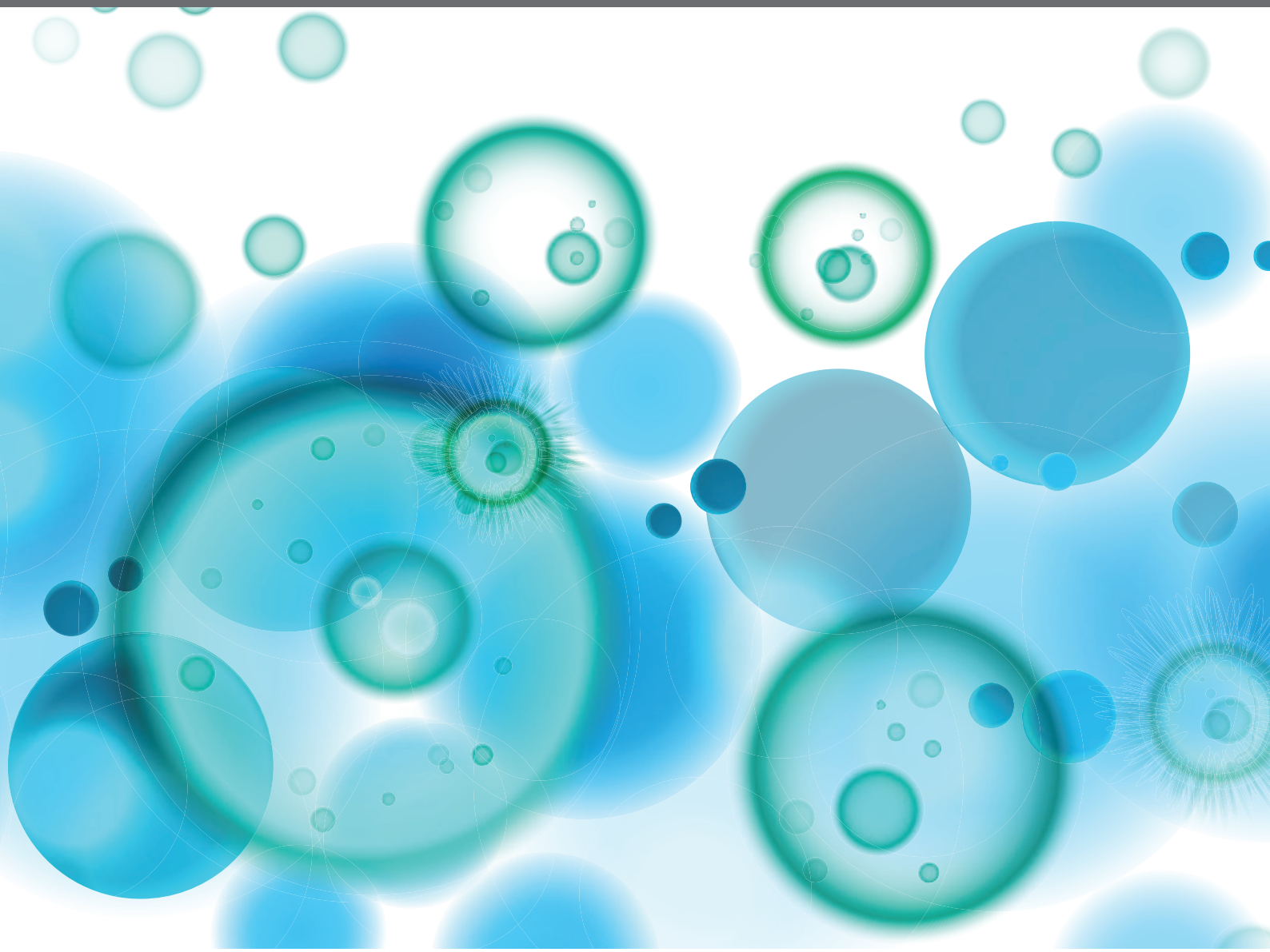
# ADVANCES IN AUTOIMMUNE MYASTHENIA GRAVIS

EDITED BY: Rozen Le Panse, Linda L. Kusner, Sonia Berrih-Aknin and Anna Rostedt Punga

PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

# frontiers



ISSN 1664-8714
ISBN 978-2-88966-107-7
DOI 10.3389/978-2-88966-107-7

## About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

## Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

## Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

## What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

# ADVANCES IN AUTOIMMUNE MYASTHENIA GRAVIS

Topic Editors:
**Rozen Le Panse,** Sorbonne Université, France
**Linda L. Kusner,** George Washington University, United States
**Sonia Berrih-Aknin,** Institut National de la Santé et de la Recherche Médicale (INSERM), France
**Anna Rostedt Punga,** Uppsala University, Sweden

**Citation:** Le Panse, R., Kusner, L. L., Berrih-Aknin, S., Punga, A. R., eds. (2020). Advances in Autoimmune Myasthenia Gravis. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-107-7

# Table of Contents

frontiers in Immunology

EDITORIAL
published: 28 August 2020
doi: 10.3389/fimmu.2020.01688

# Editorial: Advances in Autoimmune Myasthenia Gravis

*Anna Rostedt Punga*[1], *Linda Kusner*[2], *Sonia Berrih-Aknin*[3] *and Rozen Le Panse*[3]*

[1] Department of Neuroscience, Clinical Neurophysiology, Uppsala University, Uppsala, Sweden, [2] Department of Pharmacology and Physiology, The George Washington University, Washington, DC, United States, [3] Sorbonne University, INSERM, Association Institute of Myology, Center of Research in Myology, Paris, France

**Keywords: autoantibodies, CD4 T cells, thymus, therapy, miRNA**

## Editorial on the Research Topic

## Advances in Autoimmune Myasthenia Gravis

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder characterized by impaired neuromuscular transmission, which causes fluctuating fatigable muscle weakness. MG is a prototypical autoimmune disease with well-defined autoantibodies that target the neuromuscular junction. The majority of MG patients have autoantibodies against the acetylcholine receptor (AChR) and a smaller proportion of patients have autoantibodies against the muscle-specific tyrosine kinase (MuSK) or the low-density lipoprotein-related protein 4 (LRP4) (Borges and Richman) (1). Furthermore, several other antigenic targets, such as agrin, Kv1.4 potassium channel, rapsyn, cortactin, acetylcholinesterase (AChE), collagen Q (ColQ), and collagen XIII, have been reported. Although the pathogenicity and specificity of these autoantibodies for MG have not been fully characterized, their presence could help in better understanding the variability in disease severity. Moreover, they could provide a diagnostic/prognostic value for the management of MG patients (Fichtner et al.; Lazaridis and Tzartos).

The existence of B cells, which produce autoantibodies, is dependent on the interaction with $CD4^+$ T cells—both are key factors in MG. Thymic regulatory T cells are not efficiently suppressive, and T helper cells are resistant to suppression (2). Phenotypic variation of regulatory T cells and functional impairment are more pronounced in the thymus than in peripheral cells (Truffault et al.). Thymic epithelial cells from MG patients appear to play a central role in $CD4^+$ T cell defect *via* the release of soluble factors, such as TSLP (Thymic Stromal LymphoPoietin) (Truffault et al.). T helper (Th) 1, Th2, Th17, and T follicular helper (Tfh) cells are involved in MG pathogenic mechanisms. An increase in the levels of interleukin (IL)-21, IL-4, IL-10, and IL-17A is observed in $CD4^+$ T peripheral cells in AChR antibody seropositive ($AChR^+$) MG, as compared to healthy controls (Çebi et al.). Among $CD4^+$ T cells, the percentage of Th17 cells is increased in AChR+ MG patients. ICOS (Inducible T-cell COStimulator) and PD-1 (Programmed cell death protein 1), two molecules associated with Tfh cell function, are also highly expressed on $CD4^+CXCR5^+$ Tfh cells in AChR+ MG. Tfh cells can be stratified in Tfh regulatory cells, or Tfh1, Tfh2, and Tfh17 cells that differentially affect B-cell differentiation. In AChR+ MG patients, the percentage of peripheral Tfh17 cells ($CXCR3^-CCR6^+CD4^+$ T cells) is also increased but Tfh1 and Tfh2 cells remain unaffected. Some of these changes are also observed to a lesser degree in AChR- MG patients (Çebi et al.) and in MuSK antibody seropositive ($MuSK^+$) MG patients (3). Immunosuppressive treatments commonly used in MG do not affect these cells but enhance IL-10 in CD4 T cells (Çebi et al.), and also B cells (4), suggesting a role of IL-10 in favoring immuno-regulatory mechanisms.

OPEN ACCESS

***Edited and reviewed by:***
*Betty Diamond,*
*Feinstein Institute for Medical Research, United States*

***Correspondence:**
*Rozen Le Panse*
*rozen.lepanse@upmc.fr*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *18 June 2020*
***Accepted:*** *24 June 2020*
***Published:*** *28 August 2020*

***Citation:***
*Punga AR, Kusner L, Berrih-Aknin S and Le Panse R (2020) Editorial: Advances in Autoimmune Myasthenia Gravis. Front. Immunol. 11:1688. doi: 10.3389/fimmu.2020.01688*

Classical treatments of MG include chronic treatments such as AChE inhibitors and general immunosuppressive drugs (5), thymectomy (6), as well as acute treatment for deterioration such as intravenous immunoglobulin and plasmapheresis (5). A new line of molecules is now available and is used for refractory MG. One of these is Rituximab, an anti-CD20 B-cell depleting monoclonal antibody which is often used as a second-line of treatment in combination with conventional immunosuppressants. Rituximab seems more efficient in MuSK$^+$ MG patients, with the reduction in MuSK antibodies being associated with clinical improvement (Marino et al.). Other therapies aiming at targeting B cells are also emerging (Huda).

Most recently, medications inhibiting the cleavage of the complement protein C5 have been evaluated in clinical trials. Eculizumab (a monoclonal antibody) has obtained authorization from the US Food and Drug Administration to be used in MG. A second generation of C5 inhibitor, Zilucoplan (a macrocyclic), has also recently entered clinical trials (Albazli et al.). Molecules blocking the function of FcRn are of interest for autoimmune diseases. Inhibition of FcRn reduces the ability to recycle IgGs and thereby removes them from circulation. Agents such as Efgartigimod (IgG1 Fc fragment) or Nicocalimab, Rozanolixizumab, and RCT-140 (monoclonal antibodies) are in clinical trials for MG (Gable and Guptill).

Other therapeutic approaches are still at the preclinical phase and have demonstrated beneficial effects on experimental MG (EAMG) rodent models. If oral or nasal administration of AChR fragments suppress autoimmunity in EAMG (Yamada et al.) (7), the recombinant extracellular domain of MuSK may also be effective in inducing oral tolerance in MuSK$^+$ EAMG (Reuveni et al.). Oral tolerance is a phenomenon based on suppressing immune responses in the gut where microbiota could play a role. In that way, probiotics could balance the gut microbiota and have beneficial effects in EAMG (Rinaldi et al.).

Even though MG has been studied for a number of years, the understanding of the etiological mechanisms is still evolving. In AChR$^+$ MG, the thymus is known to play a central role in disease onset either in the early-onset form of the disease or in MG-associated thymoma (8). The analyses of enriched pathways from "omics" data might reveal new unexplored pathways central in MG development (Cron et al.; Yamada et al.). It is well-known that genetic predispositions exist in MG patients (9), however, additional epigenetic changes occur. The expression of small non-coding RNA, microRNA (miRNA), is dysregulated in the thymus of AChR$^+$ MG patients (10, 11) and could be involved in thymic changes associated with MG, linked to thymic inflammation and ectopic germinal center development (Bortone et al.; Cron et al.). Circulating miRNAs are also potential biomarkers since they are differentially expressed in the serum of MG patients (Sabre et al.). Specific circulating miRNAs have been associated with AChR$^+$ and MuSK$^+$ MG subtypes and their expression is regulated by treatment with immunosuppression and thymectomy (Fiorillo et al.; Sabre et al.). Investigations into the triggering events that lead to MG are still needed. Sexual hormones can affect, for example miRNAs, and in addition, favor autoimmunity in women (Fiorillo et al.) (12). Environmental factors are also candidates for driving/perpetuating autoimmunity, such as pathogen infection, endocrinal disruptors, and microbiota changes.

Although MG is a relatively well-characterized autoimmune disease, recent studies shed light on the mechanisms of development of this pathology and, most importantly, make it possible to propose more effective tools for monitoring and more effective treatments with fewer side effects. This Research Topic, dedicated to autoimmune MG, addresses these different aspects, both with Original Research articles and Reviews of the literature.

## AUTHOR CONTRIBUTIONS

AP, LK, SB-A, and RL wrote the editorial. All authors contributed to the article and approved the submitted version.

## REFERENCES


1. Mantegazza R, Bernasconi P, Cavalcante P. Myasthenia gravis: from autoantibodies to therapy. *Curr Opin Neurol.* (2018) 31:517–25. doi: 10.1097/WCO.0000000000000596
2. Gradolatto A, Nazzal D, Truffault F, Bismuth J, Fadel E, Foti M, et al. Both Treg cells and Tconv cells are defective in the Myasthenia gravis thymus: roles of IL-17 and TNF-alpha. *J Autoimmun.* (2014) 52:53–63. doi: 10.1016/j.jaut.2013.12.015
3. Li Y, Guptill JT, Russo MA, Howard JF, Massey JM, Juel VC, et al. Imbalance in T follicular helper cells producing IL-17 promotes pro- in fl ammatory responses in MuSK antibody positive myasthenia gravis. *J Neuroimmunol.* (2020) 345:577279. doi: 10.1016/j.jneuroim.2020.577279
4. Yilmaz V, Maillard S, Truffault F, Bolgert F, Behin A, Regnard JF, et al. Regulatory B cells in myasthenia gravis are differentially affected by therapies. *Ann Clin Transl Neurol.* (2018) 5:1408–14. doi: 10.1002/acn3.645
5. Wang S, Breskovska I, Gandhy S, Punga AR, Guptill JT, Kaminski HJ. Advances in autoimmune myasthenia gravis management. *Expert Rev Neurother.* (2018) 18:573–588. doi: 10.1080/14737175.2018.1491310
6. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo HC, Marx A, et al. Long-term effect of thymectomy plus prednisone versus prednisone alone in patients with non-thymomatous myasthenia gravis: 2-year extension of the MGTX randomised trial. *Lancet Neurol.* (2019). 18:P259–68. doi: 10.1016/S1474-4422(18)30392-2
7. Weiner HL. Induction of oral tolerance to the acetylcholine receptor for treatment of myasthenia gravis. *J Clin Invest.* (1999) 104:1667–8. doi: 10.1172/JCI8775
8. Marx A, Pfister F, Schalke B, Saruhan-Direskeneli G, Melms A, Ströbel P. The different roles of the thymus in the pathogenesis of the various myasthenia gravis subtypes. *Autoimmun Rev.* (2013) 12:875–84. doi: 10.1016/j.autrev.2013.03.007
9. Giraud M, Vandiedonck C, Garchon HJ. Genetic factors in autoimmune myasthenia gravis. *Ann N Y Acad Sci.* (2008). 1132:180–92. doi: 10.1196/annals.1405.027

10. Cron MA, Maillard S, Villegas J, Truffault F, Sudres M, Dragin N, et al. Thymus involvement in early-onset myasthenia gravis. *Ann N Y Acad Sci.* (2018) 1412:137–45. doi: 10.1111/nyas.13519
11. Sengupta M, Wang BD, Lee NH, Marx A, Kusner LL, Kaminski HJ. MicroRNA and mRNA expression associated with ectopic germinal centers in thymus of myasthenia gravis. *PLoS ONE.* (2018) 13:e0205464. doi: 10.1371/journal.pone.0205464
12. Dragin N, Bismuth J, Cizeron-Clairac G, Biferi MG, Berthault C, Serraf A, et al. Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. *J Clin Invest.* (2016) 126:1525–37. doi: 10.1172/JCI81894

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 December 2019
doi: 10.3389/fimmu.2019.02949

# Therapeutic Effect of Bifidobacterium Administration on Experimental Autoimmune Myasthenia Gravis in Lewis Rats

*Elena Rinaldi[1], Alessandra Consonni[1], Chiara Cordiglieri[1], Grazia Sacco[1], Camilla Crasà[1], Alessandra Fontana[2], Lorenzo Morelli[2], Marina Elli[3], Renato Mantegazza[1] and Fulvio Baggi[1]*

[1] Neurology IV – Neuroimmunology and Neuromuscular Diseases Unit, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy, [2] Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, Piacenza, Italy, [3] AAT—Advanced Analytical Technologies, Fiorenzuola d'Arda, Italy

OPEN ACCESS

**Edited by:**
Rozen Le Panse,
INSERM U974 Thérapie des Maladies du Muscle Strié (Institut de Myologie), France

**Reviewed by:**
Jie Luo,
University of Pennsylvania, United States
Maria I. Bokarewa,
University of Gothenburg, Sweden

***Correspondence:**
Fulvio Baggi
fulvio.baggi@istituto-besta.it

**Specialty section:**
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

**Received:** 27 September 2019
**Accepted:** 02 December 2019
**Published:** 19 December 2019

**Citation:**
Rinaldi E, Consonni A, Cordiglieri C, Sacco G, Crasà C, Fontana A, Morelli L, Elli M, Mantegazza R and Baggi F (2019) Therapeutic Effect of Bifidobacterium Administration on Experimental Autoimmune Myasthenia Gravis in Lewis Rats. Front. Immunol. 10:2949. doi: 10.3389/fimmu.2019.02949

Beneficial effects of probiotics on gut microbiota homeostasis and inflammatory immune responses suggested the investigation of their potential clinical efficacy in experimental models of autoimmune diseases. Indeed, administration of two bifidobacteria and lactobacilli probiotic strains prevented disease manifestations in the Lewis rat model of Myasthenia Gravis (EAMG). Here, we demonstrate the clinical efficacy of therapeutic administration of vital bifidobacteria (i.e., from EAMG onset). The mechanisms involved in immunomodulation were investigated with *ex vivo* and *in vitro* experiments. Improvement of EAMG symptoms was associated to decreased anti-rat AChR antibody levels, and differential expression of TGFβ and FoxP3 immunoregulatory transcripts in draining lymph nodes and spleen of treated-EAMG rats. Exposure of rat bone marrow-derived dendritic cells to bifidobacteria or lactobacilli strains upregulated toll-like receptor 2 mRNA expression, a key molecule involved in bacterium recognition via lipotheicoic acid. Live imaging experiments of AChR-specific effector T cells, co-cultured with BMDCs pre-exposed to bifidobacteria, demonstrated increased percentages of motile effector T cells, suggesting a hindered formation of TCR-peptide-MHC complex. Composition of gut microbiota was studied by 16S rRNA gene sequencing, and α and β diversity were determined in probiotic treated EAMG rats, with altered ratios between Tenericutes and Verrucomicrobia (phylum level), and Ruminococcaceae and Lachnospiraceae (family level). Moreover, the relative abundance of Akkermansia genus was found increased compared to healthy and probiotic treated EAMG rats. In conclusion, our findings confirms that the administration of vital bifidobacteria at EAMG onset has beneficial effects on disease progression; this study further supports preclinical research in human MG to evaluate probiotic efficacy as supplementary therapy in MG.

**Keywords: MG, EAMG, probiotics, immunoregulation, therapeutic treatment**

## INTRODUCTION

Myasthenia Gravis (MG) is a chronic autoimmune disease characterized by the presence of serum autoantibodies against the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction (NMJ) in a large proportion of patients; AChR-specific antibodies lead to the alteration and destruction of NMJ causing muscle weakness and fatigability, major clinical symptoms in MG (1). Experimental autoimmune myasthenia gravis (EAMG), induced in susceptible strains as the Lewis rat or the C57Bl/6 mouse, is a well-characterized model to study the mechanisms involved in MG and novel therapeutic treatments (2).

Over the past years, many studies have been conducted and demonstrated the benefits of probiotic treatment in animal models of inflammatory diseases, such as experimental arthritis (3), experimentally induced colitis (4) and experimental autoimmune encephalomyelitis (5). Effects of probiotic administration on clinical symptoms and on immune mechanisms of EAMG has been investigated by us and other groups, according to a preventive or prophylactic/preventive protocol (6, 7). We previously demonstrated the effects of the preventive administration of two bifidobacteria strains or two lactobacilli strains in the Lewis rats EAMG model. We observed that probiotics significantly attenuated EAMG symptoms, decreased serum anti-rat AChR antibody levels and increased muscle AChR content. Pro-inflammatory and immunoregulatory transcripts were found differentially expressed in primary and secondary immune organs, and increased levels of Transforming Growth Factor-β (TGFβ) were measured in EAMG rat serum (6).

Orally administered probiotics exert their function in the gut, interacting with epithelial cells and the gut associated lymphoid tissue (GALT). GALT defends the host from pathogenic microorganisms and it is influenced by intestinal microbiota that lives symbiotically in the human gut. Dendritic cells (DCs), IgA-producing B cells, T helper1 (Th1), T helper 17 (Th17), and T regulatory (Treg) cells are the main players of the mucosal firewall that protects gut from external threats (8). In particular, DCs keep the immune system on high alert and balance T cell responses to pathogenic and not to commensal bacteria (9). Indeed, DCs can receive antigens from $CX3CR1^+$ macrophages and intestinal epithelial cells, and extend dendrites toward the gut lumen to capture bacteria (10, 11). Upon antigen uptake, DCs migrate to mesenteric lymph nodes (LNs) to interact with T cells and shape the intestinal immune response (12).

Probiotics exert beneficial effects on gut microbiota homeostasis, and can contribute in restoring the eubiosis condition. The most abundant taxa colonizing the human gut are *Firmicutes* and *Bacteroidetes* and an altered ratio between the abundance of these taxa can be considered an index of intestinal dysbiosis. Besides this effect, probiotics also modulate inflammatory immune responses and foster the immunological surveillance; in this regards, it has been demonstrated that certain Lactobacilli strains stimulate the gamma interferon (IFNγ) and tumor necrosis factor (TNF) production, key molecules involved in the maturation and proliferation of immune cells (13), *Lactobacillus casei* Shirota induces IL12 production and promotes T helper cells development (14), *Lactobacillus rhamnosus* GG induces $CD4^+CD25^+Foxp3^+$ T cell expansion in mesenteric LNs (15), strains of *Bifidobacterium animalis* and *Bifidobacterium longum* are able to support Th1 response, whereas strains of *Bifidobacterium bifidum* induce Th17 polarization (16).

In this study, we report the clinical efficacy of the therapeutic bifidobacterium administration on EAMG course, and that vital bacteria are more potent compared with inactive (heat exposed) bacteria. Moreover, we showed probiotic interactions with immune cells in the gut (namely the Peyer's Patches), through *ex vivo* and *in vitro* immunofluorescence analyses, and that probiotic altered the motility patterns of AChR-specific effector Tcells when co-cultured with probiotic-exposed bone marrow DC (BMDC), by means of live imaging microscopy. Lastly, we investigated gut microbiota composition of probiotic-treated EAMG rats by NGS 16S rRNA analysis, showing greater α and β diversity during EAMG course.

## MATERIALS AND METHODS

### Animals

Female Lewis rats, 6–8 weeks old, were purchased from Charles River Laboratories Italia (Calco, Italy) housed at the animal facility of the Foundation IRCCS Neurological Institute Carlo Besta. Rats were housed in groups of three in cages with artificial circadian 12-h light/12-h dark cycle, maintained at air-conditioned room with temperature of 23°C at all time, with free access to a standard stock diet and water provided *ad libitum*. Procedures involving animals were approved by the Institute Ethical Board and Italian Ministry of Health (1064/2015- PR) and were performed in respect to the Italian Principle of Laboratory Animal Care (D.Lgs 116/92 and D.Lgs 26/2014), in accordance to European Communities Council Directive 86/609/EEC and 2010/63/UE. Animals were sacrificed after deep anesthesia obtained by carbon dioxide; low-grade anesthesia with 2% isoflurane (60:40 $N_2O$: $O_2$, flow rate 0.8 l/min) was induced in animals prior to immunizations and treatments.

### TAChR Preparation

TAChR was purified from *Torpedo californica* electric organ tissue (Aquatic Research Consultants), according to (17). Briefly, the electric tissue was homogenized in 10 mM sodium phosphate buffer, 1 mM EDTA, 0.02% $NaN_3$, 0.01 mM PMSF, pH 7.8 for 3 min, and then centrifuged for 1 h at 100,000 × g at 4°C. Pellet was resuspended in ice-cold water and the pH adjusted to 11.0 with NaOH; membranes were centrifuged for 30 min at 100,000 × g at 4°C. AChR-containing membranes were homogenized for 2 min and the receptor solubilized with 2% sodium deoxycholate, overnight at 4°C. The detergent was removed by progressive dialysis, and TAChR stored at −80°C. TAChR concentration was quantified by the standard radioimmunoprecipitation protocol with [$^{125}$I]-α bungarotoxin (αBTX) (PerkinElmer), according to Lindstrom et al. (18). [$^{125}$I]-αBTX in samples was determined by a gamma counter (PerkinElmer). To evaluate the aspecific binding, serum samples were pre-incubated with an excess of unlabelled αBTX and counts per minutes (cpm) were subtracted from test samples. The specific activity of TAChR preparation

used to induce EAMG was 1.19 nmol/mg, expressed as the α-BTX binding sites/mg of total protein content (micro BCA assay).

## Experimental Autoimmune Myasthenia Gravis (EAMG) Model

Experimental MG model was induced according to a consensus protocol (2) by a single subcutaneous immunization in the hind limbs (multiple sites) with 50 μg of TAChR emulsified in Complete Freud Adjuvant (CFA; Difco) supplemented with 1 mg/rat of *Mycobacterium tubercolosis* (total volume 200 μl). Each animal was weighed and scored at the beginning of the experiment, and at least twice weekly until the end of the experiment; clinical scores were taken every 24 h or less if the animals demonstrated severe weakness (2). EAMG clinical score was assessed by researchers, blinded to animal treatment, after 30 s exercise, using a manual grip strength test. Disease severity was graded as follows: grade 0, normal strength; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present before exercise (tremor, head down, hunched posture, weak grip); grade 3, severe clinical signs at rest, no grip; grade 4, sacrifice, humane endpoint. EAMG was confirmed by Piridostigmine test (i.p. injection). The experiments were concluded 8 weeks post TAChR/CFA immunization.

## Generation of R97-116 Teff Cell Line

Lewis rats were immunized with 200 μg of R97-116 peptide (CASLO), the immunogenic region of rat AChR α-subunit, in CFA. Draining lymph nodes were aseptically removed 10 days post immunization, and LNCs suspensions were stimulated with R97-116 (10 μg/ml) in complete RPMI-1640 medium (Euroclone), containing 1% Na-pyruvate, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin-streptomycin, 50 μM 2-mercaptoethanol, 2% normal rat serum (19). Antigen specific Teff were maintained by restimulation with R97-116 peptide every 15 days, and expanded with IL2 (10 U/ml) every 3 days.

## Probiotic Strains and Treatment Protocols

*Lactobacillus crispatus* LMG P-23257 (LC), *Lactobacillus rhamnosus* ATCC 53103 (LR), *B. animalis* subsp. *lactis* BB12® (BA, from CHR Hansen, Denmark) and *B. animalis* subsp. *lactis* LMG S-28195 (BL) were used. All strains were grown at AAT laboratory; briefly, lactobacilli were cultured in De Man, Rogosa & Sharp (MRS) broth (Difco) at 37°C in microearophilic conditions for 18 h, and bifidobacteria were grown in MRS broth supplemented with 0.05% cysteine at 37°C by anaerobic incubation for 24–48 h. Enumeration of viable bacterial cells was performed on selective media (MRS for lactobacilli, and Transoligosaccharide propionate agar medium added with 50 μg/ml mupirocin for bifidobacteria) by decimal counts.

