm exchange allowing conditions (0.5 mM GSH in PBS) or control conditions (PBS only) were generated with AChR mAb 637 and purified IgG4 of seven MuSK-MG patients or IgG1-3 of six MuSK MG patients (from plasmapheresis) or two healthy controls. MuSK-AChR bispecific molecules were determined. N = 1 or 2, measured in five individual experiments. One-way ANOVA, Bonferroni post-test. All test conditions had significantly lower values than the samples with purified IgG4 from MuSK patients incubated with AChR mAb IgG4-637 under reducing conditions. N = 1 or 2, measured in five individual experiments.

plasmas would contain non-MuSK IgG4 that could participate in the Fab-arm exchange, competing with the AChR monoclonal antibody for forming hybrid MuSK IgG4 antibodies. Thus the values obtained were not expected to be quantitatively reliable. Accordingly, no correlation between the degree of Fab-arm exchange and MuSK titer or MGFA grade could be found (data not shown).

To confirm that the Fab-exchange was specific for IgG4, total IgG4 and IgG1-3 were purified from seven patients (P46-52) and two healthy controls using affinity chromatography (Fig. 2D). Fab-arm exchange was induced as described for the serum, the resulting MuSK-AChR bispecific IgG4 was then measured in the SPRIA. As anticipated, only MuSK IgG4 in combination with AChR mAb IgG4-637 yielded a bispecific MuSK-AChR IgG (Fig. 2E). Notably, the IgG1 isotype of AChR mAb-637 did not exchange with the MuSK IgG4 or IgG1-3 antibodies, and no exchange occurred in the absence of GSH.

3.2. A fraction of MuSK IgG4 is monospecific in vivo but can undergo Fab-arm exchange in vitro

Next it was important to test whether divalent, monospecific MuSK IgG4 is present in serum samples and to see if *in vitro* Fab-arm exchange could reduce the proportion of such monospecific

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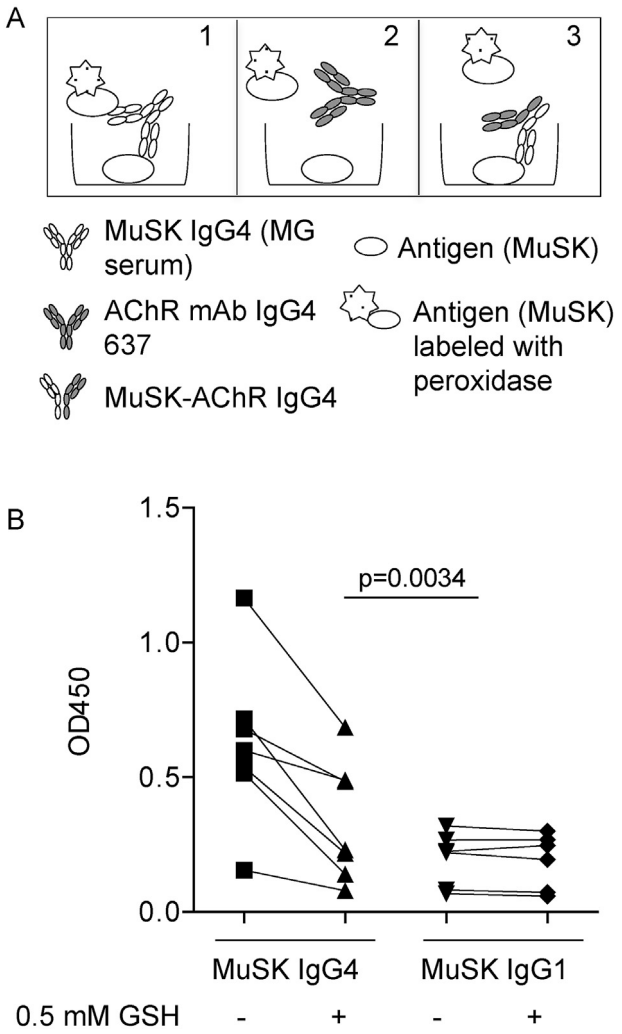

Fig. 3. Monospecific MuSK IgG4 is present *in vivo* and is reduced by Fab-arm exchange. A) Principle of the monospecific cross-linking assay. MuSK antibodies (A1, A3) are captured on immobilized MuSK, and monospecific antibodies (A1) are able to bind as well to soluble, biotinylated MuSK. B) Purified IgG4 from seven and IgG1-3 from six MuSK MG patients were subjected to *in vitro* Fab-arm exchange with AChR mAb IgG4-637 or non-reducing conditions. After Fab-arm exchange, monospecific cross-linking was measured. For statistical analysis, the data were normalized to non-reducing conditions (data not shown) and it was found that the MuSK cross-linking ability after Fab-arm exchange was reduced to 49% in the MuSK IgG4 fraction, on average. N = 1-2, measured in four individual experiments. Paired *t*-test, p = 0.0034.

antibodies. As a first step, purified IgG4 and IgG1-3 from MuSK MG patients were Fab-arm exchanged in solution under reducing conditions as described above. After the Fab-arm exchange, the samples were then applied to MuSK coated ELISA plates; MuSK antibodies bound to immobilized MuSK were tested for their ability to capture biotinylated soluble MuSK (Fig. 3A–1). Both MuSK IgG4 and IgG1-3 were able to capture some biotinylated-MuSK under non-reducing conditions (Fig. 3B), although, as expected, there were less MuSK autoantibodies in the IgG1-3 fraction available that could bind than in the IgG4 fraction, due to the predominance of the IgG4 subclass for MuSK autoantibodies [36,69].

Under reducing conditions, however, the cross-linking ability was decreased, in each of the IgG4 fractions (on average 49.6%) but not in the IgG1-3 fractions (average 4.4%; Fig. 3B). This confirms that there are divalent, monospecific IgG4 MuSK antibodies present *in vivo* but that they can be Fab-arm exchanged with other, non-MuSK binding, IgG4 *in vitro.* It should be noted that we cannot exclude the possibility that a fraction of monospecific MuSK antibodies had bound *divalently* to the immobilized MuSK in the first step, this being dependent on the coating density of MuSK protein. Thus the data must be considered as only qualitative.

3.3. *In vitro* Fab-arm exchange did not affect pathogenicity of MuSK autoantibodies in serum in the C2C12 AChR clustering assay

After demonstrating that there are monospecific MuSK IgG4s present in the patient samples, showing that not all the IgG4s have exchanged *in vivo*, we were interested to see whether Fab-arm exchange affects the pathogenicity of the autoantibodies.

To address this question, the AChR clustering assay in C2C12 mouse myotubes was used as an *in vitro* model for MuSK antibody pathogenicity. GSH treatment of healthy control serum (Fig. 4A) or GSH treatment alone did not decrease the number of AChR clusters (and even caused some increase of AChR clusters, Fig. 4C). Sera from three MuSK MG patients (P41, 43, 33), which had shown high levels of bispecific antibodies after *in vitro* Fab-arm exchange with AChR IgG4 (see Fig. 2C), were first tested to determine serum dilutions at which partial reduction of agrin-induced AChR clusters on C2C12 myotubes was achieved (data not shown). Next, *in vitro* Fab-arm exchange with purified healthy control IgG4 was induced before applying the treated patient and healthy control preparations to the C2C12 myotubes for 16 h. As expected from many previous studies, the MuSK IgG4 antibodies were found to inhibit agrin-induced AChR clustering (Fig. 4B) but inducing Fab-arm exchange did not decrease the inhibitory effect. This confirmed a previous finding regarding MuSK IgG Fabs [36] but also raised the question how large the proportion of monospecific MuSK IgG4 had been initially, and whether it could have been responsible for the inhibitory effect of MuSK antibodies.

3.4. The majority of MuSK IgG4 is Fab-arm exchanged in vivo

One possibility was that the majority of MuSK IgG4 is already bispecific, overshadowing any effect that additional Fab-arm exchange might have had on a small fraction of monospecific MuSK IgG4. To estimate the concentration of monospecific and bispecific IgG4, we used steady state calculations based on first-order kinetics using published values for metabolic half-life rates of human IgG4 and of Fab-arm exchange *in vivo* [34,35,86] (see supplementary). The resulting estimation suggests that a large proportion, perhaps even up to 99%, of MuSK IgG4 could be bispecific *in vivo*.

To confirm the prediction that *in vivo* Fab-arm exchange had already occurred in the majority of MuSK IgG4, we purified IgG4s that were hybrids for their κ and λ light chains, using affinity chromatography [24] to isolate bispecific κ/λ, as well as κ/κ and λ/λ from patient serum (Fig. 5A). MuSK IgG4 antibodies were present in the bispecific κ/λ fraction, as well as in the total IgG4, κ/κ and λ/λ fractions (Fig. 5B and C, data in Supplementary Table 4). Although the recoveries of the different fractions, in terms of total IgG4 and MuSK IgG4 antibody, were low (Supplementary Tables 3 and 4), for both patients there was a substantial proportion of MuSK IgG4s that were κ/λ. Given that hybrid κ/κ and λ/λ IgG4 antibodies would also be present, these results indicated a high proportion of Fab-exchanged IgG4 present *in vivo*.