Bacterial cells were resuspended at $10^9$ CFU/150 μl in phosphate saline buffer (PBS), 20% glucose, 10% glycerol and stored at −80° C. Loss of bacterial viability was <2–4% over 2 months storage period.

Combinations of bifidobacteria (BBmix) or lactobacilli (LBmix) strains were orally administered at a cumulative dose of $2 \times 10^9$ CFU/300 μl. Alternatively to vital bifidobacteria, EAMG rats were fed with heat exposed (90°C for 5 min) bifidobacteria (BBmix heat exposed). Twenty-two consecutive doses of probiotics were administered starting from disease onset (days 27–28); end of experiments was set at least 4 days after the last probiotic administration. Animals were randomly selected in experimental groups, and evaluation of EAMG symptoms was performed by researchers blinded to the treatment group allocation. Inguinal and popliteal lymph nodes, spleen, muscle and blood were collected at the end of the experiment.

## Anti-Rat AChR Antibodies in Serum

Anti-rat AChR antibodies were assayed in sera by radioimmunoprecipitation, according to Lindstrom et al. (18). Briefly, AChR was extracted from healthy rat muscle and labeled with 2 nM [$^{125}$I]-αBTX. Sera were incubated overnight with [$^{125}$I]-αBTX-rat AChR (0.5 pmol). Ab-AChR complexes were precipitated by adding an excess of rabbit anti-rat IgG (Sigma). Pellet was washed twice with cold 0.5% Triton X-100 (Carlo Erba) in PBS, and [$^{125}$I]-αBTX labeled-rat AChR complexes in the pellet were evaluated by γ-counter (Perkin Elmer). The non-specific binding was subtracted from each sample. Anti-AChR antibodies titres were expressed as picomole of [$^{125}$I]-αBTX binding sites precipitated per milliliter of serum (pmol/ml).

## Probiotic Interaction With GALT

To evaluate probiotic presence in the gut, bacteria were labeled with wheat germ agglutinin-Alexa Fluor 555 conjugate (WGA-AF555) (Thermo Fisher) for 10 min, followed by extensive washes. A dose of $10^9$ CFU bacteria was administered to Lewis rats, and gut samples were excised after 30–60 min, washed with PBS, fixed with paraformaldehyde (PFA, 4% in PBS) for 24 h and then transferred in sucrose (30% in PBS) for cryopreservation. Samples were included in Killik (Bio-Optica) and kept at −80°C, pending analysis. Serial 10 μm thick cryosections were stained with Hematoxilin and Eosin (images digitalized with ScanScope, Aperio technologies) or with the following antibodies: mouse anti-vimentin mAb (V9, Dako), mouse anti-cytokeratin mAb (MNF116, Dako), mouse anti-CD11c mAb (8A2; ThermoFisher), mouse anti-CD3 mAb (G4.18, eBioscience), followed by species-specific Alexa Fluor 488-conjugated secondary antibodies. Isotype control stainings were routinely performed in the immunofluorescence procedures. Nuclei were stained with DAPI (Thermo Fisher Scientific). Single plan and z-scan images were captured via confocal microscopy and Structured Illumination microscopy (SIM), using a 100X APO-TIRF (NA 1.49) objective, with 3D optical sectioning. Images were processed with Fiji software (20).

## Bone Marrow Dendritic Cells Cultures and Probiotic Interaction

Single cell suspensions of myeloid precursor cells were derived from bone marrows of femur and tibia of naïve Lewis rats and cultured in RPMI-1640 medium supplemented with 1% Na-pyruvate, 1% non-essential aminoacids, 1% penicillin-streptomycin, 1% L-glutamine, 50 μM 2-mercaptoethanol, 10% fetal bovine serum (complete RPMI-1640 medium), in presence of GM-CSF and IL4 (each at 20 ng/ml; Peprotech) for 7 days

to induce differentiation into immature bone marrow dendritic cells (BMDCs). BMDCs were seeded into 8-well chamber slides ($1 \times 10^5$ cells/chamber) for 16 h and then WGA-AF555-labeled probiotics ($1 \times 10^7$ CFU/$1 \times 10^5$ BMDCs) were added for 4 h. BMDCs cultures were then washed and fixed with 4% PFA for 10 min. BMDCs were stained with anti-CD11c mAb (8A2; Thermofisher). The interaction between BMDCs and bacteria has been visualized with rabbit anti-TLR2 pAb (AbClonal) and mouse anti-LTA mAb (3811; GeneTex), followed by species-specific Alexa Fluor-conjugated secondary antibodies.

## Live Imaging Assay

BMDCs ($2 \times 10^6$ cells) were exposed to probiotics ($2 \times 10^8$ CFU) or TGFβ (10 ng/ml) for 4 h, extensively washed with PBS, and cultured with complete RPMI-1640 medium or with R97-116 peptide (10 μg/ml in complete RPMI-1640) for further 2 h. BMDCs were detached, counted and seeded ($5 \times 10^5$ cells/dish) on glass-inserted imaging collagen-coated dishes for 16 h. Then, freshly stimulated R97-116 specific $CD4^+$Tcell lines, labeled with 5 μM CFSE for 10 min at 37°C, were added to BMDC cultures ($1.5 \times 10^6$ cells/dish).

Time-lapse video microscopy was performed using a live-imaging Nikon set-up equipped with temperature/$CO_2$ control unit (OKO lab). Differential interference contrast (DIC) and green channel images were acquired on a 512 × 512 pixel field of view, with 1.31 μm/pixel conversion. One hour recordings were performed with 30 s time-lapse interval using an inverted microscope (20X, 0.5 NA objective) and a Q-imaging Fast Camera (Roper scientific) and processed by NIS Elements AR software v3.1 (Nikon). TrackMate plug-in of Fiji software was used to automatically track T cells (21). Cells were defined as stationary if their path length was shorter than 10 μm every 10 min recording, or else they were classified as motile (22, 23).

## RT-qPCR

cDNA was synthesized from total RNA (TRIzol, Thermo Fisher Scientific) using random hexamers, and reverse transcriptase (SuperScript VILO cDNA Synthesis Kit, Thermo Fisher Scientific). Real-time quantitative PCR (qRT-PCR) was performed using Assay-on Demand Gene Expression Products (Thermo Fisher Scientific) specific for: IFNγ (Rn00594078_m1), IL6 (Rn01410330_m1), FoxP3 (Rn01525092_m1), TGFβ (Rn00572010_m1), TLR1 (Rn04181452_s1), TLR2 (Rn02133647_s1), TLR6 (Rn02121288_s1), CHRNA (Rn01278033_m1), Rapsyn (Rn014886207_m1), LRP4 (Rn01486328_m1); β-actin (Rn01515681_m1) was used as housekeeping endogenous gene. Target mRNA expression was calculated as mean $2^{-\Delta Ct} \times 100$ value, in which ΔCt is the difference between target and housekeeping Ct. Real-time PCR reactions were performed in duplicates using Viia7 Real-Time PCR System, according to the manufacturer's instructions.

## Stool Collection and Nucleic Acid Extraction

Stool samples were collected at day 0 (before immunization), day 30 (at EAMG onset) and at the end of experiment. Stool samples collected from the animals housed in the same cage were pooled and dissolved in Stool Nucleic Acid Collection and Preservation Tubes (Norgen Biotek Corp.), pending analysis. Bacterial DNA extraction was performed using Stool DNA Isolation Kit (Norgen), according to the manufacturer's instructions. Briefly, 400 μl of stool samples were mixed with lysis buffer (1:1.5 v/v) and homogenized using a flat-bed vortex. The supernatant was collected and transferred to a DNAse-free microcentrifuge tube, centrifuged to pellet any cell debris and loaded onto a spin-column. The bound DNA was washed, eluted and stored at −20°C.

Samples were: healthy rats (HD rats), EAMG rats at disease onset (EAMG onset), vehicle treated EAMG rats (EAMG chronic), EAMG rats treated with vital BBmix (EAMG BBmix vital), EAMG rats treated with heat exposed BBmix (EAMG BBmix heat exposed). Five replicates were considered for HD rats and EAMG BBmix vital samples; four replicates for EAMG chronic samples; three replicates for EAMG onset and EAMG BBmix heat exposed.

## 16S rRNA NGS Sequencing

Purity and quantity of the bacterial DNA were confirmed by Bioanalyser 2100 (Agilent Technologies, USA) and NanoDrop 2000 (ThermoFisher) devices. Bacterial 16S rRNA variable regions (V2, V3, V4, V6, V8, V7-9) were amplified using specific primers (Metagenomic kit, Invitrogen) and completed by the addition of a PGM sequencing adaptor (P1) and unique barcode to allow multiplex analyses. Prior to NGS sequencing, quality and amplicon sizes were assessed using the Bioanalyser 2100. The samples were adjusted to a final concentration of 26 pM and attached to the surface of Ion Sphere particles (ISPs) according to the manufacturer's instructions. Manual enrichment of the resulting ISPs resulted in >95% templated- ISPs. Templated-ISPs were sequenced on either "314" (10 Mb.p.) or "316" (100 Mb.p.) micro-chips using the Ion Torrent Personal Genome Machine (Life Technologies, USA) for 850 flows.

## Metagenomic Analysis

Raw data from the Ion Torrent Personal Genome Machine were analyzed with the Ion Reporter™ Software 5.10 and the workflow Metagenomics 16S w1.1 to generate fastq sequences for the different 16S rRNA variable regions and a consensus sequence for each sample. Consensus fastq sequences were elaborated with QIIME 2 2018.8 (24). Specifically, demultiplexing and quality filtering were performed using the q2-demux plugin followed by denoising with DADA2 (25). Alpha-diversity metrics (observed OTUs, Shannon and evenness), beta-diversity metric (unweighted UniFrac) (26) and Principle Coordinate Analysis (PCoA) were estimated using q2-diversity after samples were rarefied (subsampled without replacement) to 9,805 sequences per sample. Taxonomy was assigned to OTUs using the q2-feature-classifier (27) classify-consensus-vsearch taxonomy classifier against the Greengenes 13_8 99% OTUs reference sequences (28). Hierarchical clustering using Pearson distances in MeV (29) was used to create a genus-level heatmap of the relative abundances.

## Statistical Analysis

Experimental data were analyzed via one-way ANOVA or two-way ANOVA for normally distributed values, followed by Dunnett's multiple comparison test or via Kruskal–Wallis test for not normally distributed values; normal distribution of data was evaluated via Kolmogorov test. All *p*-values were corrected for multiple comparisons. $P < 0.05$ was considered statistically significant. Graph Pad Prism was used for data elaboration and statistical analyses.

# RESULTS

## Improvement of EAMG Symptoms After Therapeutic Treatment With Bifidobacteria

The therapeutic effect of bifidobacteria (BBmix) and lactobacilli (LBmix) administration was evaluated in EAMG Lewis rats, compared to vehicle-fed animals (**Figure 1A**). Probiotic treatments started at disease onset and throughout the chronic phase. BBmix was more effective than LBmix in ameliorating the disease course (**Figure 1A**; vehicle-EAMG, mean score 1.75 ± 0.27, LBmix-EAMG, mean score 1.5 ± 0.45, BBmix-EAMG, mean score 0.67 ± 0.52, corrected $p < 0.001$). The observed EAMG improvement in BBmix treated animals was confirmed by the assessment of animal weights, compared to control (vehicle fed) EAMG. The mean weights (grams ± SD) at the end of experiments were: BBmix-EAMG, 228 ± 10; vehicle-EAMG, 189 ± 33; LBmix-EAMG, 218 ± 15 (**Supplementary Figure 1A**). Next, we investigated whether the beneficial effect of BBmix could be associated to vital or heat exposed probiotics; administration of vital bacteria was significantly associated with a decreased clinical score, whereas heat exposed bifidobacteria did not modify the disease course (**Figure 1B**; vehicle-EAMG, mean score 2.2 ± 1.25, BBmix vital- EAMG, mean score 0.86 ± 1.34, corrected $p < 0.001$; BBmix heat exposed- EAMG, mean score 1.54 ± 1.19, corrected *p*-value = 0.26 vs. vehicle-EAMG). Again, EAMG improvement was paralleled by improvement of mean animal weight (**Supplementary Figure 1B**, BBmix vital-EAMG, grams 220 ± 25, vehicle-EAMG, grams 182 ± 37, BBmix heat exposed- EAMG, grams 193 ± 30).

Improvement of EAMG symptoms were confirmed by a significant reduction of serum antibody level against rat AChR in BBmix-EAMG rats (mean titer 12.73 ± 6.50 pmol/ml) compared to vehicle-fed (mean titer 32.53 ± 11.13 pmol/ml) or LBmix-EAMG rats (mean titer = 37.51 ± 5.67 pmol/ml) (**Figure 1C**). Similarly, anti-rat AChR antibody levels were found reduced in EAMG rats treated with vital bifidobacteria but not in heat exposed BBmix-fed rats (**Figure 1D**; BBmix vital-EAMG rats mean titer 9.49 ± 4.55 pmol/ml, vehicle-EAMG rats mean titer 20.53 ± 4.72 pmol/ml, corrected $p = 0.007$; BBmix heat exposed-EAMG mean titer = 13.13 ± 10.07 pmol/ml, corrected $p = 0.06$ ns). Clinical efficacy of BBmix treatment has been confirmed by RT-qPCR analysis for the expression of mRNAs encoding for CHRNA, Rapsyn and LRP4, key molecules of the NMJ involved in AChR stabilization on the postsynaptic membrane. Treatment of EAMG rats with vital BBmix is associated with decreased CHRNA1 and Rapsyn mRNAs, comparable to HD rats, whereas their expression was significantly upregulated in vehicle treated-EAMG animals (**Supplementary Figure 2**).

Since BBmix treatment, and especially BBmix vital, was associated with a significant EAMG improvement, we then studied by means of qRT-PCR the differential expression of IFNγ and IL6 (as proinflammatory markers) and of FoxP3 and TGFβ (as immunomodulatory markers) mRNA transcripts in draining lymph nodes (drLNs) and spleen isolated from BBmix treated EAMG rats (**Figure 2**). We did not detect differences for IFNγ in the different immunocompetent tissues analyzed, whereas IL6 mRNA was found increased in the spleen (corrected $p = 0.0074$). With regard to regulatory markers, we found increased expression of FoxP3 mRNA in drLNs (corrected $p = 0.0209$) and spleen (corrected $p = 0.0104$) of animals treated with BBmix vital; also TGFβ mRNA was found upregulated in drLNs (corrected $p = 0.0380$).

## Detection of Probiotic in the GALT

Probiotic interaction with the host immune system occurs in the GALT and, among the lymphoid structures located in the intestinal mucosa, Peyer's Patches (PPs) are one of the main lymphocyte priming sites in response to microbial stimulation (30). To investigate the interaction of our probiotic strains within the GALT, bifidobacteria and lactobacilli were stained with WGA-AF555, a fluorescently labeled lectin that binds specifically to polar polysaccharides (31, 32) of bacterium wall (representative images of labeled BA, **Figure 3A**, and LR, **Figure 3B**). Lewis rats received a single dose ($1 \times 10^9$ CFU) of WGA-AF555 labeled probiotic and, after 30–60 min, the small intestine was removed and processed for histological (**Figure 3C**, H&E staining) and immunofluorescence analyses (**Figures 3D–I**). Fluorescently labeled bacteria were found localized inside intestinal villi (counterstained with a green-fluorescent mAb anti-cytokeratin) with confocal microscopy (**Figure 3D**) and SIM (**Figure 3E**), and within the PPs (counterstained with a green-fluorescent mAb anti-vimentin) (**Figure 3F**, confocal microscopy, and **Figure 3G**, SIM).

It is known that PPs are constituted by three regions containing different cell types: the follicular and interfollicular areas with a germinal center of proliferating B-lymphocytes, follicular dendritic cells and macrophages, and the corona or subepithelial dome, surrounding the follicle, populated by B-cells, T-cells, macrophages, and dendritic cells (33). In order to identify the main cell types within the PPs substructures proximal to WGA-AF555 labeled probiotic, serial sections of the small intestine were stained with a AF488-conjugated anti-CD3 to detect T lymphocytes and with a AF488-conjugated anti-CD11c to detect dendritic cells; super resolution analysis confirmed that WGA-AF555 bacteria were nearby $CD3^+$ lymphocytes (**Figure 3I**) and $CD11c^+$ dendritic cells (**Figure 3H**).

## Exposure of BMDCs to Probiotic Affects R97-116 Tcells Motility

The interaction between probiotics and immune cells was further investigated by *in vitro* experiments with rat BMDCs and T lymphocytes specific for the immunodominant peptide R97-116 of the rat AChR alpha subunit (34). BMDCs were

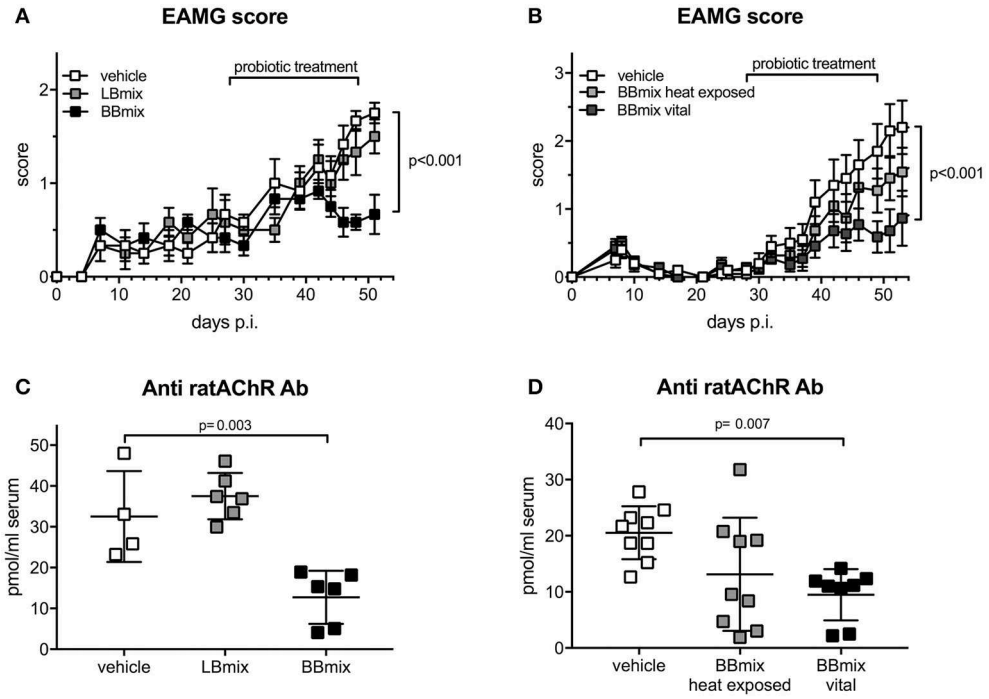



**FIGURE 1 |** Therapeutic administration of vital bifidobacteria strains ameliorates EAMG course. **(A)** Clinical EAMG score (mean ± SEM) of EAMG animals treated with vehicle, LBmix or BBmix ($n = 6$ rats/group). **(B)** Clinical EAMG score (mean ± SEM) of EAMG animals treated with vehicle, BBmix heat exposed or BBmix vital ($n = 11$ rats/group). **(C,D)** Anti-rat AChR Ab serum titer (pmol/ml of rat serum, mean ± SD) of treated-EAMG rats. Two-way ANOVA test with Tukey's *post-hoc* test for multiple-comparisons was used for clinical score. One-way ANOVA test with Dunnett's multiple comparison test was used for anti-rat AChR. Corrected *p*-values are reported.

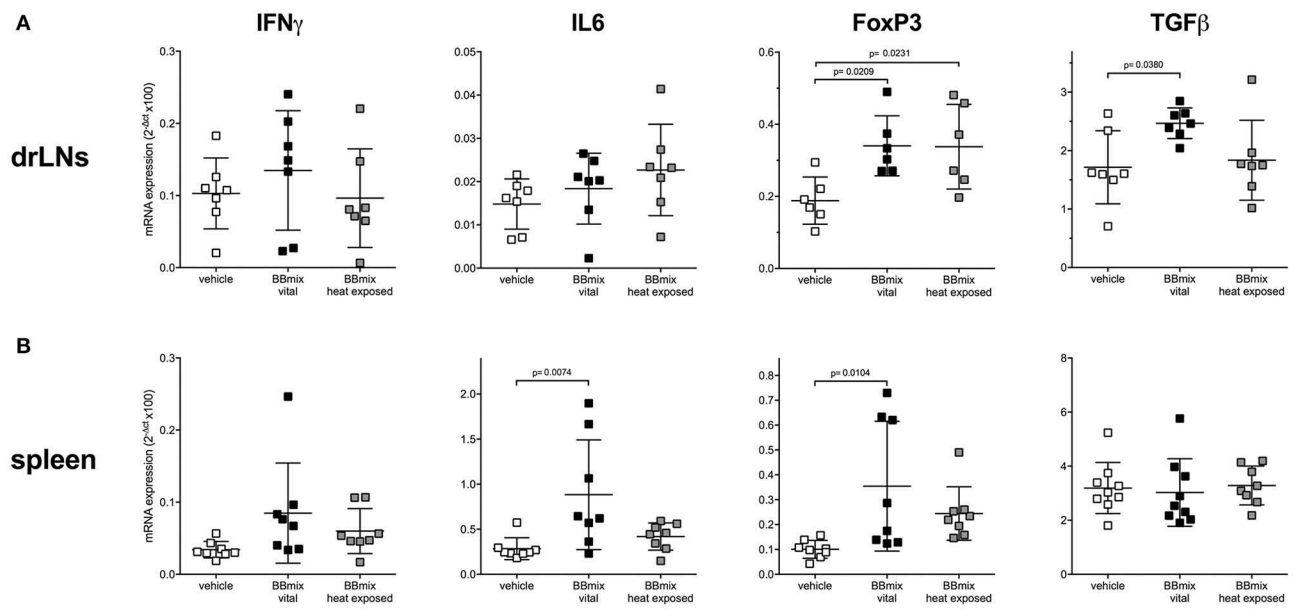



**FIGURE 2 |** Differential expression of pro-inflammatory and regulatory transcripts in primary and secondary lymphoid organs of EAMG rats. qRT-PCR analysis of IFNγ, IL6, FoxP3, and TGFβ mRNAs (mean ± SD) in drLNs **(A)** and spleen **(B)** of EAMG rats treated with vehicle, BBmix vital or BBmix heat exposed. One-way ANOVA test with Dunnett's multiple comparison test was used to assess statistical significance. Corrected *p*-values are reported.

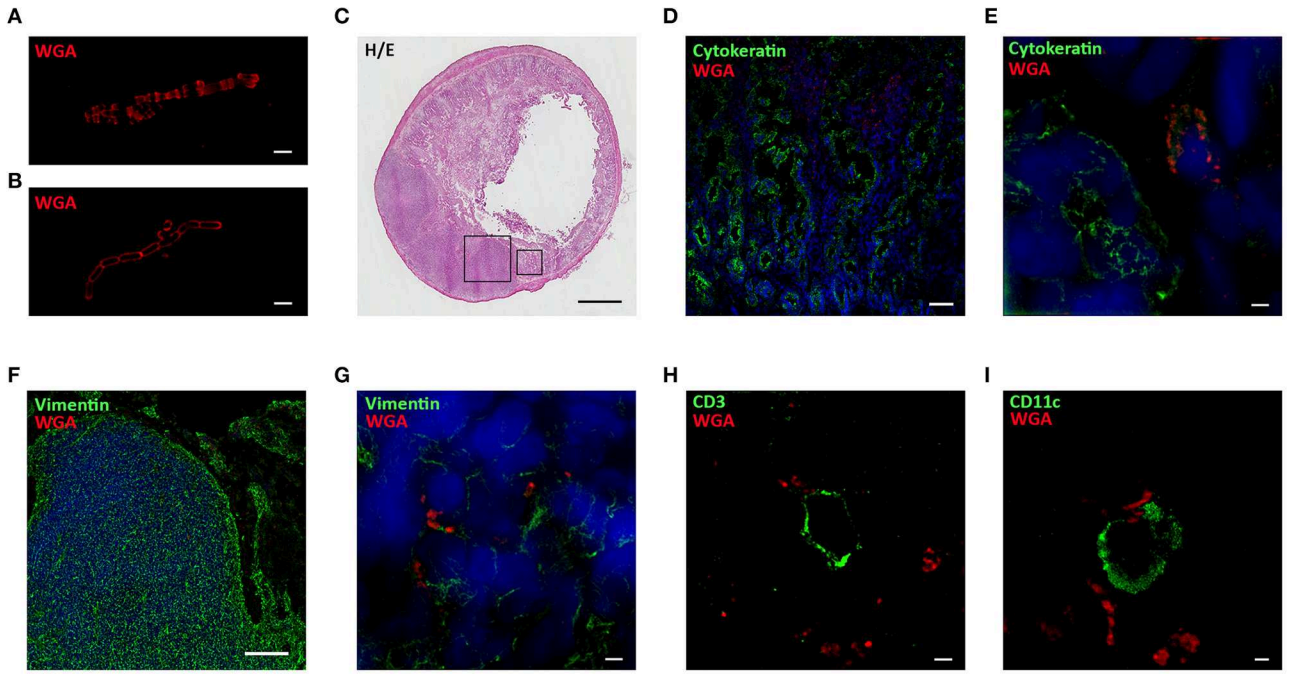



**FIGURE 3 |** Probiotics interact with immune cells located in Peyer's Patches and villi. SIM images of WGA-AF555 (red) labeled-BA **(A)** and LR **(B)**. **(C)** Hematoxilin and Eosin staining of rat small intestine. **(D)** Confocal and **(E)** SIM images of villi stained for cytokeratin (green) and nuclei (blue). WGA-AF555 LR (red) are located inside villi (confocal image scale bar: 50 μm). **(F)** Confocal and **(G)** SIM images of PP stained for vimentin (green) and nuclei (blue). WGA-AF555 LR (red) are located inside the PP (confocal image scale bar: 100 μm). SIM images of $CD3^+$ cell (green) **(H)** and $CD11c^+$ cell (green) **(I)**. WGA-AF555 LR (red) are detected nearby $CD3^+$ and $CD11c^+$ cells. SIM image scale bar: 2 μm.

exposed to WGA-AF555 labeled-probiotics for 60 min, and cultures were analyzed via live imaging microscopy (**Figure 4A**—single frame—and **Supplementary Video 1**). Most BMDCs are characterized by short movements to sample the surrounding microenvironment, while WGA-AF555-probiotics are either stably captured by BMDCs or free to move. Parallel cultures were processed for immunofluorescence analysis by SIM (**Figures 4B–D**), further confirming that single BMDCs (bright field) make multiple contacts with WGA-AF555 labeled-LR bacteria, arranged in chain (**Figure 4B**). Representative SIM images of $CD11c^+$ BMDCs contacting WGA-AF555 labeled-probiotics are reported in **Figure 4C** (LC strain, single plan) and **Figure 4D** (BA strain, single plan), and in the reconstructed 3D images (insets **Figures 4C,D**), showing probiotics contacting BMDC plasma membrane.

Toll-like receptors (TLRs) recognize multiple pathogens, including bacteria, viruses, fungi, and parasites, and their expression is regulated in both a cell type- and stimulus-dependent fashion (35). Hence, the differential expression of selected TLRs mRNA was evaluated in BMDCs exposed to bifidobacteria (BA, BL, BBmix) and lactobacilli (LC, LR, LBmix) (**Figure 5**). Increased TLR2 mRNA expression was observed in bifidobacteria- ($p < 0.001$) or lactobacilli-treated BMDCs ($p \leq 0.004$) vs. untreated BMDCs; TLR1 transcript were downregulated (BBmix, $p = 0.048$; LR, $p = 0.047$; LBmix, $p = 0.008$) whereas TLR6 expression was not altered in any experimental condition. The increased expression of TLR2 transcript was confirmed via immunofluorescence analysis (**Figures 5B–E**). Confocal and super resolution microscopy studies demonstrated increased expression of TLR2 on the membrane of probiotic exposed-$CD11c^+$ cells (**Figures 5C,E**) compared to untreated BMDCs (**Figures 5B,D**). Since TLR2 forms clusters in response to Lipoteichoic Acid (LTA), a component of the wall of Gram-positive bacteria (36), we investigated whether TLR2 upregulation observed on BMDCs could be associated with LTA, expressed on our probiotic strains, by means of SIM (**Figure 5F**). The analysis showed that LTA molecules (in green) are arranged in clusters and localized in proximity of TLR2 (in red) expressed by BMDCs, or even in close contact with TLR2 molecules (reconstructed 3D volume, **Figure 5F** inset).

To further evaluate the events associated with the probiotic-induced BMDCs immunomodulatory profile, rat BMDCs, grown in complete RPMI medium (as control) or exposed to BBmix or TGFβ, were subsequently loaded with peptide R97-116 and *in vitro* co-cultured with R97-116 specific $CD4^+$ effector T cells (Teff) (**Figure 6A**). By means of live imaging microscopy, the motility pattern of Teffs was recorded (1 frame/30 s, 60 min recording) and analyzed with Fiji software, plug-in TrackMate (21). Representative movies are included as **Supplementary Videos 2, 3**. Cells were defined as stationary if their path length was shorter than 10 μm every 10 min recording, or else they were classified as motile (22, 23).

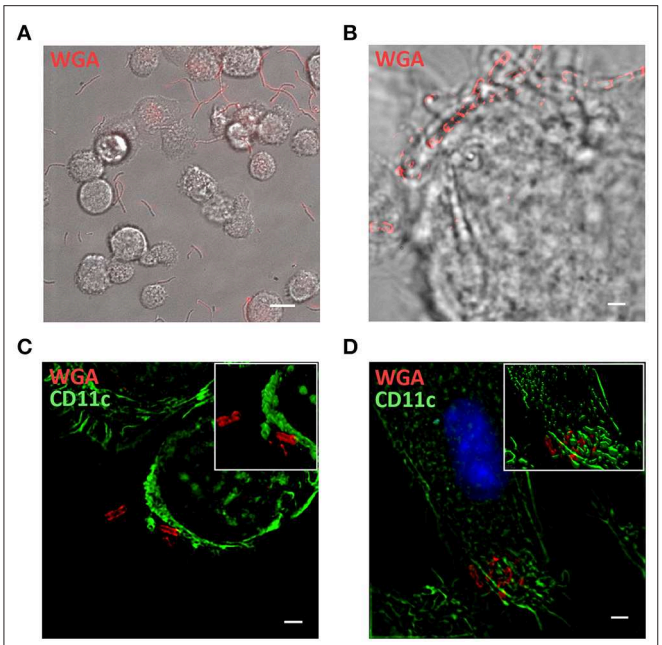



**FIGURE 4 |** *In vitro* interaction between BMDCs and probiotics. **(A)** Single frame of BMDCs co-cultured with WGA-AF555 labeled-LR (red). Scale bar 20 μm. **(B)** SIM image of a BMDC physically interacting with a chain of WGA-AF555 labeled-LR (red). **(C,D)** SIM images of CD11c$^{+}$BMDC (green) and WGA-AF555-LC and WGA-AF555-BA (red) respectively. Site of contact between BMDCs and bacterial cells are reported in insets (3D volume). SIM image scale bar: 2 μm.

When antigen-specific Teff cells were co-cultured with control BMDCs (peptide-loaded but not exposed to BBmix), 27.3 ± 8.6% of tracked cells (total number of tracked cells = 300; 4 replicates) showed a motile pattern at $t = 10$ min (**Figure 6B**, empty bar) and, conversely, 72.7 ± 8.6% had a stationary pattern (**Figure 6C**, empty bar); percentages of motile Teff (as well as stationary Teff) did not change in the subsequent time points (stationary Teff: 75.7 ± 5.5% at $t = 30$ min and 75.3 ± 4.3% at $t = 60$ min). Exposure of BMDCs to BBmix resulted in an altered Teff motility pattern: 63.7 ± 9.0% were classified as motile at $t = 10$ min ($p < 0.001$ vs. control BMDCs, **Figure 6B**, black bar), and this percentage was stable at $t = 30$ and $t = 60$.

Then, mean velocity (expressed as μm/minute) and meandering index were calculated for either motile (**Figures 6D,F**) and stationary Teffs (**Figures 6E,G**): as expected, motile Teff cells had a mean velocity greater than stationary cells (8.1 ± 1.3 μm/min vs. 4.3 ± 1.9, **Figures 6D,E** respectively, empty bars) at $t = 10$, $t = 30$ and $t = 60$. Interestingly, when BBmix-exposed BMDCs were evaluated, mean velocity of motile Teff significantly increased compared with control BMDCs ($t = 10$, 18.2 ± 12.4 μm/min vs. 8.1 ± 1.3, corrected $p = 0.01$, **Figure 6D**, black bars), and this observation was confirmed at the subsequent time points. On the contrary, mean velocity of stationary Teffs was significantly different at $t = 30$ only (**Figure 6E**, black bars), and at $t = 60$ returned similar to $t = 10$.

Meandering index is a measure of the Teff cells patrolling while moving nearby BMDCs, that reflects the intrinsic difference between motile Teff cells, with a meandering index 0.4 ± 0.1 ($t = 10$, **Figure 6F**, empty bar) and stationary Teff cells (meandering index 0.13 ± 0.04, $t = 10$, **Figure 6G**, empty bar). No differences were observed when Teffs were co-cultured with BBmix-exposed BMDCs (**Figures 6F,G**, black bars).

In a previous study, we have reported increased expression of TGFβ in tissue culture supernatants from probiotic-treated BMDCs and in the serum from probiotic-treated EAMG rats, suggesting an immunomodulatory role for this pleiotropic cytokine. Hence, we exposed BMDCs to TGFβ (10 ng/ml) prior to antigen pulsing and co-culture with Teff cells (representative movie is reported as **Supplementary Video 4**). Interestingly, the percentage of tracked cells (total number of tracked cells = 225; 3 replicates) classified as motile (**Figure 6B**, striped bars) was significantly increased compared to Teff cells co-cultured with control BMDCs (corrected $p < 0.001$, **Figure 6B**, empty bars) and similar to what observed with BBmix-exposed BMDCs (**Figure 6B**, black bars), at $t = 10$, $t = 30$ and $t = 60$ time points. Again, the mean velocity of motile Teff cells was found increased (31.2 ± 4.9 μm/min, $t = 10$, **Figure 6D**, striped bars) compared to control co-cultures (corrected $p < 0.001$), a behavior similar to that observed in co-cultures with BBmix-exposed BMDCs (**Figure 6D**, black bars).

## Probiotics Influence Gut Microbiota in EAMG

NGS analysis of gut microbiota was performed on stools collected from experimental animals at different time points: HD animals (day 0, before TAChR/CFA immunization), EAMG onset (at day 27, before treatments) and at the end of experiment, for each treatment groups (vehicle, BBmix and BBmix heat exposed). Stools collected from the animals housed in the same cage were pooled before processing for DNA extraction. Raw data from NGS sequencing were analyzed with the Ion Reporter™ Software 5.10 and the workflow Metagenomics 16S w1.1; consensus fastq sequences were then elaborated with QIIME 2 microbiome bioinformatics platform (version 2018.8). A total number of 1,255,155 reads were obtained, with an average of 59,769 reads per sample, and 934 OTUs identified. Alpha and beta diversity analyses were performed on the OTU table. The observed OTUs plot (**Figure 7A**) showed a similar number of OTUs in HD and EAMG onset groups, different from the OTU numbers in EAMG chronic, EAMG BBmix vital and EAMG BBmix heat exposed groups. The Shannon evenness analysis (**Figure 7B**) showed a more complex degree of α-diversity occurring across experimental groups, indicating a higher OTUs richness of gut microbiota in chronic, vehicle fed, EAMG animals, with a tendency to α-diversity reduction in BBmix heat exposed group. Concerning the Pielou's index for evenness, the highest value was observed in EAMG onset group (**Figure 7C**), pointing out that the microbiota species in these animals were more equally distributed compared to the other groups. β-diversity was

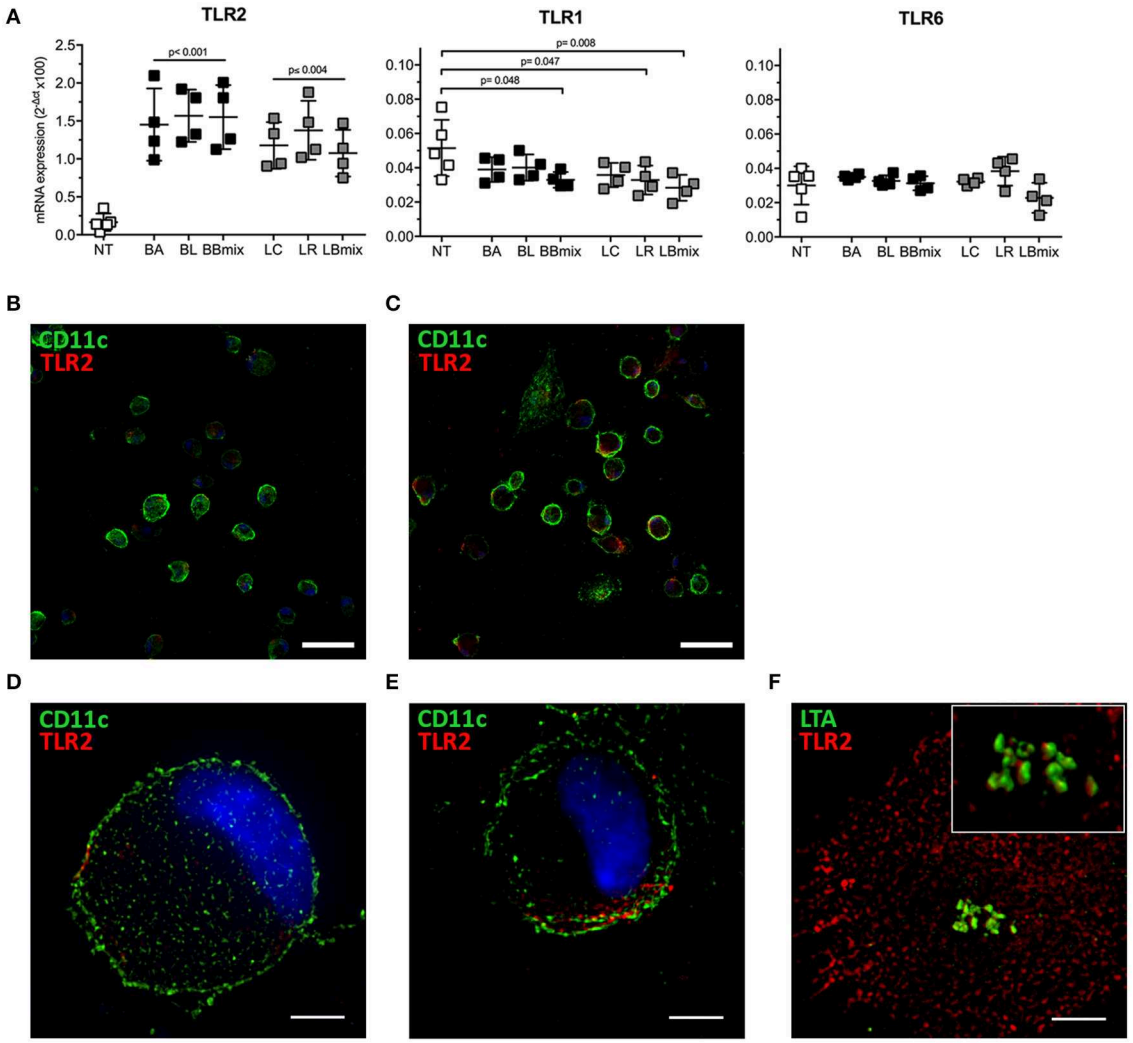



**FIGURE 5 |** Differential expression of TLRs in BMDCs exposed to probiotics. **(A)** qRT-PCR analysis of TLR2, TLR1, and TLR6 in BMDCs exposed to single strains (BA, BL, LC, LR) and combinations (BBmix, LBmix) of probiotics for 4 h (mean ± SD). **(B)** Confocal and **(D)** SIM images of untreated CD11c$^+$ cells (green) and TLR2 (red). **(C)** Confocal and **(E)** SIM images of LR-exposed CD11c$^+$ cells (green) and TLR2 (red). **(F)** SIM image of LTA (green) and TLR2 (red) of BMDCs exposed to LC (inset: 3D volume). Statistical significance was assessed by one-way ANOVA test with Dunnett's multiple comparison test. Corrected *p*-values are reported. Confocal image scale bar: 50 μm; SIM image scale bar: 5 μm.

evaluated by the unweighted UniFracPCoA analysis (**Figure 7D**), which allowed the identification of three separated clusters: HD (red dots), EAMG onset (blue dots) and a more heterogeneous cluster comprising EAMG chronic (green dots), EAMG BBmix vital (pink dots) and heat exposed (turquoise dots). Of note, HD, EAMG onset and EAMG chronic groups were quite separated, indicative of a greater degree of β-diversity, while it was possible to discriminate only a modest difference between probiotic treated EAMG rats.

At taxonomical level, *Firmicutes* (relative abundance 62–64%) and *Bacteroidetes* (relative abundance 28–34%) were the dominant phyla in all groups (**Figure 8A**), together representing ∼98% of the gut microbiota. *Firmicutes*/*Bacteroidetes* ratio (F/B, **Table 1**) did not show any difference across experimental groups. The analysis restricted to the phyla with relative abundance below 5% showed 5 main phyla: *Proteobacteria*, *Deferribacteres*, *Verrucomicrobia*, *Actinobacteria* and *Tenericutes* (**Figure 8B**). Within this subset, the *Tenericutes*/*Verrucomicrobia* ratio (T/V) was found significantly altered in EAMG onset group, compared to HD (T/V ratio 5.18 ± 1.3 vs. 2.21 ± 1.8, corrected $p = 0.007$), followed by a sharp decrease (0.1 ± 0.1) in chronic, vehicle fed, EAMG rats. Interestingly, treatment with vital probiotic partly restored the T/V ratio (0.96 ± 0.3), indicating a possible effect of the BBmix treatment on these phyla, not observed in heat exposed treated EAMG animals. At family level, *Ruminococcaceae* (relative abundance 27 ± 2%), *Prevotellaceae* (relative abundance 20 ± 3%) and an unclassified family belonging to the *Clostridiales* order

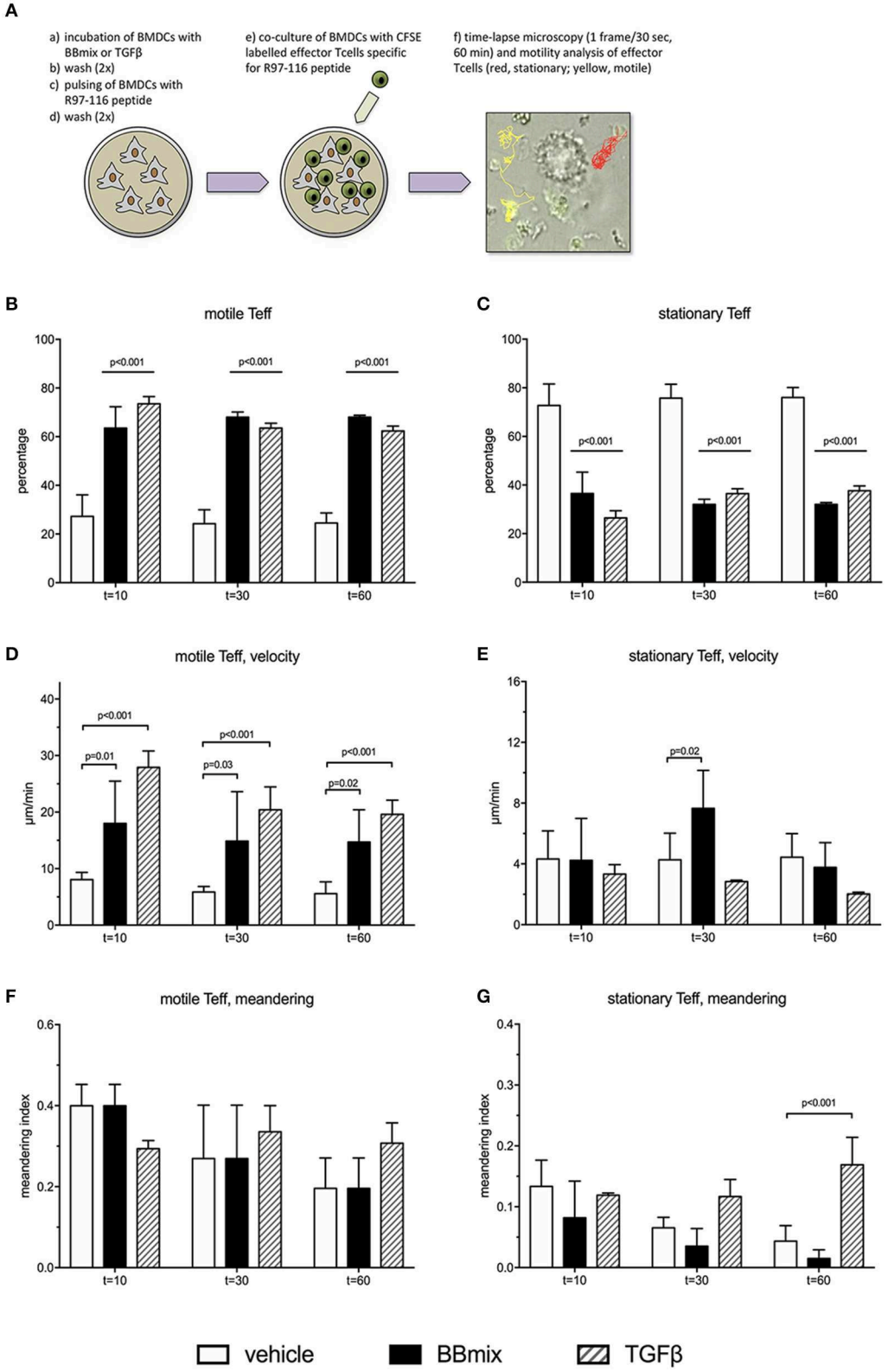



**FIGURE 6 |** Treatment of BMDCs with probiotics modifies R97-116 T cell motility. **(A)** Experimental set-up of live imaging analyses of CFSE-labeled R97-116-specific T cells co-cultured with R97-116-loaded BMDCs, and example of motility analysis. **(B)** Percentage, **(D)** velocity and **(F)** meandering index of motile Teff. **(C)**

*(Continued)*

**FIGURE 6 |** Percentage, **(E)** velocity, and **(G)** meandering index of stationary Teff. Experimental conditions were: control cultures of BMDC, loaded with R97-116 peptide (empty bars), BMDC exposed to BBmix prior to antigen loading (black bars), BMDC exposed to TGFβ prior to antigen loading (striped bars). Statistical significance was assessed by two-way ANOVA test with Dunnett's multiple comparison test. Corrected *p*-values are reported.

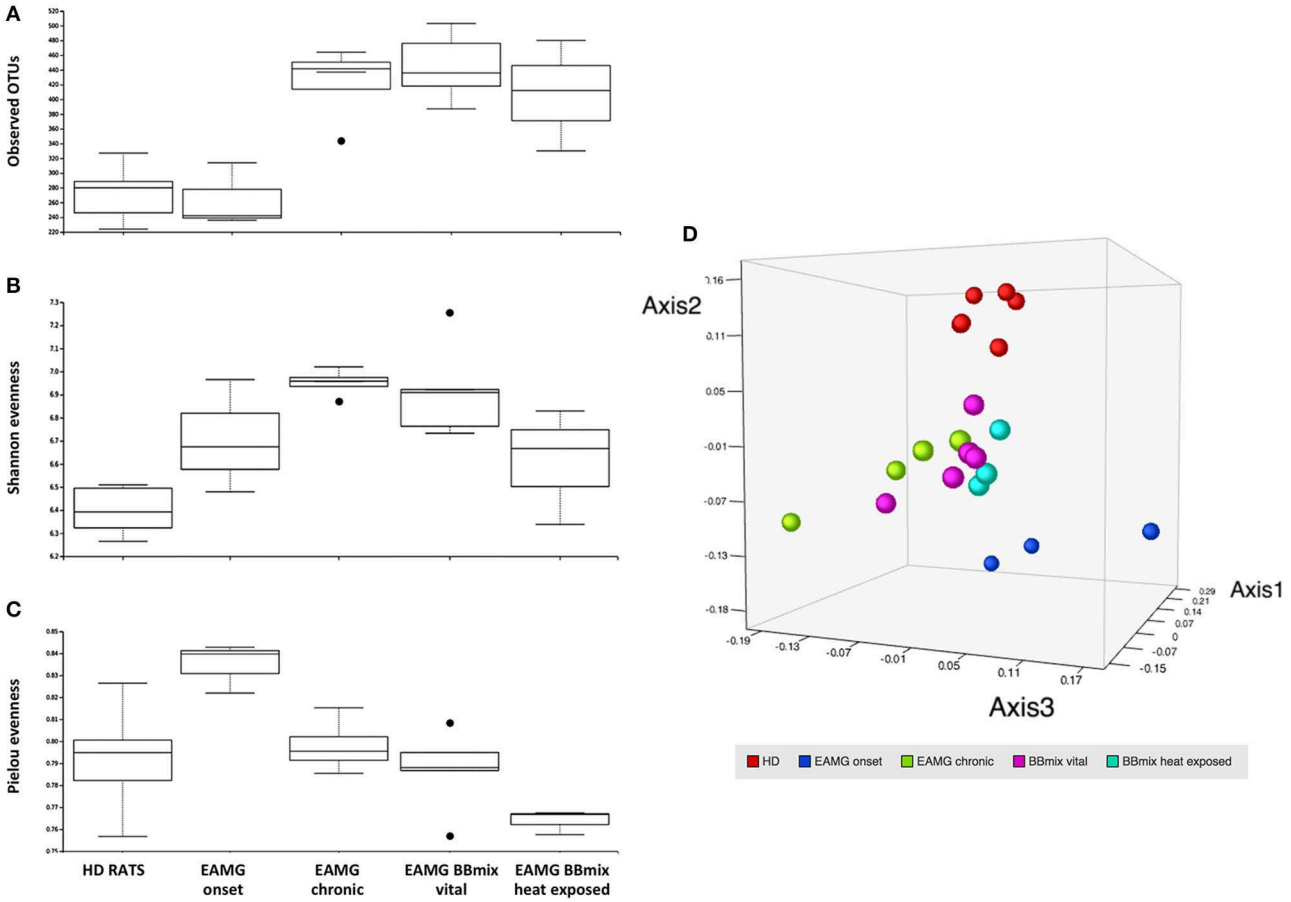



**FIGURE 7 |** Treatment with Bifidobacteria modifies alpha and beta diversity of EAMG gut microbiota. **(A)** Observed OTUs, **(B)** Shannon index, **(C)** evenness, and **(D)** unweighted UniFrac distance PCoA of healthy rats (HD rats; red dots), EAMG rats 30 days after the induction of the disease (EAMG onset; blue dots), EAMG rats in the chronic phase (EAMG chronic; green dots), EAMG rats treated with vital bifidobacteria (EAMG BBmix vital; pink dots), and EAMG rats treated with heat exposed bifidobacteria (EAMG BBmix heat exposed; turquoise dots).

(relative abundance 24 ± 1%) represented ∼70% of the gut microbiota (**Figure 8C**), without differences. Major changes were observed in the less abundant families (relative abundance lower than 15%) (**Figure 8D**), showing a strong reduction of *Lachnospiraceae* in EAMG chronic group, and an increase of the *Ruminococcaceae*/*Lachnospiraceae* (R/L) ratio (EAMG chronic R/L ratio 13.22 ± 2.4; **Table 1**). Interestingly, probiotic treatment was associated with a decreased R/L ratio (EAMG BBmix vital R/L ratio 8.49 ± 0.8). A similar trend was showed by the relative abundances for *Peptoptococcaceae* and *Peptostreptococcaceae* families (**Figure 8D**), increased in EAMG chronic, unchanged in heat exposed BBmix, but reduced in vital BBmix group to values similar to HD rats (**Figure 8D**). The relative abundance heatmap (**Supplementary Figure 3**) showed a specific cluster in the EAMG onset group comprising the genera *Sutterella, Phascolarctobacterium, Dialister, Odoribacter*, *Lachnospira*, two unclassified genera belonging to *Rikenellaceae* and *Barnesiellaceae* families and one unclassified genus belonging to the *Bacteroidales* order. On note, the increase of *Akkermansia* in the EAMG chronic group, and the *Turicibacter* genus, reduced in vital BBmix group similar to what observed at the family level. Of interest, the relative abundance of *Lactobacillus* genus was also affected by vital bifidobacteria treatment and not by inactivated probiotic strains (**Supplementary Figure 3**).