4. Discussion

Using serum from MuSK-MG patients, we demonstrated for the first time the polyclonality and heterogeneity of the MuSK antibodies even within the IgG4 subclass. MuSK IgG4 contained both κ and λ light chains, and a substantial proportion of MuSK IgG4 antibodies was already Fab-arm exchanged *in vivo*. We showed that

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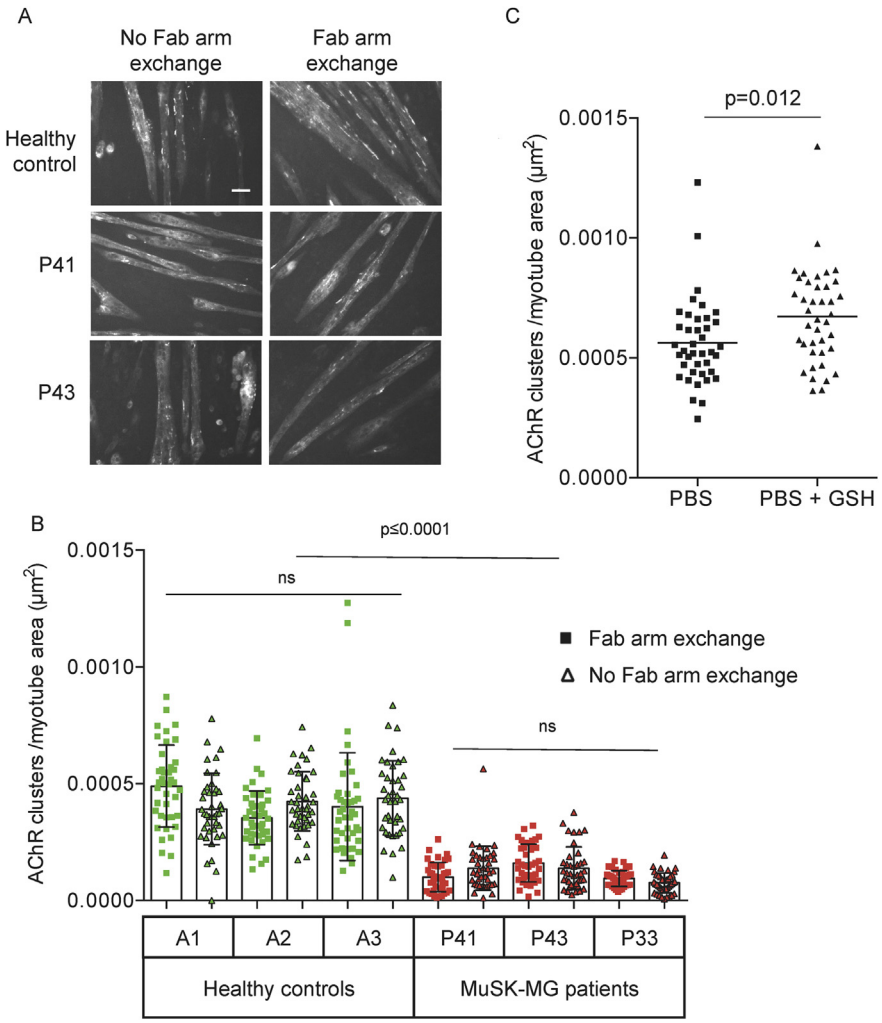

Fig. 4. Fab-arm exchange did not affect the pathogenicity of MuSK antibodies in the AChR clustering assay on C2C12 mouse myotubes. Serum from three MuSK MG patients (P33, 41, 43) that yielded high amounts of bispecific antibodies after *in vitro* Fab-arm exchange (Fig. 2C) and three healthy control sera (A1, A2, A3) were used for *in vitro* Fab-arm exchange with purified IgG4 derived from healthy control serum or using non-reducing conditions. A, B) The samples were blinded and incubated on the C2C12 mouse myotubes in the presence of agrin. AChRs were visualized with Alexa Fluor 488 labeled α-bungarotoxin. A) Representative images at 20× magnification. B) AChR clusters and myotube areas were analyzed using Micro-Manager software. Green hues: healthy controls, red hues: MuSK-MG patients. Squares: Fab-arm exchange (0.5 mM GSH in PBS), triangles: non-reducing conditions/no Fab-arm exchange (PBS). Fab-arm exchange (0.28% of total variation, $p = 0.48$) and interaction (0.59% of total variation, $p = 0.58$), in contrast to disease status (73% of total variation, $p < 0.0001$), did not affect AChR clustering. The samples treated with MuSK-MG serum had all significantly reduced numbers of AChR clusters compared to samples treated with healthy control serum ($p \leq 0.0001$). Two-way ANOVA with Bonferroni post-test. $N = 3$. C) PBS only or PBS with 0.5 mM glutathione did not reduce AChR clusters. The presence of GSH led to a small but significant increase in AChR clusters. (unpaired *t*-test, $p = 0.012$). $N = 3$.