## DISCUSSION

Modulation of the gut microbiota and its interactions with the host's immune system by probiotics has been proposed as a novel therapeutic tool for the treatment of autoimmune

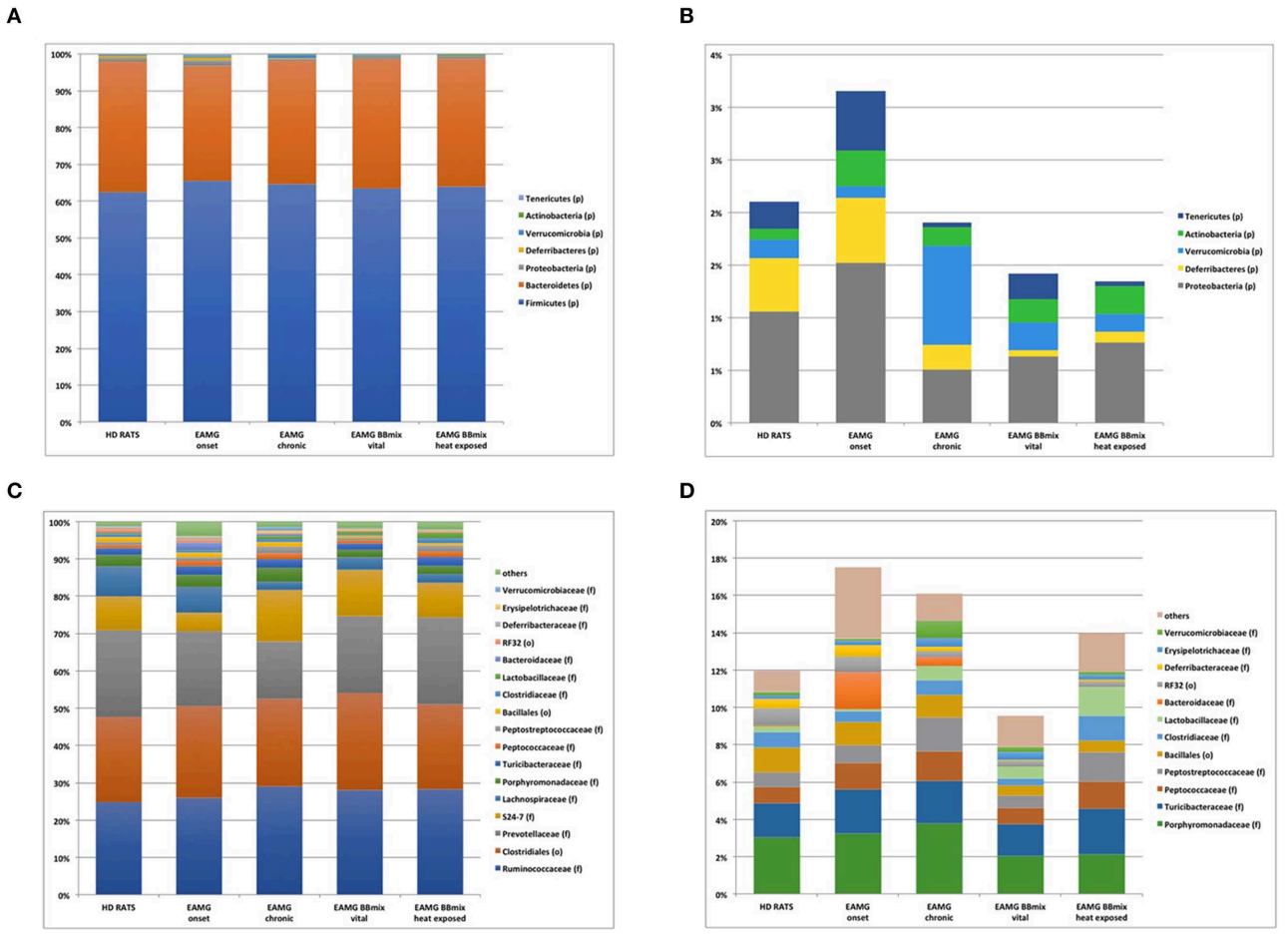



**FIGURE 8 |** Bifidobacteria reduce the dysbiosis induced by the disease. Stacked barplot representing the taxonomic assignments at *phylum* level (phyla with relative abundance higher than 0.1%). Major phyla, defining on average the 98% of the bacterial community **(A)**, and minor phyla **(B)**. Stacked barplot representing the taxonomic assignments at *family* level (families with relative abundance higher than 1%). Dominant families, defining on average the 86% of the bacterial community **(C)**, and less abundant families **(D)**.

disorders; altered immunological responses can be skewed and damped by probiotics that exhibit immunomodulatory properties (37–39). Previous reports from Chae's (7) and our (6) groups demonstrated that probiotic administration, according to a prophylactic (7) or preventive (6) treatment protocols, ameliorated EAMG symptoms and modulated AChR-specific immune responses. In this study, we evaluated the probiotic efficacy in the Lewis rat EAMG model, given to animals according to a therapeutic protocol (i.e., treatments started at disease onset). Two lactobacilli (LBmix) and two bifidobacteria (BBmix) strains were administered during EAMG chronic phase of the disease, and a significantly improvement of EAMG was observed in BBmix-treated animals, compared with vehicle- and LBmix-fed groups (**Figure 1A**), with a parallel reduction of anti-AChR antibody levels (**Figure 1C**).

Then, we wondered whether the observed clinical efficacy of bifidobacteria in our EAMG model was dependent to their vitality. Indeed, the debate concerning the efficacy of live probiotics compared to inactivated bacteria is still open, with contrasting results. Zimmermann et al. compared the effects of several bacteria strains, live or heat exposed, on a human colorectal adenocarcinoma cell line (HT-29) and observed that live probiotics induced immunosuppressive effects, whereas heat exposed bacteria caused an elevated immune response (40). By contrast, Sugahara et al. observed that both live and heat exposed *B. breve* M-16V suppressed pro-inflammatory cytokine production in spleen cells of gnotobiotic mouse model (41). However, the comparison between the effects of vital or dead cells (irradiated or heat exposed) contained in a mixture of probiotics (VSL3) administered to animals with colitis (DSS-induced colitis), revealed that the non-viable irradiated or viable probiotics attenuated the symptoms, whereas the heat exposed probiotics had no effect on the severity of disease (42). In this regard, results from *in vivo* experiments suggest that the selected bifidobacteria strains, when vital, have a greater efficacy in modulating EAMG compared with vital lactobacilli (**Figure 1A**)

**TABLE 1 |** *Firmicutes/Bacteroidetes* (F/B), *Tenericutes/Verrucomicrobia* (T/V), and *Ruminococcaceae/Lachnospiraceae* (R/L) ratios in samples.

| | F/B ratio (phylum) | T/V ratio (phylum) | R/L ratio (family) |
|---|---|---|---|
| **HD** | 1.79 ± 0.3 | 2.21 ± 1.8 | 3.11 ± 0.5 |
| **EAMG onset** | 2.21 ± 0.7 | 5.18 ± 1.3[a] | 3.93 ± 1.4 |
| **EAMG chronic** | 1.98 ± 0.5 | 0.10 ± 0.1[b] | 13.22 ± 2.4[c] |
| **EAMG BBmix vital** | 1.83 ± 0.3 | 0.96 ± 0.3 | 8.49 ± 0.8[d,f,g] |
| **EAMG BBmix heat exposed** | 1.88 ± 0.4 | 0.37 ± 0.3 | 11.92 ± 1.9[e] |

*F, Firmicutes (p); B, Bacteroidetes (p); T, Tenericutes (p); V, Verrucomicrobia (p); R, Ruminococcaceae (f); L, Lachnospiraceae (f).*
*[a]vs. HD, corrected P-value = 0.007.*
*[b]vs. HD, corrected P-value = 0.04.*
*[c,d,e]vs. HD, corrected P-value < 0.001.*
*[f]vs. EAMG vehicle, corrected P-value < 0.001.*
*[g]vs. EAMG BBmix heat exposed, corrected P-value = 0.02.*
*Statistical significance was assessed by one-way ANOVA test with Dunnett's multiple comparison test.*

and with heat exposed bifidobacteria (**Figure 1B**), when given to animals after disease onset (therapeutic protocol), and this effect was associated with reduced serum anti-AChR antibodies titers (**Figure 1D**). Thus, these data further confirm our previous study on the efficacy of preventive probiotic treatment in the rat EAMG model (6) and show, for the first time, the efficacy of the administration of probiotics to EAMG Lewis rats, following a therapeutic treatment. The beneficial effects of vital bifidobacteria could be related to the maintenance of the integrity of bacterial proteins (e.g., carbohydrate polymers exhibited on the cell surface) and DNA sequences (e.g., CpG motifs), that exhibit immunomodulatory properties (16). The minor effects of heat exposed bifidobacteria could be, therefore, linked to the partial denaturation of molecules with immunomodulatory activity. Moreover, the heat inactivation blocks the metabolite production, that could, even in a small amount, alter the cross-feeding mechanisms among commensal bacteria, important in the regulation of immune system (e.g., short chain fatty acid). However, further studies using different techniques (such as irradiation, formaldehyde, or DNase-treatment) that could inactivate bacteria while retaining the intact protein structures, may provide more insight into the mechanisms by which the probiotics act and into the mediators induced in the EAMG model by our vital or inactive probiotics.

As reported, probiotics are capable to influence the immune system, not only locally but also in the periphery (38). We observed that the therapeutic treatment with BBmix was associated with increased FoxP3 and TGFβ mRNAs expression in drLNs of EAMG animals (**Figure 2A**). This observation is in line with our previous report on the efficacy of preventive probiotic treatment in this model (6); overall, these data further support the hypothesis of TGFβ as systemic cytokine able to orchestrate the immune-modulation activated by probiotics (6), besides FoxP3$^+$cells as key players in probiotic induced-suppressive activity (43, 44).

Whether an interaction of bacteria with GALT could occur has been studied by several researchers and it has been described that DCs in the gut can even send dendrites in the intestinal lumen to sample the microenvironment (10, 45). To study possible interactions of our probiotic strains with immune elements in the gut mucosa, bacterial wall was *in vitro* labeled with WGA-AF555, that selectively binds to N-acetyl-D-glucosamine and sialic-acid containing glycoconjugates and oligosaccharides (46). Super resolution microscopy images showed slightly differences in theWGA-AF555 staining pattern (**Figures 3A,B**), suggesting a specific distribution of the two WGA ligands in bifidobacteria and in lactobacilli cell wall. Then, WGA-AF555 labeled probiotics were administered to Lewis rats and, after 30–60 min, the intestine were aseptically removed and processed for confocal and super resolution microscopy. Immunofluorescence images demonstrated that WGA-AF555-labeled probiotics were found nearby villi (**Figures 3D,E**) and in PPs (**Figures 3F,G**); within PPs, WGA-AF555-labeled probiotics were found in close contact with CD3$^+$ cells (**Figure 3H**) and CD11c$^+$ cells (**Figure 3I**).

Furthermore, by means of *in vitro* live imaging (**Figure 4A**, **Supplementary Video 1**) and super resolution microscopy (**Figures 4C,D**) we were able to confirm that bacterial cells interact BMDCs. These data suggests that DC cells and CD3$^+$ lymphocytes could indeed interact with bifidobacteria in PPs; whether these interactions are preliminary to phagocytosis by DCs (47, 48) needs to be further investigated.

DCs make contacts with microbes using pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs); among PRRs, toll-like receptors (TLRs) play a pivotal role, and are capable to discriminate different components of bacteria (49). Hence, we evaluated whether the interaction of bifidobacteria with BMDCs could modulate TLRs transcription profile. qRT-PCR analysis showed increased TLR2 transcription in probiotic-treated BMDCs and a weak down-regulation of TLR1 in BBmix-and in lactobacilli-treated BMDCs (**Figure 5A**); TLR6 mRNA was unaltered. The increased TLR2 mRNA expression was confirmed by confocal (**Figures 5B,C**) and super resolution microscopy (**Figures 5D,E**), showing increased TLR2 expression on CD11c$^+$ cells and its localization in specific plasma membrane regions. Although BMDC exposure to bifidobacteria and lactobacilli resulted in differential expression of TLR1 and TLR2 mRNAs, as a consequence of cell-cell recognition, their effects on EAMG manifestation were different. The better therapeutic efficacy of bifidobacteria could be partly explained by a differential downstream effect on pathogens recognition pathways by BMDC *in vitro*, possibly in correlation with not yet reported differences on LTA protein structure between bifidobacteria and lactobacilli and their affinity to TLR2. Indeed, we found that lactobacilli strains induce a considerable increase of IL12b mRNA (preliminary data, not shown) besides IL10 mRNA (6) suggesting that lactobacilli could drive the differentiation of naïve T cells toward a Thelper rather than Treg phenotype. All together these findings suggest that TLR2 could be one of the PRRs engaged in the bifidobacteria recognition, while TLR1 and TLR6, functionally associated with TLR2 in the discrimination of a subtle difference between triacyl- and diacyl-lipopeptides (49),

are not involved at this stage. Since lipoteichoic acid (LTA), a major cell wall component of gram-positive bacteria, is primarily recognized by TLR2 (50), probiotic exposed-BMDCs were analyzed by immunofluorescence for the co-expression of LTA and TLR2 (**Figure 5F**), further suggesting that the recognition of our probiotics is mediated by LTA-TLR2 engagement that occurs in TLR2-enriched domains on the BMDC plasma membrane. Further analysis are needed to describe the events occurring in DC cytoplasmic domain below the engaged LTA-TLR2 molecules (51), with regard to the TLR2-signal transduction cascade resulting in the release of immunomodulatory cytokines.

To summarize, results or our research suggest that $CD11c^+$ DCs contact probiotics in the GALT (**Figure 3**) and this interaction is mediated by TLR2 (**Figure 4**), upregulates immunoregulatory citokines mRNAs (i.e. IL10 and TGFβ) and induces the production of TGFβ (6). Since activated DCs in PPs migrate to mesLN (12), we sought to investigate, using a simplified *in vitro* model of DC-Teffector interactions, a plausible mechanism of immune modulation induced by probiotics *in vivo*. Thus, DCs were exposed to bifidobacteria, and loaded with an antigen involved in EAMG (R97-116). Then, DCs were co-cultured with "circulating" antigen- specific $CD4^+$ Teff cells (**Figure 6A**) and since it has been suggested that probiotics can induce immune tolerance either/or promoting Treg activity and/or suppressing T helper response (5), we wondered whether bifidobacteria treatment could influence Tcell motility, clue of the correct immunological synapse formation (22). Indeed, it has been demonstrated that the formation of a stable major histocompatibility complex (MHC)-peptide-TCR complex between DCs and antigen-specific T-lymphocytes results in a temporary engagement of T cells, characterized by a reduced motility and low meandering index (stationary phenotype), in contrast to not engaged T cells, that remains free to move, with high velocity and elevated meandering index (motile phenotype) (22, 23). Hence, bifidobacteria-exposed and antigen-loaded BMDCs were co-cultured with R97-116 specific $CD4^+$ Teff cells (**Figure 6A**) and, by means of live-imaging microscopy and motility analysis, stationary and motile R97-116 Tcells analyzed. In control co-cultures (i.e., antigen loaded BMDC) more than 70% of Teff had a stationary phenotype (**Figure 6C**, empty bars); on the contrary, when Teff cells were co-cultured with BBmix-exposed BMDCs, the majority of tracked cells displayed a motile phenotype (**Figure 6B**, black bars), and an increased mean velocity (**Figure 6D**, black bars), suggesting that antigen-specific T lymphocytes continued to patrol the microenvironment near BMDCs, presumably still searching the engagement with the proper MHC-peptide complex. Whether this different Teff behavior is due to a reduced density of MHC-peptide complexes or of co-stimulatory molecules on BMDCs, is a point that deserves further studies.

In our previous study (6), we demonstrated that TGFβ was involved in the immune-modulation observed in probiotic treated EAMG rats (preventive protocol); of note, TGFβ was found increased in drLNs from BBmix-vital treated EAMG rats (therapeutic protocol) (**Figure 2A**). Since the role of TGFβ in association to tolerance induction in EAMG has been reported in several studies (52, 53), we choose to expose BMDCs to TGFβ prior to the antigen-loading step and co-culture with R97-116 specific $CD4^+$ Teff cells; then motility analyses were performed (**Figures 6B–G**, striped bars). Our data showed that TGFβ is able to reproduce the effects observed with BBmix exposed BMDCs, i.e., increased percentages of motile Teff (**Figure 6B**, striped bars), characterized by a greater mean velocity (**Figure 6C**, striped bars). Overall, these data show that the exposition of BMDCs to bifidobacteria cause a downstream effect on Tcell motility, mimicking the same results obtained with the TGFβ. Hence, we can hypothesized that BBmix treatment could lead to a T cell phenotype more tolerant, resembling the effects induced by TGFβ in DC *in vitro* (i.e., downregulation of antigen-presenting function and expression of co-stimulatory molecules) (52). The exact molecular mechanism that lead toward a tolerant phenotype needs to be elucidated.

The effects of probiotics on the host immune system could be also mediated by a modulation of the gut microbiota; indeed, several studies reported alterations in bacterial taxonomical composition in stools from patients and from animal models (54, 55), suggesting an important role of microbiota in (auto)immune diseases. To our knowledge, this is the first study that characterize the intestinal microbiota composition in the Lewis rat EAMG model, considering also the effect of a probiotic therapeutic treatment. The analysis of the gut microbiota demonstrated differences between HD and EAMG rats, and during progression of EAMG from onset to the chronic phase (end of experiments) (**Figures 7A–C**), with an higher microbial diversity at the onset stage of the disease (**Figure 8D**).

At the *phylum* level, *Firmicutes* and *Bacteroidetes* represent almost the totality of the microbial population both in healthy and EAMG rats (**Figure 8A**), and the *Firmicutes*/*Bacteroidetes* (F/B) ratio is used to describe a pro-inflammatory microbiota (56–59); however, in the different experimental conditions of our study, the F/B ratio was unaltered (**Table 1**), suggesting the absence of pathological disequilibrium in the gut microbiota (dysbiosis). The microbial alteration observed during EAMG course mostly regarded the less abundant bacterial populations at each taxonomic level (**Figures 8B,D**). Overall, we observed an increased abundance of certain families/genera in EAMG onset compared to the other experimental groups (**Table 1**), and that the probiotic treatment is associated with modulation of the relative abundance of certain microbial community (**Supplementary Figure 3**). Of note, this disequilibrium seems to be partially reverted in BBmix vital EAMG rats, as it also occurs in the last phase of the disease (EAMG chronic). The most affected bacterial populations were *Tenericutes* and *Verrucomicrobia* phyla, *Lachnospiraceae* family, *Turicibacter*, *Lactobacillus,* and *Akkermansia* genera. The decrease of *Tenericutes* phylum was also observed in intestinal dysbiosis of rats due to inflammatory conditions (60), as well as the increase of *Verrucomicrobia* (61). Moreover, it has been reported a positive correlation of members belonging to the *Lachnospiraceae* family as immune-modulating bacteria in rats affected by experimental autoimmune encephalomyelitis (62). Thus, the decreased levels of *Lachnospiraceae* in the EAMG chronic group could be related to the inflammatory status, whereas the treatment with BBmix vital could contribute to its increase. This effect was also observed in gut microbiota of patients with inflammatory bowel disease (63). We observed the increase of *Akkermansia* in rats in the

chronic phase of the disease compared to both healthy rats and rats treated with bifidobactiera. It is known that *Akkermansia muciniphila,* belonging to the genera *Akkermansia*, is a mucin-degrader bacterium and it has been reported to have both regulatory and inflammatory properties (64). The increase of this genus has been reported in other autoimmune pathologies, such as multiple sclerosis (64, 65) and type 1 diabetes patients (66). The reasons of this increase can be found in its dual functions: it can increase to compensate for the imbalance in gut microbiota composition or it can contribute itself to the disequilibrium. However, a deepened analysis of the genus *Akkermansia* and a more complex evaluation of bacteria and their metabolites are required to understand the EAMG gut microbiota profile. We were unable to detect changes in the relative abundance of Bifidobacterium genera by 16S rRNA NGS sequencing in gut microbiota; this could be explained by the fact that NGS approach is not truly quantitative, and more specific and sensitive methods (e.g., strain specific RT-qPCR assays) should be employed (6).

Our data suggest that an ongoing immune sensitization process, from EAMG induction to EAMG onset, could contribute to the microbiota imbalance that could be counteracted during the disease course. Furthermore, microbiota analysis suggests that, even if an evident alteration of gut microbiota can be observed at EAMG onset, the administration of bifidobacteria, either vital or heat exposed, could help in restoring the normal gut microbiota balance, as observed in HD animals. However, we cannot exclude that the systemic activation of the immune system, as a consequence of the active TAChR/CFA immunization, could influence the GALT thus playing a crucial, although indirect, role in the gut microbial alterations. Administration of probiotics is not associated to major modification in the most abundant microbiota phyla present in EAMG animals, and more focused studies are necessary to specifically target the bifidobacterium genus, and particularly in relation to the probiotic administration. Moreover, the beneficial effects associated to probiotic administration still deserve proper investigation, due to heterogeneity of metabolic mediators, such as short chain fatty acid (67), produced by the gut flora and involved in the crosstalk between microbiota and the immune system.

In conclusion, our study demonstrated that the therapeutic administration of two bifidobacteria probiotic strains induced immunomodulatory effects leading to EAMG amelioration. Furthermore, inactivated probiotic by heat exposure were less effective; *in vitro* experiments demonstrate that LTA-TLR2 interaction does occur between probiotics and DCs, and TLR2 undergoes a membrane redistribution that could interfere with the formation of the MHC-Ag-TCR complex between DCs and AChR specific T cells; BMDCs exposed to TGFβ alters Tcells motility pattern similar to that observed by bifidobacteria exposed BMDCs. Finally, EAMG induction is associated to an altered gut microbiota, and probiotic intake could contribute to restore the normal microbiota.

In MG, innovative therapies counteracting the altered autoimmune attack and loss of tolerance to AChR, possibly without side effects, are still needed. To reach this aim, the selection and characterization of probiotic strains with immunomodulatory properties could be of relevance for MG and other autoimmune diseases.

## DATA AVAILABILITY STATEMENT

The raw data concerning metagenomic analysis and supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institute Ethical Board and the Italian Ministry of Health (code 1064/2015-PR).

## AUTHOR CONTRIBUTIONS

ER, AC, CCo, GS, and CCr generated the data. ER, AC, AF, CCo, and FB analyzed the data. ER, AC, AF, and FB wrote the manuscript. All authors approved the final manuscript.

## FUNDING

This research was supported by a grant from the Italian Ministry of Health (WFR PE-2011-02346818 to FB); Institutional funds for Annual Preclinical Research and for RC2018—IRCCS Network for Neurosciences and Neurorehabilitation.

## ACKNOWLEDGMENTS

We thank CHR Hansen (Denmark) for providing *Bifidobacterium animalis subsplactis*., BB12.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02949/full#supplementary-material

## REFERENCES

1. Mantegazza R, Bernasconi P, Cavalcante P. *Myasthenia gravis*: from autoantibodies to therapy. *Curr Opin Neurol.* (2018) 31:517–25. doi: 10.1097/WCO.0000000000000596
2. Losen M, Martinez-Martinez P, Molenaar PC, Lazaridis K, Tzartos S, Brenner T, et al. Standardization of the experimental autoimmune myasthenia gravis (EAMG) model by immunization of rats with *Torpedo californica* acetylcholine receptors–Recommendations for methods and experimental designs. *Exp Neurol.* (2015) 270:18–28. doi: 10.1016/j.expneurol.2015.03.010
3. So J-S, Kwon H-K, Lee C-G, Yi H-J, Park J-A, Lim S-Y, et al. *Lactobacillus casei* suppresses experimental arthritis by down-regulating T helper 1 effector functions. *Mol Immunol.* (2008) 45:2690–9. doi: 10.1016/j.molimm.2007.12.010

4. Castagliuolo I, Galeazzi F, Ferrari S, Elli M, Brun P, Cavaggioni A, et al. Beneficial effect of auto-aggregating *Lactobacillus crispatus* on experimentally induced colitis in mice. *FEMS Immunol Med Microbiol*. (2005) 43:197–204. doi: 10.1016/j.femsim.2004.08.011
5. Kwon H-K, Kim G-C, Kim Y, Hwang W, Jash A, Sahoo A, et al. Amelioration of experimental autoimmune encephalomyelitis by probiotic mixture is mediated by a shift in T helper cell immune response. *Clin Immunol*. (2013) 146:217–27. doi: 10.1016/j.clim.2013.01.001
6. Consonni A, Cordiglieri C, Rinaldi E, Marolda R, Ravanelli I, Guidesi E, et al. Administration of bifidobacterium and lactobacillus strains modulates experimental myasthenia gravis and experimental encephalomyelitis in Lewis rats. *Oncotarget*. (2018) 9:22269–87. doi: 10.18632/oncotarget.25170
7. Chae C-S, Kwon H-K, Hwang J-S, Kim J-E, Im S-H. Prophylactic effect of probiotics on the development of experimental autoimmune myasthenia gravis. *PLoS ONE*. (2012) 7:e52119. doi: 10.1371/journal.pone.0052119
8. Macpherson AJ, Slack E, Geuking MB, McCoy KD. The mucosal firewalls against commensal intestinal microbes. *Semin Immunopathol*. (2009) 31:145–9. doi: 10.1007/s00281-009-0174-3
9. Bekiaris V, Persson EK, Agace WW. Intestinal dendritic cells in the regulation of mucosal immunity. *Immunol Rev*. (2014) 260:86–101. doi: 10.1111/imr.12194
10. Farache J, Koren I, Milo I, Gurevich I, Kim K-W, Zigmond E, et al. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity*. (2013) 38:581–95. doi: 10.1016/j.immuni.2013.01.009
11. Mazzini E, Massimiliano L, Penna G, Rescigno M. Oral tolerance can be established via gap junction transfer of fed antigens from $CX3CR1^{+}$ macrophages to $CD103^{+}$ dendritic cells. *Immunity*. (2014) 40:248–61. doi: 10.1016/j.immuni.2013.12.012
12. Rescigno M. Dendritic cell-epithelial cell crosstalk in the gut. *Immunol Rev*. (2014) 260:118–28. doi: 10.1111/imr.12181
13. Galdeano CM, de Moreno de LeBlanc A, Vinderola G, Bonet MEB, Perdigón G. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clin Vaccine Immunol*. (2007) 14:485–92. doi: 10.1128/CVI.00406-06
14. Chiba Y, Shida K, Nagata S, Wada M, Bian L, Wang C, et al. Well-controlled proinflammatory cytokine responses of Peyer's patch cells to probiotic *Lactobacillus casei*. *Immunology*. (2010) 130:352–62. doi: 10.1111/j.1365-2567.2009.03204.x
15. Finamore A, Roselli M, Britti MS, Merendino N, Mengheri E. *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* MB5 induce intestinal but not systemic antigen-specific hyporesponsiveness in ovalbumin-immunized rats. *J Nutr*. (2012) 142:375–81. doi: 10.3945/jn.111.148924
16. Ruiz L, Delgado S, Ruas-Madiedo P, Sánchez B, Margolles A. Bifidobacteria and their molecular communication with the immune system. *Front Microbiol*. (2017) 8:2345. doi: 10.3389/fmicb.2017.02345
17. Elliott J, Blanchard SG, Wu W, Miller J, Strader CD, Hartig P, et al. Purification of *Torpedo californica* post-synaptic membranes and fractionation of their constituent proteins. *Biochem J*. (1980) 185:667–77. doi: 10.1042/bj1850667
18. Lindstrom J, Einarson B, Tzartos S. Production and assay of antibodies to acetylcholine receptors. *Methods Enzymol*. (1981) 74:432–60. doi: 10.1016/0076-6879(81)74031-X
19. Flügel A, Berkowicz T, Ritter T, Labeur M, Jenne DE, Li Z, et al. Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity*. (2001) 14:547–60. doi: 10.1016/S1074-7613(01)00143-1
20. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. (2012) 9:676–82. doi: 10.1038/nmeth.2019
21. Tinevez J-Y, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. TrackMate: an open and extensible platform for single-particle tracking. *Methods*. (2017) 115:80–90. doi: 10.1016/j.ymeth.2016.09.016
22. Kawakami N, Nägerl UV, Odoardi F, Bonhoeffer T, Wekerle H, Flügel A. Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. *J Exp Med*. (2005) 201:1805–14. doi: 10.1084/jem.20050011
23. Cordiglieri C, Marolda R, Franzi S, Cappelletti C, Giardina C, Motta T, et al. Innate immunity in myasthenia gravis thymus: pathogenic effects of Toll-like receptor 4 signaling on autoimmunity. *J Autoimmun*. (2014) 52:74–89. doi: 10.1016/j.jaut.2013.12.013
24. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9
25. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. (2016) 13:581–3. doi: 10.1038/nmeth.3869
26. Lozupone C, Hamady M, Knight R. UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics*. (2006) 7:371. doi: 10.1186/1471-2105-7-371
27. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*. (2018) 6:90. doi: 10.1186/s40168-018-0470-z
28. McDonald D, Price MN, Goodrich J, Nawrocki EP, Desantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J*. (2012) 6:610–8. doi: 10.1038/ismej.2011.139
29. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques*. (2003) 34:374–8. doi: 10.2144/03342mt01
30. Bron PA, van Baarlen P, Kleerebezem M. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nat Rev Microbiol*. (2011) 10:66–78. doi: 10.1038/nrmicro2690
31. Hoffman MD, Zucker LI, Brown PJB, Kysela DT, Brun Y V, Jacobson SC. Timescales and frequencies of reversible and irreversible adhesion events of single bacterial cells. *Anal Chem*. (2015) 87:12032–9. doi: 10.1021/acs.analchem.5b02087
32. Chindera K, Mahato M, Sharma AK, Horsley H, Kloc-Muniak K, Kamaruzzaman NF, et al. The antimicrobial polymer PHMB enters cells and selectively condenses bacterial chromosomes. *Sci Rep*. (2016) 6:23121. doi: 10.1038/srep23121
33. Jung C, Hugot J-P, Barreau F. Peyer's patches: the immune sensors of the intestine. *Int J Inflam*. (2010) 2010:823710. doi: 10.4061/2010/823710
34. Baggi F, Annoni A, Ubiali F, Milani M, Longhi R, Scaioli W, et al. Breakdown of tolerance to a self-peptide of acetylcholine receptor alpha-subunit induces experimental myasthenia gravis in rats. *J Immunol*. (2004) 172:2697–703. doi: 10.4049/jimmunol.172.4.2697
35. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. (2011) 34:637–50. doi: 10.1016/j.immuni.2011.05.006
36. Triantafilou M, Manukyan M, Mackie A, Morath S, Hartung T, Heine H, et al. Lipoteichoic acid and toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. *J Biol Chem*. (2004) 279:40882–9. doi: 10.1074/jbc.M400466200
37. Dargahi N, Johnson J, Donkor O, Vasiljevic T, Apostolopoulos V. Immunomodulatory effects of probiotics: can they be used to treat allergies and autoimmune diseases? *Maturitas*. (2019) 119:25–38. doi: 10.1016/j.maturitas.2018.11.002
38. Sarkar A, Mandal S. Bifidobacteria-insight into clinical outcomes and mechanisms of its probiotic action. *Microbiol Res*. (2016) 192:159–71. doi: 10.1016/j.micres.2016.07.001
39. de Oliveira GLV, Leite AZ, Higuchi BS, Gonzaga MI, Mariano VS. Intestinal dysbiosis and probiotic applications in autoimmune diseases. *Immunology*. (2017) 152:1–12. doi: 10.1111/imm.12765
40. Zimmermann C, Schild M, Kunz C, Zimmermann K, Kuntz S. Effects of live and heat-inactivated *E. coli* strains and their supernatants on immune regulation in HT-29 cells. *Eur J Microbiol Immunol*. (2018) 8:41–6. doi: 10.1556/1886.2018.00004
41. Sugahara H, Yao R, Odamaki T, Xiao JZ. Differences between live and heat-killed bifidobacteria in the regulation of immune function and the intestinal environment. *Benef Microbes*. (2017) 8:463–72. doi: 10.3920/BM2016.0158
42. Rachmilewitz D, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology*. (2004) 126:520–8. doi: 10.1053/j.gastro.2003.11.019