further exchange could easily be induced under reducing conditions *in vitro*. MuSK antibodies inhibit agrin-induced clustering, an *in vitro* measure of pathogenicity. Nevertheless, this ability was not altered by Fab-arm exchange with healthy serum IgG4, confirming results from our previous study [36] that a monovalent Fab is sufficient for pathogenicity.

It is unknown how Fab-arm exchange is regulated *in vivo*, but it has been shown to be inducible *in vitro* using reducing agents, such as glutathione (GSH) [25,33,87–89]. Concentrations of GSH up to 1 mM can be found in human red blood cells [90], and suggested to be physiological relevant [25,29]. Under similar reducing conditions (0.5 mM GSH), MuSK IgG4 from MuSK MG patients was able to exchange half-molecules with a monoclonal IgG4, originally derived from an AChR MG patient [25,83], such that the resulting IgG4 antibodies were able to bind to two different antigens, MuSK on the ELISA plate and AChR in solution, in a solid phase radioimmunoassay.

No correlation between newly formed MuSK-AChR bispecific IgG4 and MuSK antibody titer could be found. This was unexpected since correlations between MuSK antibody titers and disease severity [91] and between MuSK titer and MuSK IgG4 levels have been described [36]. However, it has to be recognized that the non-MuSK binding IgG4s in the sera will have competed to varying extents with the AChR monoclonal that provided the detection end-point in the Fab-arm exchange assay.

As the detection methods were different between the three assays – peroxidase-labeled anti-human IgGs for the MuSK ELISA, bound ^{125}I-AChR for the Fab-arm exchange and biotinylated MuSK for the demonstration of preexisting cross-linking MuSK antibodies – it was not possible to calculate directly the proportions of MuSK antibodies that were divalent for MuSK, or those that were already Fab-exchanged *in vivo*. A different approach was to look at the presence of hybrid IgG4 antibodies that contained both κ and λ light chains. The values obtained must depend not only on the extent of Fab arm exchange but also on the proportions of κ and λ light chains within the specific IgG4 MuSK antibodies. These were different

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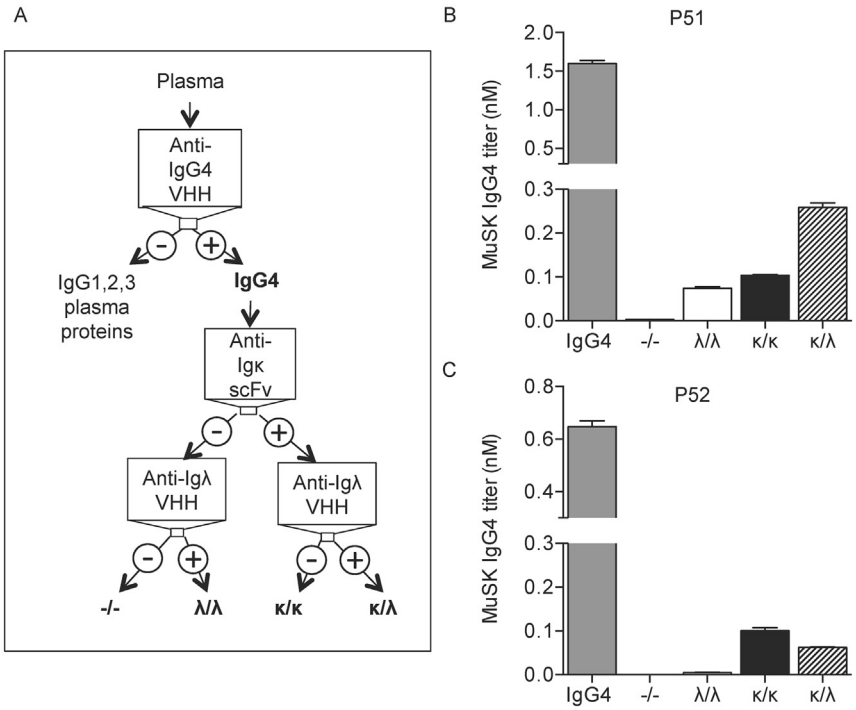