43. Kwon H-K, Lee C-G, So J-S, Chae C-S, Hwang J-S, Sahoo A, et al. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci USA*. (2010) 107:2159–64. doi: 10.1073/pnas.0904055107
44. Dwivedi M, Kumar P, Laddha NC, Kemp EH. Induction of regulatory T cells: a role for probiotics and prebiotics to suppress autoimmunity. *Autoimmun Rev*. (2016) 15:379–92. doi: 10.1016/j.autrev.2016.01.002
45. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol*. (2001) 2:361–7. doi: 10.1038/86373
46. Ryva B, Zhang K, Asthana A, Wong D, Vicioso Y, Parameswaran R. Wheat germ agglutinin as a potential therapeutic agent for Leukemia. *Front Oncol*. (2019) 9:100. doi: 10.3389/fonc.2019.00100
47. Nagl M, Kacani L, Müllauer B, Lemberger E-M, Stoiber H, Sprinzl GM, et al. Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells. *Clin Diagn Lab Immunol*. (2002) 9:1165–8. doi: 10.1128/CDLI.9.6.1165-1168.2002
48. Uribe-Querol E, Rosales C. Control of phagocytosis by microbial pathogens. *Front Immunol*. (2017) 8:1368. doi: 10.3389/fimmu.2017.01368
49. Gómez-Llorente C, Muñoz S, Gil A. Role of toll-like receptors in the development of immunotolerance mediated by probiotics. *Proc Nutr Soc*. (2010) 69:381–9. doi: 10.1017/S0029665110001527
50. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*. (1999) 11:443–51. doi: 10.1016/S1074-7613(00)80119-3
51. Michelsen KS, Aicher A, Mohaupt M, Hartung T, Dimmeler S, Kirschning CJ, et al. The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCS). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. *J Biol Chem*. (2001) 276:25680–6. doi: 10.1074/jbc.M011615200
52. Esebanmen GE, Langridge WHR. The role of TGF-beta signaling in dendritic cell tolerance. *Immunol Res*. (2017) 65:987–94. doi: 10.1007/s12026-017-8944-9
53. Drachman DB, Okumura S, Adams RN, McIntosh KR. Oral tolerance in myasthenia gravis. *Ann N Y Acad Sci*. (1996) 778:258–72. doi: 10.1111/j.1749-6632.1996.tb21134.x
54. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep*. (2016) 6:28484. doi: 10.1038/srep28484
55. Gandy KAO, Zhang J, Nagarkatti P, Nagarkatti M. The role of gut microbiota in shaping the relapse-remitting and chronic-progressive forms of multiple sclerosis in mouse models. *Sci Rep*. (2019) 9:6923. doi: 10.1038/s41598-019-43356-7
56. Qiu D, Xia Z, Jiao X, Deng J, Zhang L, Li J. Altered gut microbiota in Myasthenia gravis. *Front Microbiol*. (2018) 9:2627. doi: 10.3389/fmicb.2018.02627
57. Haro C, Montes-Borrego M, Rangel-Zúñiga OA, Alcalá-Díaz JF, Gómez-Delgado F, Pérez-Martínez P, et al. Two healthy diets modulate gut microbial community improving insulin sensitivity in a human obese population. *J Clin Endocrinol Metab*. (2016) 101:233–42. doi: 10.1210/jc.2015-3351
58. Andoh A, Imaeda H, Aomatsu T, Inatomi O, Bamba S, Sasaki M, et al. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol*. (2011) 46:479–86. doi: 10.1007/s00535-010-0368-4
59. Walker AW, Sanderson JD, Churcher C, Parkes GC, Hudspith BN, Rayment N, et al. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. *BMC Microbiol*. (2011) 11:7. doi: 10.1186/1471-2180-11-7
60. Huang C, Chen J, Wang J, Zhou H, Lu Y, Lou L, et al. Dysbiosis of intestinal microbiota and decreased antimicrobial peptide level in paneth cells during hypertriglyceridemia-related acute necrotizing pancreatitis in rats. *Front Microbiol*. (2017) 8:776. doi: 10.3389/fmicb.2017.00776
61. Liang Y, Liang S, Zhang Y, Deng Y, He Y, Chen Y, et al. Oral administration of compound probiotics ameliorates HFD-induced gut microbe dysbiosis and chronic metabolic inflammation via the G protein-coupled receptor 43 in non-alcoholic fatty liver disease rats. *Probiotics Antimicrob Proteins*. (2019) 11:175–85. doi: 10.1007/s12602-017-9378-3
62. Stanisavljević S, Lukić J, Soković S, Mihajlovic S, Mostarica Stojković M, Miljković D, et al. Correlation of gut microbiota composition with resistance to experimental autoimmune encephalomyelitis in rats. *Front Microbiol*. (2016) 7:2005. doi: 10.3389/fmicb.2016.02005
63. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA*. (2007) 104:13780–5. doi: 10.1073/pnas.0706625104
64. Jangi S, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, et al. Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun*. (2016) 7:12015. doi: 10.1038/ncomms12015
65. Berer K, Gerdes LA, Cekanaviciute E, Jia X, Xiao L, Xia Z, et al. Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc Natl Acad Sci USA*. (2017) 114:10719–24. doi: 10.1073/pnas.1711233114
66. Kihl P, Krych L, Deng L, Kildemoes AO, Laigaard A, Hansen LH, et al. Oral LPS dosing induces local immunological changes in the pancreatic lymph nodes in mice. *J Diabetes Res*. (2019) 2019:1649279. doi: 10.1155/2019/1649279
67. Haase S, Haghikia A, Wilck N, Müller DN, Linker RA. Impacts of microbiome metabolites on immune regulation and autoimmunity. *Immunology*. (2018) 154:230–8. doi: 10.1111/imm.12933


**Conflict of Interest:** ME was employed by company AAT—Advanced Analytical Technologies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 10 January 2020
doi: 10.3389/fimmu.2019.03052

# Antagonism of the Neonatal Fc Receptor as an Emerging Treatment for Myasthenia Gravis

*Karissa L. Gable*[1] *and Jeffrey T. Guptill*[1,2]*

[1] Department of Neurology, Duke University School of Medicine, Durham, NC, United States, [2] Duke Clinical Research Institute, Durham, NC, United States

Myasthenia gravis is an autoimmune disease in which immunoglobulin G (IgG) autoantibodies are formed against the nicotinic acetylcholine receptor (AChR) or other components of the neuromuscular junction. Though effective treatments are currently available, many commonly used therapies have important limitations and alternative therapeutic options are needed for patients. A novel treatment approach currently in clinical trials for myasthenia gravis targets the neonatal Fc receptor (FcRn). This receptor plays a central role in prolonging the half–life of IgG molecules. The primary function of FcRn is salvage of IgG and albumin from lysosomal degradation through the recycling and transcytosis of IgG within cells. Antagonism of this receptor causes IgG catabolism, resulting in reduced overall IgG and pathogenic autoantibody levels. This treatment approach is particularly intriguing as it does not result in widespread immune suppression, in contrast to many therapies in routine clinical use. Experience with plasma exchange and emerging phase 2 clinical trial data of FcRn antagonists provide proof of concept for IgG lowering in myasthenia gravis. Here we review the IgG lifecycle and the relevance of IgG lowering to myasthenia gravis treatment and summarize the available data on FcRn targeted therapeutics in clinical trials for myasthenia gravis.

**Keywords: FcRn antibodies, myasthenia (myasthenia gravis–MG), novel therapeutic, IgG, autoimmune, treatment, clinical trial review**

OPEN ACCESS

***Edited by:***
*Anna Rostedt Punga, Uppsala University, Sweden*

***Reviewed by:***
*Emanuela Bartoccioni, Agostino Gemelli University Polyclinic, Italy*
*Konstantinos Lazaridis, Pasteur Hellenic Institute, Greece*

***Correspondence:**
*Jeffrey T. Guptill*
*jeffrey.guptill@duke.edu*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *07 November 2019*
***Accepted:*** *12 December 2019*
***Published:*** *10 January 2020*

***Citation:***
*Gable KL and Guptill JT (2020) Antagonism of the Neonatal Fc Receptor as an Emerging Treatment for Myasthenia Gravis. Front. Immunol. 10:3052. doi: 10.3389/fimmu.2019.03052*

Myasthenia gravis (MG) is an autoimmune disease affecting the neuromuscular junction that manifests in clinical symptoms, such as dyspnea, dysphagia, diplopia, dysarthria, ptosis, and fatigable muscle weakness. Symptoms often fluctuate in severity, are generally fatigable, and improve with rest. It is estimated that this disorder affects ∼60,000 people in the United States (1). Patients with mild disease experience ocular symptoms of diplopia and intermittent ptosis and, on the other end of the spectrum, patients with severe disease experience generalized weakness that can progress into myasthenic crisis resulting in respiratory insufficiency and need for ventilatory support.

Neuromuscular junction function involves acetylcholine release from the motor nerve, binding of acetylcholine to the acetylcholine receptor (AchR) on the post-synaptic membrane, followed by generation of muscle end plate potentials. Once the end plate potential reaches threshold an action potential is generated, resulting in normal muscle contraction. The etiology of the autoimmune process in myasthenia gravis is unclear in most cases, however, the autoantibodies generated in the disease target the nicotinic AchR or other components of the post-synaptic neuromuscular junction. Interference with downstream signaling at the post-synaptic junction reduces the ability of the end plate potential to reach the threshold needed to trigger an action potential, ultimately resulting in the clinical manifestation of fatigable or persistent muscle weakness (2).

Current pharmacologic approaches to treat MG either try to control the symptoms (e.g., cholinesterase inhibitors) or suppress or modulate the immune system. Corticosteroids, steroid–sparing agents, therapeutic plasma exchange (TPE), and immunoglobulin infusions (IVIg) are currently the most common treatment modalities. However, each of these treatments can be associated with various side effects. Corticosteroid treatment, especially at high doses over the long term, is associated with a myriad of potential complications, such as steroid-induced diabetes, bone density loss, accelerated cataract formation, gastrointestinal ulcer formation, hypertension, and peripheral edema. Commonly used steroid-sparing agents in the United States, such as azathioprine or mycophenolate mofetil, suppress the immune system and increase risk of infection as well as slightly increase the incidence of certain cancers, such as squamous cell cancer and lymphoma, respectively.

IVIg infusions, TPE, and in some countries, immunoadsorption are used in the setting of myasthenic exacerbations and crisis. Immunoglobulin infusions do not widely suppress the immune system but rather modulate the autoimmune response to minimize the effect of the autoantibodies directed against the post-synaptic receptors. IVIg is associated with rare, but severe adverse events, such as thrombosis, aseptic meningitis, and allergic reactions. In fact, it has been reported that though adverse systemic reactions are rare with subcutaneous IG infusions, they are relatively common with IVIg infusions, occurring in 20–50% of patients and 5–15% of all IVIg infusions (3). There can also be supply issues given that IVIg is a blood product and requires healthy donors. In many cases it is also not a viable long term treatment given relatively common issues with intravenous access needed for the infusions or the need for a long-term indwelling line; subcutaneous administration may help alleviate those issues.

TPE effectively lowers circulating IgG blood levels, including pathogenic autoantibodies, and provides proof of concept for the clinical effectiveness of lowering IgG levels to treat MG (4, 5). One plasma volume exchange reduces serum immunoglobulin levels by 60% and total body immunoglobulin stores by 20% (6). Five sessions of TPE can lower IgG and autoantibody levels by ∼75–80%. Recovery of IgG levels close to a baseline level occurs ∼6 weeks after a course of TPE (5). TPE has limitations as a maintenance treatment given intravenous access limitations and many centers perform the procedure using a central line. Also, there is also the potential for intolerance due to allergic reactions to the solutions used in the exchange procedure, blood pressure fluctuations or hematologic side effects. TPE removes coagulation factors and complement proteins, requires colloid replacement, most often with albumin replacement, and requires special equipment and expertise (7). Due to the depletion of coagulation factors, treatment is often performed every other day to allow for natural recovery of these clotting factors. On occasion, during the course of TPE treatment, infusion with fresh frozen plasma is required to replace these clotting factors if they are significantly depleted. The required spacing of TPE adds to the time consuming nature of this type of treatment. Given the limitations and risks of the currently available treatments of myasthenia gravis, there is need for treatments that could provide benefits similar to TPE or IVIg and that do not suppress the immune system.

## THE NEONATAL FC RECEPTOR AS A THERAPEUTIC TARGET

A novel potential treatment approach is targeting the neonatal Fc Receptor (FcRn). FcRn mediated IgG recycling accounts for passive short-term humoral immunity that is provided *in utero* from mother to offspring (8). In adults, FcRn is expressed in muscle, skin, and vascular endothelium and is critically important to the life cycle of IgG (9–12). In summary, the normal mechanism of directional transport and recycling involves IgG binding to FcRn on the surface of an endothelial cell. This is followed by passive pinocytosis of IgG bound to FcRn into the cell via an acidified endosome. Unbound protein is relegated to lysosomal degradation, whereas IgG bound to FcRn is transcytosed and released back into the serum at physiologic pH. IgG and albumin make up 90% of the serum protein content and the FcRn-mediated recycling process extends the serum half-life of both proteins and is responsible for the 21 days half-life of IgG (**Figure 1A**) (8, 13, 14). It has been estimated that the FcRn-mediated IgG recycling rate is 42% greater than the rate of IgG production, indicating that recycling of IgG, not its production, is the dominant process for maintaining IgG plasma concentrations in humans (14). Thus, FcRn serves a vital function in maintaining serum IgG levels. Other immunoglobulins, such as IgM, are not involved in FcRn mediated recycling. Inhibiting FcRn recycling is overall expected to be a promising therapeutic target for lowering all IgG subclasses, including IgG4, which has unique properties, such as the ability of IgG half-molecules to recombine randomly with other half-molecules via Fab arm exchange (15).

If binding of IgG to FcRn is inhibited, the expected effect is enhanced IgG catabolism and a reduction in serum IgG concentrations, an effect similar to TPE (**Figure 1B**). This mechanism of action is potentially quite promising, as therapeutics targeting FcRn inhibition could provide a rapid and selective IgG lowering effect in a much less cumbersome method as compared to TPE. Thus, FcRn inhibitors could potentially be thought of as potential treatments for myasthenic crisis or as maintenance therapy.

Myasthenia gravis is an autoantibody-mediated disease with a favorable response to TPE treatment, so it is a prime disease for testing whether FcRn targeted treatments would be beneficial for antibody-mediated disease patient populations. Preclinical studies in the experimental animal model of MG support this treatment approach. A high affinity, pH-independent rat anti-FcRN inhibitor enhanced the clearance of pathogenic AChR antibodies and demonstrated a dose-dependent improvement in disease symptoms in both passive and active models of induced autoimmune MG. Therapeutic potential for FcRn agents was also demonstrated in a mouse model for muscle-specific kinase (MuSK) myasthenia gravis (16, 17). In addition to MG, this target for drug development is also being explored to expand treatment options for other autoimmune diseases, such

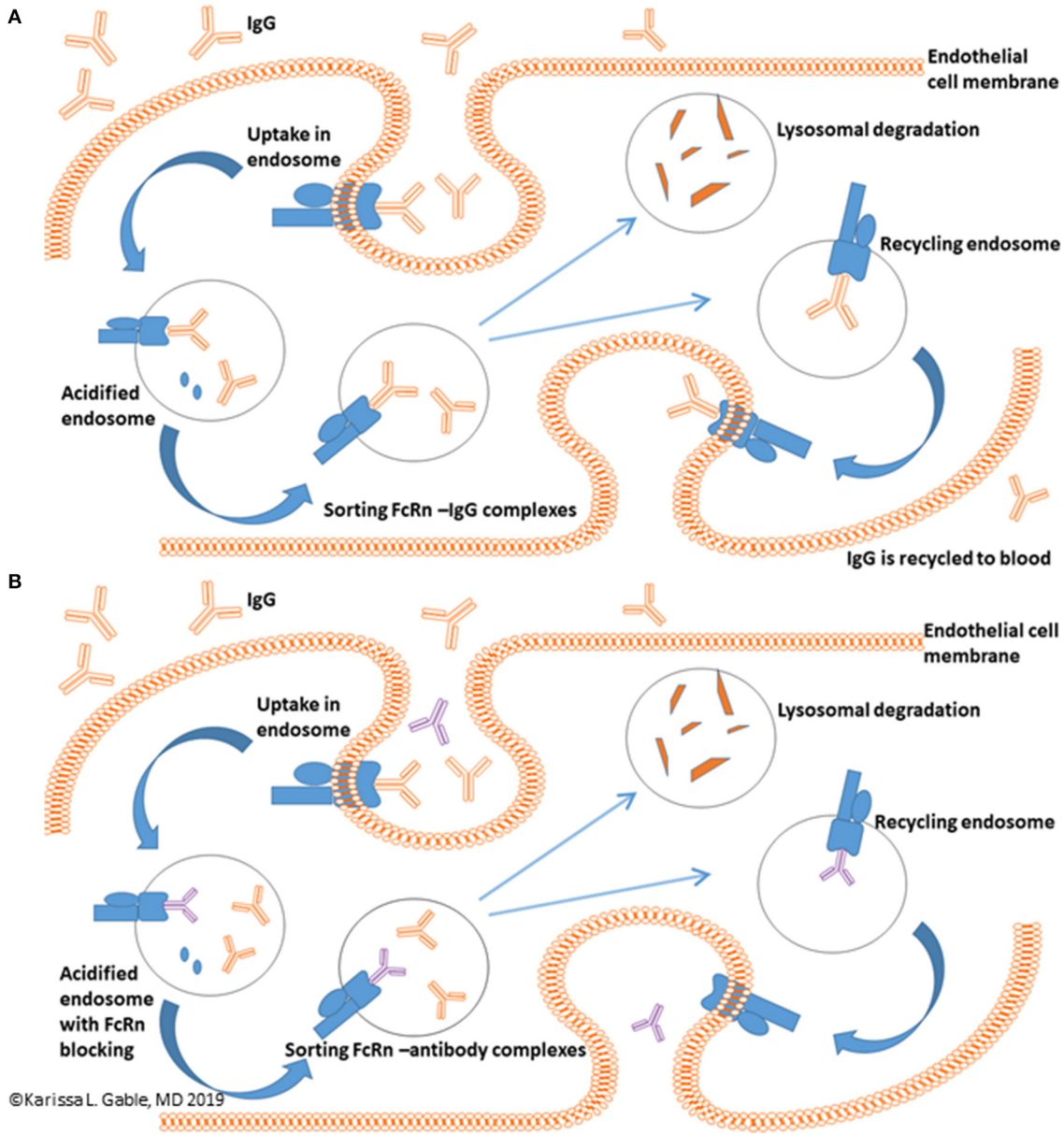



**FIGURE 1 |** FcRn mediated recycling of IgG. **(A)** IgG recycling begins with IgG binding to the FcRn with IgG uptake into an endothelial cell via an acidified endosome with ultimate release of IgG back into the blood. **(B)** Binding of anti-FcRn therapeutic to the FcRn receptor leaves unbound IgG in the endosome which undergoes lysosomal degradation and reduces circulating IgG levels. Blue receptor, FcRn protein; Blue oval, albumin; Orange, IgG; Purple, anti-FcRn therapeutic.

as chronic inflammatory demyelinating polyneuropathy and idiopathic thrombocytopenic purpura.

## SAFETY CONSIDERATIONS FOR FcRn TARGETED THERAPEUTICS

Therapeutics targeting FcRn in clinical development for MG are human monoclonal antibodies or Fc fragments (**Table 1**). Given the specificity associated with these therapeutics, limited off-target effects are expected or have been observed in trials completed to date. In comparison to TPE, which removes many serum proteins, anti-FcRn therapeutics are expected to confer significant benefits in terms of fewer off-target effects. The current primary safety considerations with anti-FcRn therapies focus on the role of FcRn binding of albumin and the potential clinical implications of a reduction in serum albumin levels. Modest post-treatment reductions in albumin have been observed in preclinical studies and early phase studies in humans. However, to date there have been no demonstrable adverse clinical effects observed in the human clinical trials. In addition, severe depletion of IgG could theoretically increase

**TABLE 1 |** Overview of FcRn targeted therapeutics in development for MG including Phase 1 trial results.

| Therapeutic Name | Company | Molecule Summary | SAD/MAD Dosing* | Maximum % IgG lowering from baseline in phase 1 |
|---|---|---|---|---|
| Efgartigimod (ARGX-113) | Argenx BVBA | Humanized IgG1 Fc fragment | **SAD**<br>IV: 0.2, 2, 10, 25, 50 mg/kg<br>**MAD**<br>**IV: 10,** 25 **mg/kg** every 4 or 7 days | 10, 25 mg/kg IV: 75% |
| Nipocalimab (M281) | Momenta | Human deglycosylated IgG1 monoclonal Ab | **SAD**<br>**IV:** 0.3, 3, 10, 30, **60 mg/kg**<br>**MAD**<br>**IV:** 15, **30 mg/kg** weekly† | 15, 30 mg/kg IV: 85% |
| Rozanolixizumab (UCB7665) | UCB Biopharma | Humanized IgG4 monoclonal Ab | **SAD**<br>IV: 1, 4, 7 mg/kg **SC:** 1, **4, 7 mg/kg**<br>**MAD**<br>NA, SAD only | 4 mg/kg SC: 26%<br>7 mg/kg SC: 43% |
| RVT-1401(HL161) | Immunovant Sciences GmbH | Human IgG1 monoclonal Ab | **SAD**<br>SC: 0.05, 1.5, 5 mg/kg, 340, 500, 765 mg<br>IV: 1.5 mg/kg, 100 mg, 340, 765, 1,530 mg<br>**MAD**<br>**SC: 340, 680 mg** weekly | 340 mg SC: 63%<br>680 mg SC: 78% |

*Ab, antibody; AChR, acetylcholine receptor; Ig, immunoglobulin; IV, intravenous; kg, kilogram; MAD, multiple ascending dose; mg, milligram; NA, not applicable; SAD, single ascending dose; SC, subcutaneous.*

**Bolded doses used in MG phase 2 studies.*

*†A 5 mg/kg dose was also tested in phase 2.*

the risk of infection. However, IgA, IgD, IgE, and IgM are not dependent on FcRn-mediated recycling and preliminary studies have not demonstrated any effect on the frequencies of immune cells (e.g., T, B cells, NK cells), complement, or peripheral cytokines (18). Furthermore, initiation of a primary immune response through IgM and IgG is expected to proceed intact (19). It is important to note that the long term effects of severe IgG depletion, particularly in the setting of additional immunosuppressive therapies as would be expected in many MG patients, remain uncertain and require additional study.

## FcRn THERAPEUTICS IN DEVELOPMENT FOR MG

The remainder of the review focuses on four therapeutics currently in clinical testing for MG. One is a Fc fragment and three are anti-FcRn monoclonal antibodies. **Table 1** summarizes each therapeutic and includes dosing and pharmacodynamic (PD) information from the phase 1 clinical trials that were critical to inform the subsequent clinical trials in MG patients. Pharmacokinetic (PK) parameters for each therapeutic may be found in the phase 1 and phase 2 manuscripts cited below. Other FcRn targeted strategies, such as recombinant Fc multimers and FcγR targeted therapeutics, and additional FcRn monoclonal antibodies are in development but have not yet entered clinical testing for MG (20, 21).

## EFGARTIGIMOD

This molecule is a modified human anti-IgG1 derived Fc fragment engineered to increase Fc/FcRn binding at neutral and acidic pH. Flow cytometry and microscopic data indicate a high affinity and avidity of efgartigimod for FcRn as evidenced by greater retention of efgartigimod in FcRn-positive compartments within cells, combined with increased lysosomal accumulation. Cynomolgus monkey studies demonstrated a maximal 75% reduction in IgG following multiple dosing up to 20 mg/kg and no significant safety concerns.

### Phase 1 Study

The safety, PK, and PD of efgartigimod was evaluated in a placebo-controlled single- and multiple ascending dose (SAD/MAD) study in 62 healthy adult volunteers (44 received efgartigimod) (21). In the SAD study, doses of 0.2–50 mg/kg administered as 2 h intravenous infusions were explored in 5 cohorts of healthy volunteers (**Table 1**). During the MAD portion of the study, 10 and 25 mg/kg doses administered every 4 or 7 days were studied. No dose-limiting toxicity was observed. Headache occurred in the highest dose of the SAD, was predominantly mild, and resolved with minimal interventions. There were no serious adverse events (SAEs) related to efgartigimod. A single dose reduced total IgGs about 50%, while repeated dosing lowered IgG levels by ∼75% (21). The maximum IgG lowering effect was seen beginning with the 10 mg/kg dose and this dose was selected for further clinical development. There was no effect on other immunoglobulins or

albumin at any of the studied doses. There was no significant anti-drug antibody production in the phase 1 study.

## NIPOCALIMAB

This molecule is a human deglycosylated IgG1 anti-FcRn monoclonal antibody with no effector function. Nipocalimab binds with picomolar affinity to FcRn at both endosomal pH 6.0 and extracellular pH 7.6 allowing occupancy of FcRn throughout the recycling pathway and has a specificity designed to minimize off target effects (22). It is not expected to cross the placenta and a clinical trial is underway in pregnant women at high risk for early onset severe hemolytic disease of the fetus and newborn (23, 24). Phase 1 data supports infusion rates of 7.5 or 15 min for 30 and 60 mg/kg doses, respectively (25).

### Phase 1 Study

The phase 1 placebo-controlled study in healthy adult volunteers consisted of both SAD and MAD components and 50 subjects were enrolled (36 nipocalimab) (**Table 1**) (22). Doses of 0.3–60 mg/kg over a 2-h infusion were studied in the SAD cohorts, and the MAD cohorts included 4 weekly doses of 15 or 30 mg/kg. Greater than 90% FcRn receptor occupancy was achieved with ≥3 mg/kg doses within 2 h of dosing. Following single doses, maximum IgG reductions of 74–80% were observed with 30 or 60 mg/kg doses, and a ≥50% reduction in IgG levels were maintained for 18 and 27 days for the 30 or 60 mg/kg doses, respectively. During multiple doses, IgG levels were reduced ∼85% below baseline by day 14. Treatment emergent AEs were similar in both the nipocalimab and placebo groups and most were mild or moderate. There were no severe or serious treatment emergent AEs, and there was no increase in the incidence of infections. Three subjects in the 15 mg/kg MAD experienced transient elevations in creatine phosphokinase and one of these cases was clearly related to exercise. Mild, transient reductions in total protein and albumin were observed in the highest SAD dose and in the MAD doses. The overall frequency of headache was similar between the nipocalimab and placebo groups in the trial.

## ROZANOLIXIZUMAB

This molecule is a high affinity human anti-FcRn IgG4 monoclonal antibody. Rozanolixizumab dosing in animals demonstrated marked decreases in plasma IgG concentrations (75–90% from baseline) at 50 and 150 mg/kg doses with maximal effects achieved by about day 10. Rozanolixizumab does not strongly block albumin binding to FcRn, and small not clinically significant albumin decreases were observed in animals, possibly related to steric hindrance by antibody bound to FcRn. There was no increase in infection rates, no effects on plasma concentrations of acute-phase proteins, no changes in IgM and IgA serum concentrations and immunophenotyping did not show a significant treatment effect on absolute lymphocyte count or lymphocyte subsets (18).

### Phase 1 Study

Rozanolixizumab was studied in a placebo-controlled phase 1 trial in 49 healthy volunteer subjects (36 rozanolixizumab) administered as a single 1 h intravenous or subcutaneous infusion at doses of 1, 4, or 7 mg/kg (**Table 1**) (18). The intravenous formulation demonstrated a dose-dependent increase in headaches and back pain, including 4 severe treatment emergent AEs, compared to the subcutaneous formulation. Dose-dependent and treatment-related vomiting, nausea, and pyrexia were also seen more frequently in the intravenous formulation compared to placebo, and were less frequent with the subcutaneous formulation. As a result of these findings, subsequent clinical development has focused on the better tolerated subcutaneous formulation. The mean maximum IgG reduction following single doses of rozanolixizumab occurred at day 10 and was ∼48% for the highest intravenous dose and 43% for the subcutaneous formulation.

## RVT-1401

### Phase 1 Study

Publically available preclinical and clinical trial data for RVT-1401 are more limited. RVT-1401 is a fully human monoclonal antibody formulated for intravenous or subcutaneous injection. A phase 1 SAD/MAD study in healthy volunteers has been completed (**Table 1**) (26). The SAD portion of the study included weight-based and fixed intravenous and subcutaneous doses (fixed doses 100–765 mg), while the MAD cohorts included administration of weekly subcutaneous doses of 340 or 680 mg RVT-1401 or placebo for 4 weeks. IgG levels were reduced by 47% after single doses of 765 mg, with the nadir being reached 8–10 days after dosing. Weekly subcutaneous dosing with 680 mg reduced total IgG levels by 78%. IgG reductions ≥35% were maintained for more than 1 month after the last dose. Reversible dose dependent reductions in albumin were observed (31% with 680 mg subcutaneous dosing) and were asymptomatic. Single and multiple doses of RVT-1401 were well-tolerated with no subjects terminating the study early due to AEs. The most common AEs in the phase 1 study were injection site erythema and swelling. No subjects in the MAD cohorts developed anti-drug antibodies.

## CLINICAL TRIAL RESULTS IN MG

The profound and rapid reductions in IgG concentrations and favorable PK and safety observed in the preclinical studies and healthy volunteer phase 1 studies supported further investigation anti-FcRn therapeutics in clinical trials in patients with MG. Of note, the rapid PD effects of the FcRn therapeutics confer the potential advantage of shorter duration clinical trials to demonstrate clinically meaningful results. Two therapeutics, efgartigimod and rozanolixizumab, have completed phase 2 clinical trials in patients with generalized MG and have active phase 3 programs. Phase 2 clinical trials for Nipocalimab and RVT-1401 are active. In summary, the phase 2 and phase 3 clinical trials studied adult patients with generalized MG. All trials included AChR antibody positive patients, while MuSK antibody positive patients were eligible for the nipocalimab and

**TABLE 2 |** Summary of completed/active studies of anti-FcRn monoclonal antibodies for MG.

| Therapeutic name | Phase | Study design | Study population | Sample size | Dosing | Efficacy outcome measures and endpoints | Safety summary | Results summary** |
|---|---|---|---|---|---|---|---|---|
| Efgartigimod (ARGX-113) | 2 | 2-arm parallel RCT (1:1) | AChR-Ab | 24 | 10 mg/kg efgartigimod or PBO weekly × 4 IV | Δ baseline to week 11:<br>MGADL<br>QMG<br>MG-Composite<br>MG-QoL15r | No SAE<br>No severe TEAE<br>One withdrawal due to lack of efficacy | **Efficacy week 11**<br>MGADL: −3.5<br>QMG: −4.8<br>MG-Composite: −7.1<br>**Max PD**<br>IgG: −71%<br>AChR-Ab: −40–70% |
| Efgartigimod* (ARGX-113) | 3 | 2-arm parallel RCT (1:1) | AChR-Ab<br>AChR-ab (includes MuSK-Ab, LRP4-Ab, Seronegative) † | 150 | 10 mg/kg efgartigimod or PBO, dose adjustments allowed according to patient symptoms IV | Δ baseline to week 8:<br>Primary:<br>% MGADL responders in AChR-Ab pts<br>Secondary:<br>% QMG responders (AChR-Ab pts)<br>% MGADL (all pts) | NA | NA |
| Nipocalimab* (M281) | 2 | 5-arm parallel RCT (1:1:1:1:1) | AChR-Ab<br>MuSK-Ab | 60 | 5 mg/kg M281 Q4 weeks, 30 mg/kg M281 Q4 weeks, 60 mg/kg M281 x1, 60 mg/kg M281Q2 week or PBO Q2 weeks × 4 IV | Δ baseline to week 8:<br>Primary:<br>MGADL<br>Secondary:<br>QMG<br>MG-QoL15r | NA | NA |
| Rozanolixizumab (UCB7665) | 2 | Randomized controlled 2-period crossover study (1:1)‡ | AChR-Ab<br>MuSK-Ab | 43 | Period 1:<br>7 mg/kg rozanolixizumab or PBO weekly × 3<br>Period 2:<br>4 or 7 mg/kg rozanolixizumab weekly × 3 SC | Δ baseline to week 4:<br>QMG<br>MGADL<br>MG-Composite | 43.5% increase in TEAE of HA in active group; 3 active treatment subjects withdrew due to HA per protocol | **Efficacy week 4**<br>QMG: −1.8<br>MGADL: −1.8<br>MG-Composite: −3.1<br>**Max PD**<br>IgG Period 1: −56%<br>IgG Period 2: −68%<br>AChR-Ab Period 2: −68% |
| Rozanolixizumab* (UCB7665) | 3 | 3-arm parallel RCT (1:1:1) | AChR-Ab<br>MuSK-Ab | 240 | 7 mg/kg rozanolixizumab or PBO weekly × 3 SC | Δ baseline to week 6:<br>Primary:<br>MGADL<br>Secondary:<br>MG-composite<br>QMG<br>PRO fatigability | NA | NA |
| RVT-1401* | 2 | 3-arm parallel RCT (1:1:1) | AChR-Ab | 21 | 340 or 680 mg RVT-1401 or PBO Q2 weeks × 4 SC | Δ baseline to week 7:<br>MGADL<br>QMG<br>MG-Composite<br>MG-QoL15r | NA | NA |

*Ab, antibody; AChR, acetylcholine receptor; ADL, activities of daily living; HA, headache; Ig, immunoglobulin; IV, intravenous; LRP4, low density lipoprotein receptor-related protein 4; MuSK, muscle-specific tyrosine kinase; NA, not available; PBO, placebo; PRO, patient-reported outcome; QMG, quantitative MG score; PD, pharmacodynamics QoL, quality of life; RCT, randomized controlled trial; SAE, serious adverse event; SC, subcutaneous; TEAE, treatment emergent adverse event.*

**Study ongoing.*

***Efficacy results should be interpreted with caution due to the different study designs and timing of primary efficacy endpoints assessments in the phase 2 trials.*

*† Limited to 30 patients.*

*‡ After period 1, subjects were re-randomized to 7 or 4 mg/kg rozanolixizumab.*

rozanolixizumab phase 2 trials. All these trials excluded patients with seronegative MG, with the exception of the efgartigimod phase 3 trial which included a limited number AChR- and MuSK antibody negative MG patients. Primary efficacy outcome measures included the MG-ADL and QMG score (**Table 2**). Several of the anti-FcRn clinical trial programs in MG include open-label extension studies.

# EFGARTIGIMOD CLINICAL DEVELOPMENT

## Phase 2 Study

The first data to directly support anti-FcRn therapy in human MG came through the phase 2 randomized, double-blind, placebo-controlled, multi-center clinical trial investigating

efgartigimod in patients with AChR antibody positive generalized MG (27). Twenty-four patients were randomized 1:1 to placebo or 10 mg/kg efgartigimod intravenous infusions administered over 2 h on days 1, 8, 15, and 22. Patients were followed for 8 weeks after the last infusion.

Key eligibility criteria included a MG-ADL of at least 5 and a MGFA-severity class of II–IVa and stable immunosuppressive treatments. A history of malignancy and thymectomy within 3 months of screening were exclusionary. The primary endpoint was safety and secondary endpoints included change from baseline in validated MG clinical outcome measures, PK, and PD markers (**Table 2**).

Overall, efgartigimod was well-tolerated with no SAEs or treatment emergent AEs that led to discontinuation. No safety signals were identified. One patient with concomitant immunosuppressive drugs experienced a moderate AE of shingles. Maximum IgG level reductions were ∼70% and AChR antibodies were reduced to a similar extent. AChR antibody levels returned to normal within 8 weeks of the last dose. Coinciding with maximal IgG lowering, improvements were observed in multiple MG outcome measures within 1–2 weeks of the last dose. The maximum change from baseline in MG outcome measures were: QMG −5.7 points, MG-ADL −4.4, MG-Composite −9.4, and MG-QOL15r −6.0. Reductions in the QMG and MG-ADL were statistically significant at day 8 (QMG) and days 29 and 36 (MG-ADL) (25). Efficacy endpoint reductions at the end of the study are shown in **Table 2**. In addition, 75% of patients who received efgartigimod had a ≥2 point improvement in the MG-ADL score for ≥6 weeks, whereas only 25% of placebo patients experienced a similar effect (28).

Given these promising results, a phase 3 randomized, double-blind, placebo-controlled, multi-center clinical trial is currently ongoing with a primary endpoint assessing the percentage of MG-ADL responders at 8 weeks among AChR antibody positive patients (29).

## NIPOCALIMAB CLINICAL DEVELOPMENT

### Phase 2 Study

In the phase 2, randomized, double-blind, placebo-controlled study, 60 patients with generalized AChR- or MuSK antibody positive MG are planned to be enrolled in 4 active and 1 placebo treatment arms (**Table 2**) (30). Each participant will receive a total of 5 study intravenous infusions administered every 2 weeks. The primary endpoints are safety and the change from baseline in the MG-ADL score at day 57. Secondary outcome measures include the change from baseline in the QMG and MG-QOL15r scores, as well as the change in serum IgG levels. The active treatment arms will evaluate multiple dosing regimens thereby providing PD data in patients that should help optimize dosing for this therapeutic in patients.

## ROZANOLIXIZUMAB CLINICAL DEVELOPMENT

### Phase 2 Study

UCB Pharma has completed a phase 2, randomized, double-blind, placebo-controlled, clinical trial in patients with generalized MG. Forty-three patients were randomized to three once per week subcutaneous infusions of placebo or 7 mg/Kg rozanolixizumab on days 1, 8, and 15 (Period 1). Patients were followed for 4 weeks after the last infusion and then were re-randomized to 3 doses of either 4 or 7 mg/kg rozanolixizumab (Period 2). Standard of care MG treatments were kept stable during the study.

Key eligibility criteria included adult patients with AChR- and MuSK antibody positive generalized MG patients who could be considered for IVIg or PLEX treatment in the opinion of the investigator and who had a QMG score of at least 11 (31).

Similar to the efgartigimod phase 2 trial, the primary outcome was safety. Of note, 57% of patients treated with rozanolixizumab experienced headache and three patients were withdrawn from the study due to headache (32). There was no difference in the rate of infections between the active and placebo treatment groups. At the end of Period 1, there was a statistically significant, but marginally clinically significant, improvement in the change from baseline MG-ADL score in the rozanolixizumab group. The MG-ADL responder rate, defined as a reduction of three or more points from baseline, more robustly favored rozanolixizumab (47.6 vs. 13.6% for placebo). Other MG outcome measures were not significant. During Period 2 further improvements were observed in the rozanolixizumab group, where the high dose group experienced improvements of −5.1, −8.5, and −3.9 points on the QMG, MG-Composite, and MG-ADL scores, respectively. The phase 2 trial demonstrated a 68% decrease in serum IgG and AChR autoantibodies at the end of Period 2 (33).

A 240 patient, phase 3, parallel design, randomized, double-blind, placebo-controlled, multi-center clinical study of rozanolixizumab is currently ongoing (34). Patients will be randomized to one of two rozanolixizumab doses or placebo. The primary endpoint is the MG-ADL score change from baseline at day 43 among AChR antibody positive patients (**Table 2**).

## RVT-1401 CLINICAL DEVELOPMENT

### Phase 2 Study

The phase 2 parallel group clinical trial will evaluate the safety and PD effects of subcutaneous RVT-1401 in 21 adult AChR antibody MG patients (35). The two active drug arms and a placebo arm will treat patients for 6 weeks. Patients must have a QMG score ≥12 prior to randomization. Efficacy endpoints include the MG-ADL, QMG, and MG-Composite (**Table 2**).

## CLINICAL DEVELOPMENT SUMMARY

The available phase 1 and phase 2 clinical trial data for anti-FcRn monoclonal antibodies consistently demonstrate the ability to reduce and maintain total IgG and/or AChR autoantibodies at levels associated with efficacy for PLEX (5, 36). In general, all of the available therapeutics reduce IgG levels by 60–80% from baseline at the doses studied, with modest effects on albumin. As expected, all of the therapeutics show a selective effect on IgG with no significant changes in IgA, IgD, IgE, and IgM.

At the moment, differences in therapeutic administration, such as the route and infusion duration, and side effects are the primary clinical features distinguishing these therapeutics in

the early phase clinical trials. All were generally well-tolerated in phase 1 studies with headaches from rozanolixizumab being the most potentially limiting AE identified to date. In the short term studies to date, no serious infections were observed. The ongoing phase 2 and phase 3 programs will undoubtedly add critical information to our understanding of these therapies and their distinguishing features.

## DISCUSSION AND CONCLUSIONS

Preliminary results of the completed rozanolixizumab and efgartigimod phase 2 trials suggest proof of concept for IgG lowering strategies to treat MG. The ongoing phase 2 and phase 3 trials will provide additional needed efficacy and safety data, though the long term safety profile of >70% reductions in total plasma IgG levels will not be available in the near term. Of particular interest is the risk of hypogammaglobulinemia associated infections in patients with MG who are typically receiving chronic immunosuppressive agents. Determination of dosing and the degree of IgG lowering needed for chronic therapy are essential and the nipocalimab phase 2 program, which has four active dosing arms, should provide important data in that regard. Immunogenicity is also uncertain, but preliminary results suggest low immunogenic potential for all of the therapeutics in development for MG.

If the efficacy and safety of anti-FcRn therapeutics are confirmed in pivotal trials, this class of therapy may be able to address limitations of the existing rapidly acting treatments, plasma derived immunoglobulins and TPE, which include limited supply/availability (IVIg), prolonged treatment durations (TPE/IVIg), large infusion volumes (IVIg), and adverse effects. In addition, clinicians will be very interested in comparative trial data for FcRn targeted therapeutics and TPE/IVIg for the inpatient treatment of MG, which is the setting where IgG lowering is presently used most commonly and which has not been studied to date. Multiple ongoing clinical trials with FcRn antibodies and complement therapeutics in MG patients have predictably established that there are a limited number of patients available for traditional clinical trials (37). How this competition for clinical sites and eligible patients will play out in future clinical trials remains to be seen. When viewed from the broader context of emerging therapeutics for MG, targeted combination therapies with distinct and complementary mechanisms, such as FcRn targeted therapies in combination with complement therapeutics, should be studied to determine whether they provide additional efficacy with favorable safety over existing regimens.

## AUTHOR CONTRIBUTIONS

JG and KG wrote and revised the manuscript.

## REFERENCES


1. Sanders DB, Guptill JT. Myasthenia gravis and Lambert-Eaton myasthenic syndrome. *Continuum (Minneap Minn).* (2014) 20:1413–25. doi: 10.1212/01.CON.0000455873.30438.9b
2. Sanders DB. Clinical neurophysiology of disorders of the neuromuscular junction. *J Clin Neurophysiol.* (1993)10:167–80. doi: 10.1097/00004691-199304000-00004
3. Stiehm ER. Adverse effects of human immunoglobulin therapy. *Transf Med Rev.* (2013) 27:171–8. doi: 10.1016/j.tmrv.2013.05.004
4. Barth D, Nabavi Nouri M, Ng E, Nwe P, Bril V. Comparison of IVIg and PLEX in patients with myasthenia gravis. *Neurology.* (2011) 76:2017–23. doi: 10.1212/WNL.0b013e31821e5505
5. Guptill JT, Juel VC, Massey JM, Anderson AC, Chopra M, Yi JS, et al. Effect of therapeutic plasma exchange on immunoglobulins in myasthenia gravis. *Autoimmunity.* (2016) 49:472–9. doi: 10.1080/08916934.2016.1214823
6. Kaplan AA. Therapeutic plasma exchange: core curriculum 2008. *Am J Kidney Dis.* (2008) 52:1180–96. doi: 10.1053/j.ajkd.2008.02.360
7. Kaplan AA. Therapeutic plasma exchange: a technical and operational review. *J Clin Apheresis.* (2013) 28:3–10. doi: 10.1002/jca.21257
8. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol.* (2007) 7:715–25. doi: 10.1038/nri2155
9. Borvak J, Richardson J, Medesan C, Antohe F, Radu C, Simionescu M, et al. Functional expression of the MHC class I-related receptor, FcRn, in endothelial cells of mice. *Int Immunol.* (1998) 10:1289–98. doi: 10.1093/intimm/10.9.1289
10. Ghetie V, Hubbard JG, Kim JK, Tsen MF, Lee Y, Ward ES. Abnormally short serum half-lives of IgG in beta 2-microglobulin-deficient mice. *Eur J Immunol.* (1996) 26:690–6. doi: 10.1002/eji.1830260327
11. Campbell RM, Cuthbertson DP, Matthews CM, McFarlane AS. Behaviour of 14C- and 131I-labelled plasma proteins in the rat. *Int J Appl Radiat Isot.* (1956) 1:66–84. doi: 10.1016/0020-708X(56)90020-5
12. Mc FA. The behavior of I 131-labeled plasma proteins *in vivo*. *Ann N Y Acad Sci.* (1957) 70:19–25. doi: 10.1111/j.1749-6632.1957.tb35374.x
13. Cohen S. Metabolic heterogeneity of human gamma-globulin. *Biochem J.* (1960) 76:475. doi: 10.1042/bj0760475
14. Xiao JJ. Pharmacokinetic models for FcRn-mediated IgG disposition. *J Biomed Biotechnol.* (2012) 2012:282989. doi: 10.1155/2012/282989
15. Vidarsoon G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol.* (2014) 5:520. doi: 10.3389/fimmu.2014.00520
16. Liu L, Garcia AM, Santoro H, Zhang Y, McDonnell K, Dumont J, et al. Ameloriation of experimental autoimmune myasthenia gravis in rats by neonatal FcR blockade. *J Immunol.* (2007) 178:5390–8. doi: 10.4049/jimmunol.178.8.5390
17. Huijbers MG, Plomp JJ, van Es IE, Fillie-Grijpma YE, Kamar-Al Majidi S, Ulrichts P, et al. Efgartigimod improves muscle weakness in a mouse model for muscle-specific kinase myasthenia gravis. *Exp Neurol.* (2019) 317:133–43. doi: 10.1016/j.expneurol.2019.03.001
18. Kiessling P, Lledo-Garcia R, Watanabe S, Langdon G, Tran D, Bari M, et al. The FcRn inhibitor rozanolixizumab reduces human serum IgG concentration: a randomized phase 1 study. *Sci Transl Med.* (2017) 9:eaan1208. doi: 10.1126/scitranslmed.aan1208
19. Nixon AE, Chen J, Sexton DJ, Muruganandam A, Bitonit AJ, Dumont J, et al. Fully human monoclonal antibody inhibitors of the neonatal Fc Receptor reduce circulating IgG in non-human primates. *Front Immunol.* (2015) 6:176. doi: 10.3389/fimmu.2015.00176
20. Zuercher AW, Spirig R, Baz Morelli A, Rowe T, Kasermann F. Next-generation Fc receptor-targeting biologics for autoimmune diseases. *Autoimmun Rev.* (2019) 18:102366. doi: 10.1016/j.autrev.2019.102366
21. Ulrichts P, Guglietta A, Dreier T, van Bragt T, Hanssens V, Hofman E, et al. Neonatal Fc receptor antagonist efgartigimod safely and sustainably reduces IgGs in humans. *J Clin Invest.* (2018) 128:4372–86. doi: 10.1172/JCI97911
22. Ling LE, Hillson JL, Tiessen RG, Bosje T, van Iersel MP, Nix DJ, et al. M281, an anti-FcRn antibody: pharmacodynamics, pharmacokinetics, and safety across

the full range of IgG reduction in a first-in-human study. *Clin Pharmacol Ther.* (2019) 105:1031–9. doi: 10.1002/cpt.1276
23. Roy S, Nanovskaya T, Patrikeeva S, Cochran E, Parge V, Guess J, et al. M281, an anti-FcRn antibody, inhibits IgG transfer in a human *ex vivo* placental perfusion model. *Am J Obstet Gynecol.* (2019) 220:498 e1–9. doi: 10.1016/j.ajog.2019.02.058
24. Momenta Pharmaceuticals, Inc. *A Study to Evaluate the Safety, Efficacy, Pharmacokinetics and Pharmacodynamics of M281 Administered to Pregnant Women at High Risk for Early Onset Severe Hemolytic Disease of the Fetus and Newborn (HDFN)*. (2019). Available online at: https://clinicaltrials.gov/ct2/show/NCT03842189?term=M281&rank=2
25. Momenta. *Momenta Pharmaceuticals Reports Second Quarter 2019 Financial and Operating Results*. (2019). Available online at: http://ir.momentapharma.com/node/20341/pdf2019
26. Collins J, Jones L, Snyder M, Sicard E, Griffin P, Webster L, et al. RVT-1401, A novel anti-FcRn monoclonal antibody, is well tolerated in healthy subjects and reduces plasma IgG following subcutaneous or intravenous administration. In: *2019 American Academy of Neurology Annual Meeting*. Philadelphia, PA (2019).
27. Howard JF Jr, Bril V, Burns TM, Mantegazza R, Bilinska M, Szczudlik A, et al. Randomized phase 2 study of FcRn antagonist efgartigimod in generalized myasthenia gravis. *Neurology*. (2019) 92:e2661–73. doi: 10.1212/WNL.0000000000007600
28. Ulrichts P, Guglietta A, Beauchamp J, De Haard H, Parys W. Efgartigimod in myasthenia gravis: update on clinical development and phase 3 ADAPT study. In: *2019 Muscle Study Group Meeting*. Snowbird, UT: Muscle Nerve (2019).
29. ArgenxBVBA. *An Efficacy and Safety Study of ARGX-113 in Patients With Myasthenia Gravis Who Have Generalized Muscle Weakness (ADAPT)*. Clinicaltrials.gov (2018). Available online at: https://clinicaltrials.gov/ct2/show/NCT03669588?term=Efgartigimod&rank=3
30. Momenta. Pharmaceuticals, Inc. *A Study to Evaluate the Safety, Tolerability, Efficacy, Pharmacokinetics and Pharmacodynamics of M281 Administered to Adults With Generalized Myasthenia Gravis*. Clinicaltrials.gov (2018). Available online at: https://clinicaltrials.gov/ct2/show/NCT03772587?term=M281&rank=5
31. UCB Pharma. *Study to Test the Safety, Tolerability and Efficacy of UCB7665 in Subjects With Moderate to Severe Myasthenia Gravis*. Clinicaltrials.gov (2017). Available online at: https://clinicaltrials.gov/ct2/show/NCT03052751?term=ucb&cond=myasthenia&rank=1
32. UCB. *UCB Accelerates Anti-FcRn Rozanolixizumab in Myasthenia Gravis Into Confirmatory Development Phase*. Available online at: https://eu.vocuspr.com/ViewAttachment.aspx?EID=hciRJLtApyzzYk8gYkD3ES2vKHWfTowcr2GXk9W%2f6xM%3d (2018).
33. Bril V, Benatar M, Brock M, Greve B, Kiessling P, Woltering F, et al. Proof-of-concept and safety of the anti-FcRn antibody rozanolixizumab in patients with moderate-to-severe generalized myasthenia gravis (GMG): a phase 2a study. In: *2019 American Academy of Neurology Annual Meeting*. Philadelphia, PA (2019).
34. UCB Pharma. *Study to Test Efficacy and Safety of Rozanolixizumab in Adult Patients With Generalized Myasthenia Gravis*. Clinicaltrials.gov (2019). Available online at: https://clinicaltrials.gov/ct2/show/NCT03971422?term=ucb&cond=myasthenia
35. Immunovant. Sciences GmbH. *A Study of RVT-1401 in Myasthenia Gravis (MG) Patients*. Clinicaltrials.gov (2019). Available online at: https://clinicaltrials.gov/ct2/show/NCT03863080?term=Immunovant&rank=3
36. Raja SM, Howard JF Jr, Juel VC, Massey JM, Chopra M, Guptill JT. Clinical outcome measures following plasma exchange for MG exacerbation. *Ann Clin Transl Neurol.* (2019) 6:2114–9. doi: 10.1002/acn3.50901
37. Benatar M, Sanders DB, Burns TM, Cutter GR, Guptill JT, Baggi F, et al. Recommendations for myasthenia gravis clinical trials. *Muscle Nerve.* (2012) 45:909–17. doi: 10.1002/mus.23330

**Conflict of Interest:** JG has served as a consultant for companies developing therapies described in the manuscript, served as an investigator for FcRn clinical trials in MG sponsored by Momenta and UCB, and served as DSMB member for Argenx.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors JG.

frontiers in Immunology

REVIEW
published: 14 February 2020
doi: 10.3389/fimmu.2020.00212

# Autoantibody Specificities in Myasthenia Gravis; Implications for Improved Diagnostics and Therapeutics

*Konstantinos Lazaridis*[1]* *and Socrates J. Tzartos*[2,3]

[1] Department of Immunology, Hellenic Pasteur Institute, Athens, Greece, [2] Department of Neurobiology, Hellenic Pasteur Institute, Athens, Greece, [3] Tzartos NeuroDiagnostics, Athens, Greece

Myasthenia gravis (MG) is an autoimmune disease characterized by muscle weakness and fatiguability of skeletal muscles. It is an antibody-mediated disease, caused by autoantibodies targeting neuromuscular junction proteins. In the majority of patients (∼85%) antibodies against the muscle acetylcholine receptor (AChR) are detected, while in 6% antibodies against the muscle-specific kinase (MuSK) are detected. In ∼10% of MG patients no autoantibodies can be found with the classical diagnostics for AChR and MuSK antibodies (seronegative MG, SN-MG), making the improvement of methods for the detection of known autoantibodies or the discovery of novel antigenic targets imperative. Over the past years, using cell-based assays or improved highly sensitive immunoprecipitation assays, it has been possible to detect autoantibodies in previously SN-MG patients, including the identification of the low-density lipoprotein receptor-related protein 4 (LRP4) as a third MG autoantigen, as well as AChR and MuSK antibodies undetectable by conventional methods. Furthermore, antibodies against other extracellular or intracellular targets, such as titin, the ryanodine receptor, agrin, collagen Q, $K_v$1.4 potassium channels and cortactin have been found in some MG patients, which can be useful biomarkers. In addition to the improvement of diagnosis, the identification of the patients' autoantibody specificity is important for their stratification into respective subgroups, which can differ in terms of clinical manifestations, prognosis and most importantly their response to therapies. The knowledge of the autoantibody profile of MG patients would allow for a therapeutic strategy tailored to their MG subgroup. This is becoming especially relevant as there is increasing progress toward the development of antigen-specific therapies, targeting only the specific autoantibodies or immune cells involved in the autoimmune response, such as antigen-specific immunoadsorption, which have shown promising results. We will herein review the advances made by us and others toward development of more sensitive detection methods and the identification of new antibody targets in MG, and discuss their significance in MG diagnosis and therapy. Overall, the development of novel autoantibody assays is aiding in the more accurate diagnosis and classification of MG patients, supporting the development of advanced therapeutics and ultimately the improvement of disease management and patient quality of life.