Fig. 5. A large proportion of MuSK IgG4 in patient plasma is Fab-arm exchanged *in vivo*. A) Bispecific IgG4 κ/λ as well as κ/κ, λ/λ, and −/− fractions were isolated from plasma derived from plasmapheresis of patient P51 and P52 using affinity chromatography. First IgG4 was purified with an IgG4 affinity matrix, and then the κ positive fraction was isolated with a human κ specific affinity matrix. IgG4 containing λ was then isolated from the κ depleted flow-through yielding in the λ/λ and −/− fraction. From the κ fraction, the κ/κ and κ/λ fractions were purified with the λ specific affinity matrix. B, C) Using MuSK ELISA, it was found that the λ/λ,κ/κ and κ/λ contained MuSK antibodies. MuSK was coated on the ELISA plates, and bound antibodies were detected with HRP conjugated anti-IgG4. The titers were determined using a standard curve of MuSK serum with known MuSK titer. N = 3, representative experiments are shown with duplicate measurements.

between the two patients examined, but in each case the proportion of κ/λ hybrids suggested a high proportion, which is in line with previous reports in healthy individuals [24,30]. Moreover, the values would represent an underestimate since some of the exchanges would, of necessity, have involved half-IgG4 molecules containing the same light chains. In theory, with a 3:1 ratio of κ: λ light chain usage in IgG4 [24], if 100% of IgG4 undergo random Fab-arm exchange, a light chain distribution of κ/κ: κ/λ: λ/λ of 9:6:1 would be expected, or in other words, only 37.5% of completely exchanged IgG4 would be κ/λ hybrids. Based on our observations, we can assume that the majority of MuSK antibodies was bispecific *in vivo*.

That bispecificity of MuSK IgG4 does not protect from pathogenicity would be in line with previous observations. Fab fragments derived from patients or from MuSK immunized animals were able to reduce agrin-induced AChR clustering [36,80], and indeed the MuSK MG derived IgG4 Fab fragments were, if anything, more effective at blocking binding of Lrp4 to MuSK and reducing AChR clustering than the whole MuSK IgG4's, clearly suggesting that cross-linking is not a prerequisite for pathogenicity of this autoantibody [36].

Whether divalent MuSK IgG4 antibodies survive long enough to induce some MuSK cross-linking and internalization is not yet clear. While one study suggested that MuSK cross-linking and endocytosis also occurs in MuSK MG [92], two further studies found no evidence for MuSK endocytosis even by the smaller proportion of IgG1-3 antibodies [36,37] perhaps because the epitopes on adjacent MuSK molecules are not accessible by a single MuSK IgG antibody. By contrast, it is well established that AChR antibodies, which are mainly of IgG1 and IgG3 subclass, can bind to either of the two main immunogenic regions on adjacent receptors and lead to internalization and complement-mediated damage.

An intriguing possibility is that Fab-arm exchange might generate bi-specific MuSK antibodies. Due to the dynamic nature of Fab-arm exchange *in vivo*, MuSK antibodies could be recombined with any other existing specificity in the IgG4 repertoire. Since we show here that the immune response against MuSK is polyclonal, involving both kappa and lambda light chains, this mechanism could potentially (re)generate bispecific IgG4 molecules that recognize the same or distinct MuSK epitopes; this could then lead to MuSK cross-linking and internalization. However, given the relatively low proportion of MuSK antibodies within the total IgG4 (<1% even in those with highest titres), any spontaneous regeneration of divalent MuSK antibodies would be infrequent, although if they accessed the neuromuscular synaptic space they could be highly effective.

It will be interesting to look at other diseases that are mediated by or associated with IgG4 to determine whether Fab-arm exchange may affect IgG4 pathogenicity or if it may be useful as a more general serological marker for IgG4 autoimmunity. It is likely that this mechanism occurs in other IgG4 autoimmune diseases, such as pemphigus [52,54,56], limbic encephalitis [58,61,93] and other, neurological disorders [94].

5. Conclusions

Autoantibodies in MuSK MG undergo Fab-arm exchange and a high proportion of MuSK IgG4 is bispecific *in vivo*. This exchange can be induced *in vitro*, but it was found that it did not reduce the pathogenicity of MuSK antibodies in the AChR clustering assay in C2C12 mouse myotubes. This suggests that Fab-arm exchange has no protective function in MuSK MG, in contrast to AChR MG and allergies. A likely explanation is that the main pathogenic function of IgG4 autoantibodies is conveyed by a functional block of protein-

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protein interaction, for which cross-linking binding apparently is unnecessary.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.11.005.

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