**Keywords: autoimmunity, myasthenia gravis, autoantibody, diagnosis, therapy, acetylcholine receptor, MuSK, LRP4**

OPEN ACCESS

***Edited by:***
*Sonia Berrih-Aknin, Institut National de la Santé et de la Recherche Médicale (INSERM), France*

***Reviewed by:***
*Janine Adele Lamb, University of Manchester, United Kingdom*
*Ruben Dario Motrich, National Council for Scientific and Technical Research (CONICET), Argentina*

***Correspondence:**
*Konstantinos Lazaridis*
*klazaridis@pasteur.gr*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *09 November 2019*
***Accepted:*** *27 January 2020*
***Published:*** *14 February 2020*

***Citation:***
*Lazaridis K and Tzartos SJ (2020) Autoantibody Specificities in Myasthenia Gravis; Implications for Improved Diagnostics and Therapeutics. Front. Immunol. 11:212. doi: 10.3389/fimmu.2020.00212*

## INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease, characterized by muscle weakness and fatiguability of skeletal muscles (1, 2). MG is antibody-mediated, caused by autoantibodies targeting components of the neuromuscular junction (NMJ). Autoantibody binding causes impaired neuromuscular transmission, either by damage of the postsynaptic muscle membrane or by disruption of its normal organization.

The NMJ is responsible for transmission of the signal from the axon terminals of motor neurons to the muscle, rapidly translating neuron action potentials into muscle contraction. Acetylcholine released from the axon terminals binds to and activates the muscle acetylcholine receptors (AChRs), triggering opening of the receptor channel and depolarization of the muscle membrane. The AChRs are clustered at the NMJ resulting in localized high density of receptor clusters, which increases the efficiency of signal transmission. AChR clustering is driven by agrin, which upon release from the nerve terminals binds to low-density lipoprotein receptor-related protein 4 (LRP4), activating it to form a complex with muscle specific kinase (MuSK), thus causing the autophosphorylation and activation of MuSK. This results in a signaling cascade that promotes rapsyn-mediated AChR clustering at the NMJ (3, 4) (**Figure 1**).

MG is heterogeneous in terms of symptom presentation, with focal or generalized weakness, as well as in terms of pathophysiology, since different NMJ antigens can be targeted (5, 6). The symptoms usually initially manifest at the ocular muscles; in some patients they remain localized (ocular MG, OMG), while in the majority of patients the symptoms progress to other skeletal muscles within a couple of years (generalized MG, GMG). The disease presents with two peaks of incidence, below or above the age of 50, termed early-onset MG (EOMG) and late-onset MG (LOMG), respectively.

Although MG is a rare disease, with a prevalence of 150–300 per million population and an incidence of ∼10 per million per

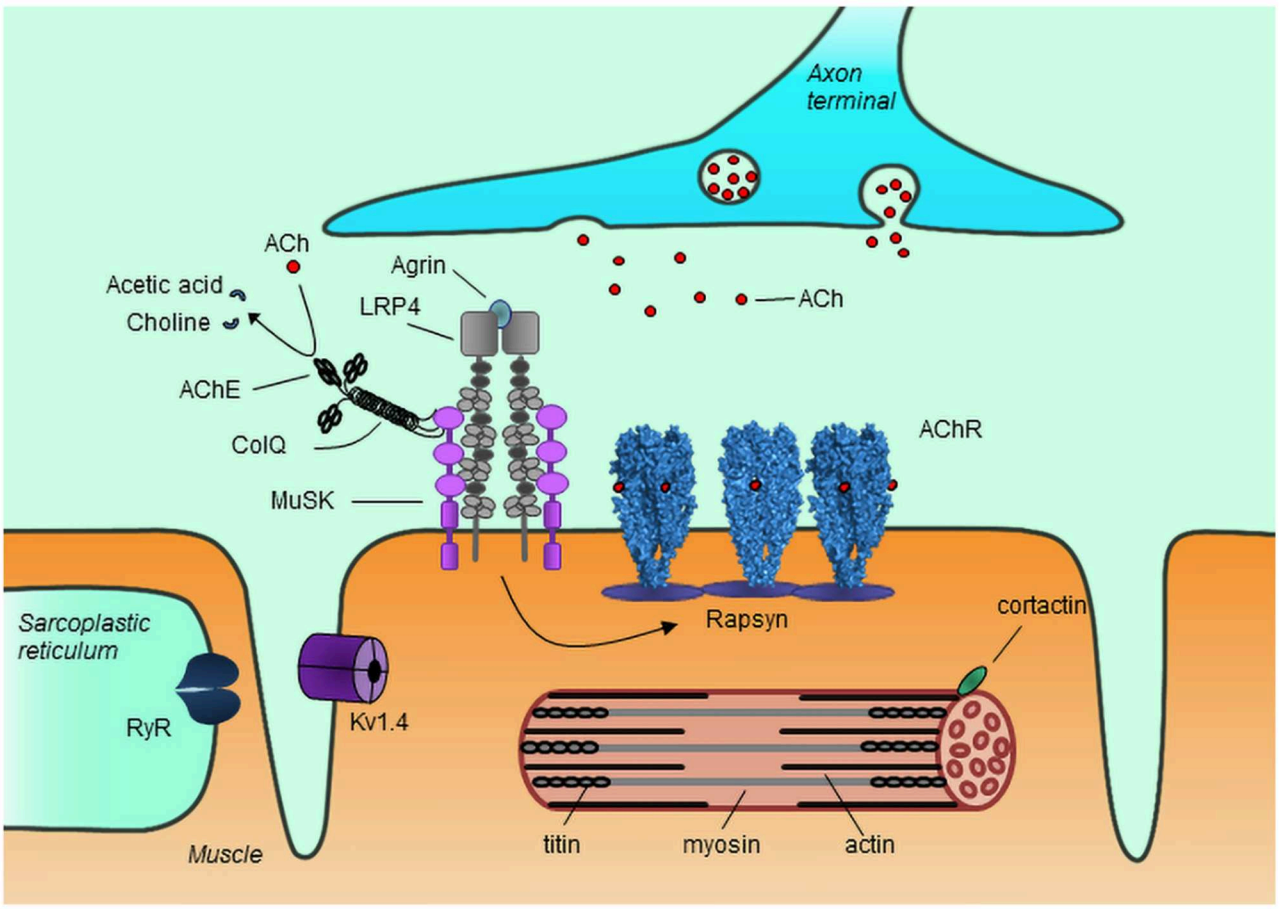



**FIGURE 1 |** Schematic representation of the neuromuscular junction and myotube components. Agrin released from the nerve terminal binds to LRP4, which in turn binds to and activates MuSK, causing rapsyn-mediated AChR clustering. Acetylcholine (Ach) released from the nerve terminal binds to AChR causing opening of the receptor channel and triggering muscle contraction. Unbound acetylcholine in the synaptic cleft is broken down into choline and acetic acid by AChE, thus terminating its action. The antigenic targets for autoantibodies in MG known so far are depicted, though not all have been shown to be implicated in pathology. AChR, acetylcholine receptor; MuSK, muscle specific kinase; LRP4, low-density lipoprotein receptor-related protein 4; RyR, ryanodine receptor; ColQ, collagen Q; AChE, acetylcholinesterase; Kv1.4, voltage gated potassium channel 1.4.

year (7), it is considered a model antibody-mediated autoimmune disease, since in most cases the autoantibodies and target antigens are well-characterized. The majority of patients (∼85%) have antibodies against the muscle AChR. Furthermore, antibodies against MuSK are found in approximately 6% of the patients, while relatively recently antibodies against LRP4 have been found in about 2% of MG patients. The pathogenicity of all these autoantibodies has been shown by the development of passive transfer experimental autoimmune MG (EAMG) when injected into laboratory animals and by the improvement of patients' symptoms following plasmapheresis (8–10). Some patients do not have detectable antibodies against any of these antigens, being referred to as seronegative (SNMG). Antibodies against various other extracellular or intracellular targets are found in several MG patients. Although the pathogenicity of these molecules is often uncertain or unlikely, they can still be highly informative disease biomarkers.

The detection of autoantibodies is crucial for MG diagnosis and for the differential diagnosis of many disorders with similar presentation. We will review the main autoantibodies found in MG, the advances toward development of increasingly sensitive detection methods and the identification of new antibody targets in MG. Furthermore, since the antigen targeted can dictate the response to treatment and novel advanced therapeutics aim to be antigen-specific, we will discuss their significance in therapy.

## MG AUTOANTIBODIES AND THEIR DETECTION

### Antibodies Against the AChR

The autoantibodies in the majority of MG patients are directed against the muscle AChR of the NMJ. The muscle AChR is composed of five homologous subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ in fetal or denervated muscles and $\alpha_2\beta\delta\varepsilon$ in adult muscles (11). Each subunit has a highly structured extracellular domain (ECD), four transmembrane domains and a partly structured intracellular domain. The autoantibodies target the ECDs of the AChR subunits and are very heterogeneous, since autoantibodies against all five subunits can be found in the same patient, including the γ subunit of the fetal AChR (12–15). Despite this, approximately half of the autoantibodies bind to the α subunit and especially the main immunogenic region (MIR), formed by overlapping epitopes located on the α1 subunit ECD, whose central core lies between amino acids 67–76, although other segments contribute as well (16–18). Furthermore, the autoantibodies against the α subunit are more pathogenic than those against the other subunits (10).

The AChR antibodies belong primarily to the IgG1 and IgG3 subclasses (19, 20). They can, therefore, activate complement at the postsynaptic membrane and thus cause AChR loss and destruction of its characteristic architecture, which is necessary for efficient signal transduction (21). Additionally, being bivalent, they can cross-link receptors leading to their endocytosis and destruction (antigenic modulation) (22). Finally, autoantibodies that bind close to the ligand binding site can directly interfere with receptor activation by acetylcholine (23).

Serological testing for the detection of AChR antibodies is often the first step for MG diagnosis, along with electrophysiological examination and assessment of response to acetylcholinesterase (AChE) inhibitors. The titer of AChR antibodies does not correlate with disease severity, although some evidence suggests that such a correlation emerges when the titer of only the MIR-directed, or the IgG1 subclass antibodies is considered (20, 24). In individual patients, on the other hand, the titer is associated with symptom severity and with response to therapy (25). Indeed, in a recent case study, gradually increasing AChR antibody titers were detected retrospectively up to 2 years before the onset of typical MG symptoms (26). Therefore, testing serial samples from the same patient attains added importance for monitoring their progress and guiding disease management. Additionally, the AChR antibody titer could provide information with respect to the risk of transient neonatal MG (TNMG), since it appears that TNMG is probable when the mother's titer is above 100 nM, but unlikely when it is below 10 nM (27).

The most widely used method for AChR antibody detection currently is the radioimmunoprecipitation assay (RIPA) (28). It is based on the indirect labeling of human AChR with $^{125}$I-α-bungarotoxin, which is a highly specific antagonist for the AChR (29). Sources of AChR can be human muscle or, more commonly, AChR-expressing cell lines, such as the CN21 cell line, which has been engineered to express both the fetal and adult types of the receptor (i.e., ε-expressing TE671 cells) (30). The AChR antibody RIPA has been the golden standard in MG diagnosis for many years due to its very high specificity (approximately 99%), as well as sensitivity, which is about 85% in the case of generalized MG and about 50% in ocular MG (31). In rare cases AChR antibodies can be found in patients with other autoimmune disorders or with thymoma without MG (32). The RIPA is also quantitative, allowing for detailed autoantibody titer determination, which is useful for monitoring individual patients.

A simple but much more sensitive RIPA than the classical one, has also been developed, which allows decreasing the cut-off for positivity from 0.5 to 0.6 nM AChR antibodies to <0.1 nM. It involves the use of 16 times larger serum volumes mixed with the usual amount of radiolabeled AChR followed by precipitation with the minimum required amount of semi-purified anti-human IgG antibody, to avoid increasing the background. By this approach, 20 of 81 tested SN-MG Chinese patients were found AChR antibody positive (33).

Enzyme-linked immunosorbent assay (ELISA) for AChR antibodies is also available (34), but it is not as sensitive as the standard RIPA (35) and consequently it has not gained as much traction in routine diagnosis as RIPA. Furthermore, radiological and ELISA assays have been developed to specifically detect modulating or blocking antibodies, but they marginally increased the sensitivity compared to the standard RIPA (36, 37). Another promising non-radioactive alternative to the RIPA is a fluorescence immunoprecipitation assay (FIPA), whereby the target antigen is labeled with fluorescence. This method has been shown to have relatively good overall sensitivity and specificity, but it is still not as good as the RIPA, and although it circumvents the hazards of radioactivity, it requires specialized equipment and expertise, making it difficult for routine diagnosis (38). Finally,

an approach based on labeling of the recombinant AChR α subunit with *Renilla* luciferase and measuring the precipitated fluorescence by serum autoantibodies was able to detect AChR antibodies in 32% of MG patients (39). The low sensitivity could be in part due to the use of a fragment of the α subunit and further investigation is needed to assess its potential role in MG diagnosis.

Over the last few years, the application of cell-based assays (CBAs) has been gaining ground as previously undetected antibodies can be identified. The antigen is expressed in a suitable cell line, usually HEK293 cells, and the binding of autoantibodies is detected by a secondary fluorescently labeled antibody by microscopy. Specifically, in the case of AChR antibody CBAs the cells are also transfected with rapsyn in order to promote clustering of the receptors at the cell surface. This allows the detection of antibodies that will only bind to high density AChRs, resembling their organization at the NMJ, or of antibodies whose epitopes are destroyed or altered by the detergent solubilization of membranes for the isolation of AChR antigen. Initially, using this CBA 60% of previously SN-GMG and 50% of SN-OMG patients were found to have AChR antibodies (40, 41), though subsequent studies had varying results ranging between 4 and 38% of previously SNMG patients (33, 42–44). Routine diagnosis indicates that the overall fraction of SNMG sera positive for clustered AChR antibodies may be ∼20% (45). The sensitivity of the assay is greater when both the adult and fetal form of the receptor are used (46). The CBA-detected antibodies were shown to belong to the same subclasses as the RIPA-detected antibodies and to potentiate complement depositions on the cell surface, indicative of a similar pathogenesis. However, patients with AChR antibodies detectable only by CBA seem to present with milder symptoms and better response to treatment (43).

Several studies have shown that the CBA can detect AChR antibodies which are not detectable by the classical diagnostics (38, 43, 47, 48). The CBA also has the advantage to be able to distinguish between antibodies against the fetal or adult form of the receptor (46). This becomes relevant in the diagnosis of cases of transient neonatal MG not associated with maternal MG, whereby the antibodies only recognize the fetal AChR leaving the adult AChR practically unaffected and the mother without signs of MG (13, 49, 50). On the other hand, in our own experience, the CBA lacks the quantitative resolution of the RIPA and thus cannot provide detailed titers for disease monitoring, while it often fails to detect autoantibodies in sera of very low but positive titer by the RIPA.

Finally, efforts are made toward the establishment of easy to perform instrument-free rapid assays, that could be used in non-specialized facilities (small clinics or neurologists' offices), since this could greatly reduce the time to diagnosis and improve patient management. To this end, we have developed a modified ELISA based on immobilization of AChR onto a solid support stick (immunostick), which has shown to have good specificity and sensitivity (99 and 91%, respectively) for AChR antibodies (51). Moreover, the immobilization of additional antigens in different zones on the immunostick could allow the simultaneous detection of more than one MG autoantibodies by this method. A similar approach based on blotting AChR preparations onto a nitrocellulose membrane, resulting in a dot-blot method, achieved the same sensitivity as the ELISA (52). Although such methods could be beneficial for MG diagnosis, they require further evaluation before clinical application.

## Antibodies Against MuSK

MuSK is a muscle membrane protein, which has an extracellular domain, a transmembrane helix domain and a cytoplasmic domain with tyrosine kinase activity. The extracellular domain includes three immunoglobulin-like regions and a cysteine-rich domain, also called Frizzled-like domain. The majority of MuSK antibodies bind to the Ig-like regions of the MuSK extracellular domain (53, 54). MuSK antibodies are detected in ∼6% of all MG patients, or 40% among the AChR antibody negative patients. This ratio varies among countries with a lower prevalence in Northern Europe and higher toward the Mediterranean, probably owing to geographical and genetic differences (53, 55–59). In Japanese populations MuSK-MG seems to be less common with an overall prevalence of 2–3% (60). Until recently, detection of MuSK antibodies in AChR antibody positive patients was very rare (61, 62).

Unlike AChR antibodies, MuSK antibodies belong primarily to the IgG4 subclass, which do not activate complement and are largely functionally monovalent due to Fab arm exchange (63, 64). Their pathogenicity appears to stem from inhibition of interactions between MuSK and collagen Q or LRP4 via binding to the first Ig-like domain of MuSK and subsequent reduction of both agrin-induced and agrin-independent AChR clustering (65–67). The titer of MuSK antibodies appears to correlate with disease severity, both in individual patients and in the population (68, 69).

MuSK antibodies are routinely detected by RIPA using directly $^{125}$I-labeled MuSK (70). In an effort to increase the sensitivity of the RIPA, an alternative two-step method has been developed, whereby in the first step the MuSK antibodies, which may be at very low titers, are concentrated from large serum volumes by means of affinity chromatography, while the second step is effectively the standard RIPA (71). This approach allows the use of up to 50 times larger serum volumes, which would otherwise cause increased non-specific binding.

Commercially available ELISAs for the detection of MuSK antibodies are available but less commonly used. As a non-radioactive alternative to RIPA, FIPA seems very promising as the two assays have been shown to have the same sensitivity (38). Importantly, the FIPA could be performed so that both AChR and MuSK antibodies are measured simultaneously by labeling each antigen with a different fluorescent dye, thus potentially reducing the cost and time for diagnosis.

CBAs for MuSK antibodies have also been developed over the last years, which have detected MuSK antibodies in previously SNMG patient sera, including Asian populations (38, 40, 47, 72). Screening of 633 SNMG sera from 13 European countries revealed a prevalence of 5–22% for MuSK antibodies depending on the country (44). Interestingly, most of the detected MuSK antibodies in this study belonged to the IgM and not the IgG class. The CBA allowed the detection of MuSK antibodies in SN-OMG patients as well, which is not common with the classical assays (38, 44). Furthermore, using CBAs the percentage of sera positive for antibodies to more than one antigen has increased.

In more detail, AChR antibody positive patients were also found positive for MuSK antibodies in 0.5–12.5% of the patients (44, 73). It is conceivable that some double positive patients were not identified in the past, since those found seropositive for AChR antibodies were not routinely tested for MuSK antibodies.

## Antibodies Against LRP4

LRP4 has a central role in synaptic development and maintenance. It is a transmembrane protein, containing several low-density lipoprotein domains. LRP4 acts as the muscle receptor for neural agrin, propagating the signal to MuSK for AChR clustering at the NMJ (74). LRP4 autoantibodies are detected in some MG patients. Inhibition of the LRP4-agrin interaction appears to be responsible, at least in part, for their pathogenicity (75–78). However, LRP4 antibodies belong mostly to the IgG1 subclass (75, 78), and they have been shown to cause *in vitro* complement-mediated cell lysis of C2C12 myotubes (78), so complement activation could also play a role in MG patients.

Initial reports varied significantly with respect to the prevalence of LRP4 antibodies, reported from 2 to 45%, possibly due to variations in the detection assays (ELISA, CBA or immunoprecipitation), the source of the antigen used (animal or human) and the populations examined (Japanese or Caucasian) (75–77). Indeed, studies in Chinese populations suggested that LRP4 antibodies are less frequent than in Western countries, as they were only found in 1–2.9% of SNMG and 0.8–1.7% of the total MG patients, while they were associated mostly with OMG (73, 79). We used CBA to perform a multinational study with samples from 635 patients without detectable AChR or MuSK antibodies. We found that 19% had LRP4 antibodies, corresponding to 2% of all MG patients, with considerable variability among the various countries (from 7% for Norway and Turkey to 33% for Poland) (80). Again, the use of these assays has revealed several cases of double positive patients; 15–20% of MuSK antibody positive and 7.5% of AChR antibody positive sera have been found positive for LRP4 antibodies as well (44, 80, 81). In addition to the NMJ, LRP4 is also present on motor neurons in the brain. Interestingly, LRP4 antibodies have also been detected in 10–23% of amyotrophic lateral sclerosis (ALS) patients and are thus not exclusively specific for MG (82, 83). Nevertheless, their detection can aid in the diagnosis of MG in parallel with the clinical presentation of the patients.

## Striational Antibodies

Striational antibodies were originally identified by staining of sarcomeres with patients' sera, which produced characteristic striational patterns. They are directed against several muscle fiber proteins, including titin, the ryanodine receptor (RyR), actin, myosin, tropomyosin, filamin, and others (84–86). Although all these proteins are important players in muscle contraction, their intracellular localization makes it unlikely for the respective autoantibodies to have a directly pathogenic role in MG. Nonetheless, titin and RyR antibodies are useful biomarkers and their detection can provide invaluable prognostic information.

Titin is a filamentous intracellular protein, the largest known so far, with a molecular weight between 3,000 and 4,200 kDa (87). Despite its size and repetitive nature, titin autoantibodies bind to a specific 30 kDa domain corresponding to 1% of titin's mass. This domain, known as MGT30, has been expressed as a recombinant titin fragment and is located near the A/I band junction (88). Titin antibodies are currently mostly detected in routine diagnosis by means of commercially available ELISA kits with the MGT30 domain. Overall, 20–40% of all AChR antibody positive patients have also been found positive for titin antibodies, with a marked age-related pattern; the prevalence is as low as 6% in EOMG and rises to 50–80% in non-thymomatous patients with LOMG (89–93). In EOMG patients titin antibodies are a strong indication for the presence of thymoma, as they are found in 50–95% of EOMG patients with thymoma, but only in few non-thymoma EOMG patients (86, 89, 90, 94–97). On the other hand, the presence of titin antibodies in all age groups appears to be related with more severe symptom manifestation (90, 95, 96, 98), although this relation has not been confirmed by all relevant studies (93). Using the aforementioned ELISA, titin antibodies have not been found in MG patients negative for AChR antibodies (93, 95, 98). More recently, we developed a RIPA method for the detection of titin antibodies using $^{125}$I-labeled MGT30, which has been used to screen a large cohort of European MG patients (99). The RIPA detected all the positive sera found by the ELISA, but it also detected titin antibodies in 13.4% of SNMG patients, as well as in patients with MuSK and LRP4 antibodies (14.6 and 16.4%, respectively). Interestingly, the titin antibody titers were higher in sera also positive for AChR antibodies. Low titer titin antibodies found in SNMG did not correlate with the presence of thymoma. This is in agreement with the finding that patients without AChR antibodies irrespective of age group were very unlikely to present with thymoma (97). The symptom severity was the same among the titin antibody positive and negative SNMG patients, suggesting that the detection of titin antibodies in SNMG is not prognostic for more severe disease, but they are a valuable biomarker for MG diagnosis. Recently, despite its intracellular localization, a "cytometric CBA" was developed for the detection of titin antibodies, based on incubation of stably titin-transfected HEK293 cell with serum samples and secondary labeled antibodies, followed by FACS analysis for quantitation of the results (100). This method showed increased sensitivity for titin antibodies compared to the ELISA when it was used to screen MG patients with myositis or myocarditis.

The RyR is a calcium channel located in the sarcoplasmic reticulum membrane and is involved in the excitation-contraction coupling mechanism by mediating $Ca^{2+}$ release from the sarcolemma to the cytoplasm. RyR antibodies can be detected by western blot using crude sarcoplasmic reticulum or by ELISA using a fusion protein containing the main immunogenic domain of the RyR (101). The presence of RyR antibodies in patients has been found to differ between MG subgroups. Similar to titin antibodies, they are usually absent in EOMG, while they can be found in up to 40% of LOMG patients. Moreover, they are present in up to 75% of MG patients with thymoma (95, 102, 103). Overall, their presence has been correlated with more severe disease manifestation (104, 105).

## Antibodies Against Other Antigens

In addition to the aforementioned, several other antigenic targets have been reported in MG, although their pathogenicity, specificity for MG and diagnostic or prognostic value have not been fully characterized. These include the proteins agrin, Kv1.4 potassium channel, rapsyn, cortactin, acetylcholinesterase (AChE), collagen Q (ColQ) and collagen XIII.

Agrin is a proteoglycan secreted by the motor neuron, which then binds to muscle LRP4 and activates a signaling cascade resulting in AChR clustering. Antibodies against agrin have been detected in sera of MG patients ranging from 2 to 15% by ELISA or CBA (81, 106–108). Although most agrin antibody positive sera were also positive for AChR, MuSK or LRP4 antibodies, some were SNMG. This finding, together with the absence of detectable agrin antibodies among the samples from healthy controls or patients with other neurological diseases (such as multiple sclerosis, ALS, and neuromyelitis optica), support their diagnostic value as MG-specific autoantibodies. Importantly, patients with agrin antibodies presented with mild to severe symptoms and moderate response to treatment, thus their early detection could aid in disease management (81). Agrin antibodies appear to be pathogenic, since in *in vitro* studies they were capable of inhibiting MuSK activation by agrin and AChR clustering (108), while immunization of mice with neural, but not muscle, agrin induced MG-like symptoms (109).

The voltage gated potassium channel α-subunit Kv1.4 is expressed mainly in neurons of the central nervous system, where they control presynaptic release of acetylcholine. They are also found in skeletal and heart muscles. Studies of antibodies against Kv1.4 in Japanese MG populations, revealed that they were present in 11–18% of the patients and their presence was correlated with severe symptoms, myasthenic crises, and thymoma (110–112). Furthermore, it was found that 11–27% of Kv1.4 antibody positive Japanese MG patients also suffered from or had clinically suspected myocarditis, the clinical onset of which was always preceded by the detection of Kv1.4 antibodies, while 36–60% presented with abnormal ECG findings. On the other hand, investigation of a Caucasian population revealed the same Kv1.4 antibody prevalence among MG patients (17%), but their presence was associated with female LOMG patients with mild symptoms, in many cases purely OMG (113). It appears therefore, that only in Japanese populations, the Kv1.4 antibodies are an important biomarker indicating increased risk of myocarditis or cardiac dysfunction among MG patients. However, their detection is difficult as it involves the immunoprecipitation of $^{35}$S-labeled cell extracts by patient sera followed by SDS-PAGE electrophoresis: the presence of a 70 kDa Kv1.4 band in rabdomyosarcoma extracts but not in leukemic cell extracts is considered a positive finding (110). The application of a cytometric CBA could prove a useful alternative, as it has recently been successfully used to detect Kv1.4 antibodies with similar efficiency to the RIPA (100).

Rapsyn is an intracellular muscle protein, which acts as a scaffold, linking the intracellular domain of the AChR with the cytoskeleton and thus mediating receptor clustering at the NMJ (114). Rapsyn antibodies are found in up to 15% of MG patients, including SNMG (115). However, the fact that no associations have been identified with disease severity or MG subgroups, while they are also detected in several other autoimmune diseases, such as systemic lupus erythematosus (116), diminishes their diagnostic potential.

Cortactin is a cytoplasmic protein involved in actin assembly and MuSK-induced AChR clustering at the NMJ. Cortactin antibodies were first identified in patient sera using a human protein array (117). Further analyses using ELISA and western blot for confirmation of the results, detected cortactin antibodies in up to 23.7% of SNMG samples and 9.5% of seropositive MG, suggesting that they can be valuable in SNMG diagnosis and prognostic of mild disease (117–119). However, cortactin antibodies have also been found in up to 12.5% of patients with other autoimmune diseases and 5.2% of healthy controls, while they have been described as myositis-associated, since they are found in 7.6–26% of patients with polymyositis, dermatomyositis and immune-mediated necrotizing myopathy (117, 120). Therefore, their relevance for MG diagnosis and contribution to pathogenesis still requires further investigation.

AChE is an enzyme localized at the synaptic cleft of the NMJ, where it catalyzes the breakdown of acetylcholine, thus terminating its action on AChRs. Antibodies against AChE have been reported in 5-50% of MG patients (121–123). No significant correlation was observed with sex, age of onset, or thymic pathology, while they were not MG-specific, as they were also found in several patients with other autoimmune diseases.

ColQ is found in the extracellular matrix at the NMJ, probably via interactions with MuSK, where it mediates the anchoring of AChE (124). Recently, antibodies against ColQ were found in a fraction of MG patients (3%) using CBA, including some SNMG patients, although they were also present in a similar fraction of the control cohort used in the study (125). The pathogenicity of ColQ antibodies has not been assessed so far. Therefore, their diagnostic value and potential pathogenic role remains to be elucidated.

Collagen XIII antibodies have been detected in 7.1% of AChR antibody positive MG patient and 15.8% SNMG sera screened (126). No discernible differences were seen among patients with and without collagen XIII antibodies in terms of symptom manifestation. Moreover, these antibodies are not MG specific and have been also associated with Grave's ophthalmopathy (127).

The above observations with respect to the different MG autoantibodies are summarized in **Table 1**, while **Figure 2** shows the percentage of previously seronegative MG patients in which autoantibodies are found using some of the novel detection assays described, in a European cohort.

# RELEVANCE FOR THERAPY

The determination of the autoantibody specificity, in addition to its diagnostic value, is also very important for correct management of MG patients. Firstly, the detection of serum autoantibodies, especially in the case of AChR and MuSK, can

**TABLE 1 |** Summary of autoantibody prevalence, usual detection method and major clinical associations where known*.

| Autoantigen | Detection method | % of MG patients | % of dSN-MG patients | Other diseases | Clinical associations | Representative references |
|---|---|---|---|---|---|---|
| AChR | RIPA | 80–85% | N.A. | Rare | Thymic abnormalities, thymoma | Several references, reviewed in Gilhus et al. (6) |
| Clustered AChR | CBA | N.T. | ∼20% (4–60%) | N.T. | Milder symptoms than AChR+ MG, thymic abnormalities | (40, 45) |
| MuSK | RIPA | ∼6% (2–3% in Japanese) | N.A. | Rare | Bulbar symptoms common, no thymic abnormalities | (53, 56, 60) |
| MuSK | CBA | N.T. | 13% | 5% | Milder symptoms | (44) |
| LRP4 | CBA | ∼2% | ∼19% | 3.6% (10–23% in ALS) | Milder symptoms than AChR+ MG, no thymoma | (80) (83) |
| Titin | ELISA | 20–30% (90% in thymoma EOMG | 0–3%) | Some | Correlation with thymoma in AChR+ EOMG | (86, 90, 94, 128) |
| Titin | RIPA | ∼41% | 13.4% | 0–3.6% | No correlation with thymoma | (99) |
| RyR | ELISA | ∼ 14% in LOMG (75% in thymoma MG) | N.T. | N.T. | Correlation with thymoma in AChR+ MG | (95, 103, 104) |
| Agrin | ELISA/CBA | 2–15% | 0–50% | 13.8% in ALS | Mild to severe symptoms, moderate response to treatment | (83, 106) |
| Kv1.4 | IP and SDS-PAGE | 10–20% | 0% | 0% | Japanese: Severe symptoms, myasthenic crises, thymoma, cardiac involvement Caucasian: Mild symptoms in LOMG | (110, 112, 113) |
| Rapsyn | Immunoblots | 11% | 17% | 10% OND 78% SLE | Not known associations | (115, 116) |
| Cortactin | ELISA, WB | 5–10% | ∼20% | 12.5% | Not known associations | (117, 118) |
| ColQ | CBA | 3% | 3.4% | 5% | Not known associations | (125) |

*Some studies on potential antigens with small cohort sizes and non-MG-specific findings are not included in the table.
N.T., not tested or not extensively tested; N.A., not applicable; SLE, systemic lupus erythematosus.*

provide a practically certain diagnosis for MG, allowing the initiation of appropriate treatment. Moreover, monitoring of the antibody titer can be very useful in following disease progression and response to therapy.

Importantly, the therapeutic regime can differ between the MG subgroups. Patients with MuSK antibodies tend to have more severe symptoms and generalized weakness (129), whereas treatment withdrawal in these patients can often lead to disease exacerbation. In addition, MuSK-MG patients can present with adverse effects when treated with pyridostigmine, an AChE inhibitor commonly used as a first-line treatment for MG, while there is little evidence to support the usefulness of thymectomy in these patients (130). On the other hand, they usually greatly benefit from plasma exchange (PLEX) (131), and they have a very good response to the administration of rituximab, possibly more pronounced than the other MG subgroups (132, 133). AChR antibody positive patients who also have titin or RyR antibodies tend to have more severe disease, while in the case of EOMG they are indicative of thymoma (93). The benefit of thymectomy is questionable in patients with SNMG, MuSK-MG and LRP4-MG since they usually lack the typical thymus pathology seen in AChR-MG (134). Especially in the case of Japanese patients, the presence of Kv1.4 antibodies has been associated with cardiac dysfunction and severe complications, so they should be monitored accordingly. It is, therefore, important to be able to diagnose the patients, not only based on clinical and electrophysiological examination, but also serologically. The detection of the autoantigen targeted in each patient is crucial to adopt the best treatment options.

The most common treatment strategies for MG currently include the use of AChE inhibitors, immunosuppressive agents, thymectomy, intravenous immunoglobulin (IVIG) and plasmapheresis (2, 135). These, however, are largely non-specific and thus may be accompanied by a variety of side effects, especially given the often life-long immunosuppressive treatment required. Novel therapies should aim to be antigen-specific, i.e., target the specific autoimmune components of the immune system, which are mostly well-known in MG. For the application of such tailor-made therapies the characterization of the patients' autoantibody specificities by serological tests is crucial.

One approach would be the selective removal of only the pathogenic autoantibodies (antigen-specific immunoadsorption). The method is similar to plasmapheresis, but in this case the plasma is passed through a suitable matrix, such as sepharose-immobilized autoantigens, to which the autoantibodies bind, while the rest of the plasma, free of autoantibodies, is returned to the patient (136, 137). Several studies have shown the efficiency of the approach, with significant fractions of the autoantibodies being removed from AChR and MuSK antibody positive MG patient sera

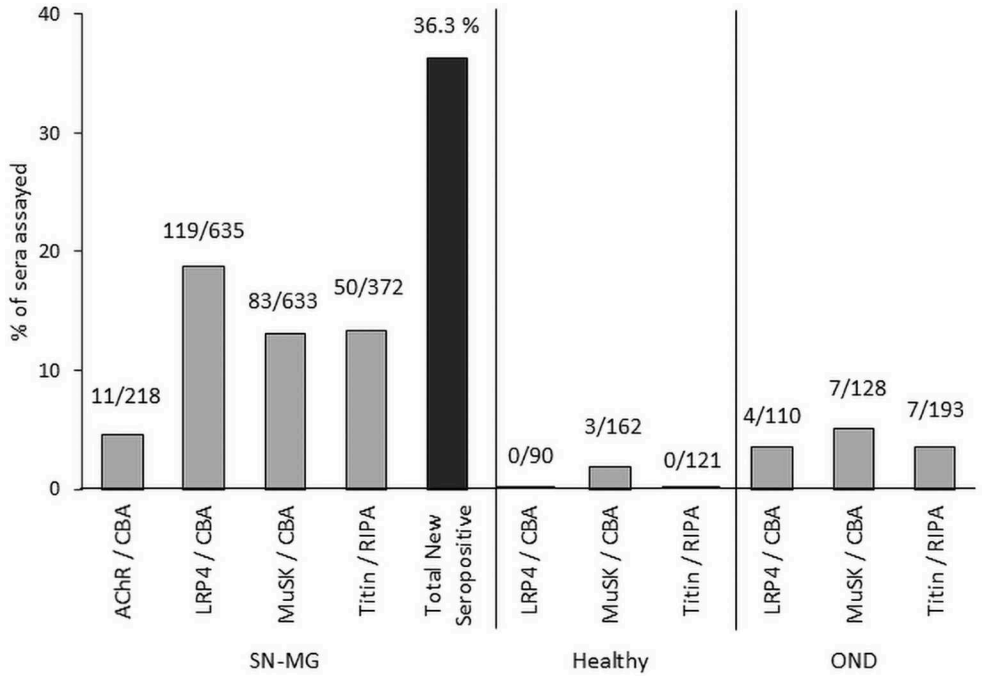



**FIGURE 2 |** Detection of autoantibodies in SNMG by novel assays. We have used CBA and RIPA for screening a large number of MG patients without detectable autoantibodies by the classical assays, as well as several control samples from healthy individuals or patients with other neuroimmune diseases (OND), from 10 to 13 different European countries (44, 80, 99). The numbers above the bars indicate the number of positive samples and the total tested with each assay. The cumulative percentage (black bar) of new positives among the SNMG samples that were positive in more than one assays were taken into account, so as to avoid overestimation of the total new seropositive patients.

*in vitro*, or from laboratory animals with EAMG *ex vivo* (54, 138). In the *ex vivo* studies immunoadsorption was shown to lead to significant amelioration of the symptoms within a few treatment sessions, while no adverse effects were seen (139, 140). No similar studies have been performed so far with LRP4 autoantibodies. Further tests are needed before clinical application of this approach, which should provide a solution when an immediate relief from MG symptoms is required (e.g., myasthenic crises, preoperatively) or for patients refractory to other treatments (132).

A different approach, aiming at treating the underlying pathology of MG, is to induce antigen-specific immunosuppression or immune tolerance for the targeted antigen, depending on the antibody specificities detected in each patient. To this end, several studies have shown that mucosal administration of AChR domains can lead to prevention or amelioration of ongoing EAMG (141–143). Prevention of EAMG was likewise achieved when T cell dominant peptides of the AChR ECDs were given orally or nasally (144, 145). Interestingly, when T cell dominant epitopes were administered in the form of subcutaneous immunization in the presence of adjuvant, a beneficial effect was also observed (146). A similar strategy relied on the use of peptide constructs incorporating only the intracellular sequences from all the AChR subunits (147). Although oral or nasal administration of the intracellular polypeptides was able to prevent and, in some cases, treat ongoing EAMG, the effect was greater when treatment was given as subcutaneous vaccination (148, 149). The therapeutic effect in the studies using ECD domains or their peptides was mediated by a shift of Treg cell responses from Th1 to Th2, a reduction in IFN-γ, IL-2, and IL-10 production levels and a switch of autoantibody subclass from IgG2b to IgG1. On the other hand, administration of the AChR intracellular domains relied on diverting the immunological response away from producing ECD-targeting pathogenic antibodies, toward epitopes of the intracellular domains, and possibly causing apoptosis of AChR-specific plasma cells. In our experience and several published studies, the therapeutic efficacy appears to depend on the conformation of the administered antigens and the route of administration (150–152). Given the advances in the heterologous expression of the AChR domains in various systems (15, 153), the elucidation of the precise mechanism and the specific immune cells involved would allow the design of increasingly targeted and specific therapeutic tools.

## CONCLUSIONS

Serological tests for the detection of autoantibodies are central in MG diagnosis. MG pathogenesis, its clinical presentation and the response of patients to therapy vary depending on the pattern of autoantibodies detected. In fact, the autoantibody specificity

pattern is often more informative for symptom severity than the autoantibody titer.

The very high specificity of AChR and MuSK antibodies for MG, which are the predominant antigens, and their successful use so far justify their use as early diagnostics in cases of clinically suspected MG. Despite the requirement for radioactivity and, consequently, specialized laboratories, RIPAs provide very sensitive results with reliable antibody titer information, and are thus proposed as the initial tests for routine MG diagnosis. Due to increases in antibody concentration during disease progression and/or epitope spreading, repeated tests should be performed when a suspected MG patient is initially seronegative.

A percentage of MG patients remain seronegative, but since autoimmune MG is most likely mediated by autoantibodies in all patients, SN-MG patients probably have autoantibodies against yet unidentified target proteins, low affinity or low concentration antibodies against the known antigens, requiring different diagnostic tests. More sensitive assays for known antigens or the discovery and validation of novel autoantibodies is thus necessary. To this end, considerable efforts have been made toward improvement of the tests; CBA for MuSK, LRP4 and clustered AChRs, RIPA for titin and two-step RIPA for AChR and MuSK have contributed significantly toward the reduction of the percentage of SNMG patients and are thus increasingly used in routine diagnosis for the detection of MG autoantibodies (33, 42, 154, 155). Furthermore, the discovery of antibodies against several other antigens whose diagnostic relevance remains to be fully assessed, should aid in the elimination of MG patients without a classical serological marker for diagnosis. Interestingly, several CBAs have recently been developed for intracellular MG antigens. However, due to the inability of CBAs to provide titer information and the lack of available commercial kits they are proposed as second line tests in patients that remain seronegative by the standard RIPAs. The use of cytometric CBAs could be a useful alternative, should they prove to reliably and specifically produce quantitative results (100). Efforts are also being made to develop diagnostic assays that can be easily performed in non-specialized and equipment-free settings to further decrease the time from sample collection to diagnosis (51). Assays based on CBAs or immunosticks could be adopted to simultaneously detect more than one antigen. The use of several antigen-expressing cells immobilized on different dots on a slide, or immunosticks onto which antigens have been immobilized in different zones could allow testing for all the major MG autoantibodies. Such an approach would decrease the time and the cost for diagnosis. The expansion of proteomic methods could result in the screening for binding to several MG and other autoimmune disease antigens aiding not only in faster diagnosis for MG but also in the differential diagnosis of related disorders. However, such approaches are still far from being used in the routine diagnosis for MG.

The sensitive and accurate detection of autoantibodies in MG patients' sera also has important implications for therapy, supporting the development of advanced therapeutics. Personalized treatment for MG patients would be highly beneficial, and it would rely on characterization of MG pathogenic antibody specificities. Antigen-specific therapies, such as immunoadsorption or induction of immunological tolerance against AChR, MuSK, and LRP4 should be the focus of efforts for future treatments (156). Many studies have shown the proof of concept for various such approaches, but their therapeutic efficacy and mechanism of action needs to be fully elucidated with vigorous preclinical and clinical trials, before they can progress into clinical practice.

## AUTHOR CONTRIBUTIONS

KL and ST researched the bibliography for the review, made substantial contributions to the content, and reviewed and edited the manuscript. KL wrote the first draft.

## REFERENCES


1. Verschuuren JJ, Huijbers MG, Plomp JJ, Niks EH, Molenaar PC, Martinez-Martinez P, et al. Pathophysiology of myasthenia gravis with antibodies to the acetylcholine receptor, muscle-specific kinase and low-density lipoprotein receptor-related protein 4. *Autoimmun Rev.* (2013) 12:918–23. doi: 10.1016/j.autrev.2013.03.001
2. Gilhus NE, Skeie GO, Romi F, Lazaridis K, Zisimopoulou P, Tzartos S. Myasthenia gravis - autoantibody characteristics and their implications for therapy. *Nat Rev Neurol.* (2016) 12:259–68. doi: 10.1038/nrneurol.2016.44
3. Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci.* (2001) 2:791–805. doi: 10.1038/35097557
4. Kummer TT, Misgeld T, Sanes JR. Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. *Curr Opin Neurobiol.* (2006) 16:74–82. doi: 10.1016/j.conb.2005.12.003
5. Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J Autoimmun.* (2014) 52:90–100. doi: 10.1016/j.jaut.2013.12.011
6. Gilhus NE, Tzartos S, Evoli A, Palace J, Burns TM, Verschuuren J. Myasthenia gravis. *Nat Rev Dis Primers.* (2019) 5:30. doi: 10.1038/s41572-019-0079-y
7. Carr AS, Cardwell CR, McCarron PO, McConville J. A systematic review of population based epidemiological studies in Myasthenia Gravis. *BMC Neurol.* (2010) 10:46. doi: 10.1186/1471-2377-10-46
8. Lindstrom JM, Engel AG, Seybold ME, Lennon VA, Lambert EH. Pathological mechanisms in experimental autoimmune myasthenia gravis. II Passive transfer of experimental autoimmune myasthenia gravis in rats with anti-acetylcholine recepotr antibodies. *J Exp Med.* (1976) 144:739–53. doi: 10.1084/jem.144.3.739
9. Newsom-Davis J, Wilson SG, Vincent A, Ward CD. Long-term effects of repeated plasma exchange in myasthenia gravis. *Lancet.* (1979) 1:464–8. doi: 10.1016/S0140-6736(79)90823-7
10. Kordas G, Lagoumintzis G, Sideris S, Poulas K, Tzartos SJ. Direct proof of the *in vivo* pathogenic role of the AChR autoantibodies from myasthenia gravis patients. *PLoS ONE.* (2014) 9:e108327. doi: 10.1371/journal.pone.0108327
11. Kalamida D, Poulas K, Avramopoulou V, Fostieri E, Lagoumintzis G, Lazaridis K, et al. Muscle and neuronal nicotinic acetylcholine receptors. *Structure, function and pathogenicity FEBS J.* (2007) 274:3799–845. doi: 10.1111/j.1742-4658.2007.05935.x
12. Vrolix K, Fraussen J, Losen M, Stevens J, Lazaridis K, Molenaar PC, et al. Clonal heterogeneity of thymic B cells from early-onset myasthenia gravis patients with antibodies against the acetylcholine receptor. *J Autoimmun.* (2014) 52:101–12. doi: 10.1016/j.jaut.2013.12.008

13. Vincent A, Newland C, Brueton L, Beeson D, Riemersma S, Huson SM, et al. Arthrogryposis multiplex congenita with maternal autoantibodies specific for a fetal antigen. *Lancet.* (1995) 346:24–5. doi: 10.1016/S0140-6736(95)92652-6
14. Kostelidou K, Trakas N, Tzartos SJ. Extracellular domains of the beta, gamma and epsilon subunits of the human acetylcholine receptor as immunoadsorbents for myasthenic autoantibodies: a combination of immunoadsorbents results in increased efficiency. *J Neuroimmunol.* (2007) 190:44–52. doi: 10.1016/j.jneuroim.2007.07.018
15. Zisimopoulou P, Lagoumintzis G, Kostelidou K, Bitzopoulou K, Kordas G, Trakas N, et al. Towards antigen-specific apheresis of pathogenic autoantibodies as a further step in the treatment of myasthenia gravis by plasmapheresis. *J Neuroimmunol.* (2008) 201–202:95–103. doi: 10.1016/j.jneuroim.2008.06.020
16. Tzartos SJ, Lindstrom JM. Monoclonal antibodies used to probe acetylcholine receptor structure: localization of the main immunogenic region and detection of similarities between subunits. *Proc Natl Acad Sci U.S.A.* (1980) 77:755–9. doi: 10.1073/pnas.77.2.755
17. Tzartos SJ, Barkas T, Cung MT, Mamalaki A, Marraud M, Orlewski P, et al. Anatomy of the antigenic structure of a large membrane autoantigen, the muscle-type nicotinic acetylcholine receptor. *Immunol Rev.* (1998) 163:89–120. doi: 10.1111/j.1600-065X.1998.tb01190.x
18. Luo J, Lindstrom J. Antigenic structure of the human muscle nicotinic acetylcholine receptor main immunogenic region. *J Mol Neurosci.* (2010) 40:217–20. doi: 10.1007/s12031-009-9271-y
19. Lefvert AK, Cuenoud S, Fulpius BW. Binding properties and subclass distribution of anti-acetylcholine receptor antibodies in myasthenia gravis. *J Neuroimmunol.* (1981) 1:125–35. doi: 10.1016/0165-5728(81)90015-1
20. Rodgaard A, Nielsen FC, Djurup R, Somnier F, Gammeltoft S. Acetylcholine receptor antibody in myasthenia gravis: predominance of IgG subclasses 1 and 3. *Clin Exp Immunol.* (1987) 67:82–8.
21. Engel AG, Arahata K. The membrane attack complex of complement at the endplate in myasthenia gravis. *Ann N Y Acad Sci.* (1987) 505:326–32. doi: 10.1111/j.1749-6632.1987.tb51301.x
22. Drachman DB, Angus CW, Adams RN, Michelson JD, Hoffman GJ. Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. *N Engl J Med.* (1978) 298:1116–22. doi: 10.1056/NEJM197805182982004
23. Drachman DB, Adams RN, Josifek LF, Self SG. Functional activities of autoantibodies to acetylcholine receptors and the clinical severity of myasthenia gravis. *N Engl J Med.* (1982) 307:769–75. doi: 10.1056/NEJM198209233071301
24. Masuda T, Motomura M, Utsugisawa K, Nagane Y, Nakata R, Tokuda M, et al. Antibodies against the main immunogenic region of the acetylcholine receptor correlate with disease severity in myasthenia gravis. *J Neurol Neurosurg Psychiatry.* (2012) 83:935–40. doi: 10.1136/jnnp-2012-302705
25. Oosterhuis HJ, Limburg PC, Hummel-Tappel E, The TH. Anti-acetylcholine receptor antibodies in myasthenia gravis. Part 2. Clinical and serological follow-up of individual patients. *J Neurol Sci.* (1983) 58:371–85. doi: 10.1016/0022-510X(83)90096-5
26. Strijbos E, Verschuuren J, Kuks JBM. Serum acetylcholine receptor antibodies before the clinical onset of myasthenia gravis. *J Neuromuscul Dis.* (2018) 5:261–4. doi: 10.3233/JND-180313
27. Eymard B, Vernet-der Garabedian B, Berrih-Aknin S, Pannier C, Bach JF, Morel E. Anti-acetylcholine receptor antibodies in neonatal myasthenia gravis: heterogeneity and pathogenic significance. *J Autoimmun.* (1991) 4:185–95. doi: 10.1016/0896-8411(91)90017-7
28. Lindstrom J. An assay for antibodies to human acetylcholine receptor in serum from patients with myasthenia gravis. *Clin Immunol Immunopathol.* (1977) 7:36–43. doi: 10.1016/0090-1229(77)90027-7
29. Patrick J, Lindstrom J, Culp B, McMillan J. Studies on purified eel acetylcholine receptor and anti-acetylcholine receptor antibody. *Proc Natl Acad Sci U.S.A.* (1973) 70:3334–8. doi: 10.1073/pnas.70.12.3334
30. Beeson D, Jacobson L, Newsom-Davis J, Vincent A. A transfected human muscle cell line expressing the adult subtype of the human muscle acetylcholine receptor for diagnostic assays in myasthenia gravis. *Neurology.* (1996) 47:1552–5. doi: 10.1212/WNL.47.6.1552
31. Benatar M. A systematic review of diagnostic studies in myasthenia gravis. *Neuromuscul Disord.* (2006) 16:459–67. doi: 10.1016/j.nmd.2006.05.006
32. Meriggioli MN, Sanders DB. Muscle autoantibodies in myasthenia gravis: beyond diagnosis? *Expert Rev Clin Immunol.* (2012) 8:427–38. doi: 10.1586/eci.12.34
33. Hong Y, Zisimopoulou P, Trakas N, Karagiorgou K, Stergiou C, Skeie GO, et al. Multiple antibody detection in 'seronegative' myasthenia gravis patients. *Eur J Neurol.* (2017) 24:844–50. doi: 10.1111/ene.13300
34. Hewer R, Matthews I, Chen S, McGrath V, Evans M, Roberts E, et al. A sensitive non-isotopic assay for acetylcholine receptor autoantibodies. *Clin Chim Acta.* (2006) 364:159–66. doi: 10.1016/j.cccn.2005.05.035
35. Oger J, Frykman H. An update on laboratory diagnosis in myasthenia gravis. *Clin Chim Acta.* (2015) 449:43–8. doi: 10.1016/j.cca.2015.07.030
36. Howard FM Jr, Lennon VA, Finley J, Matsumoto J, Elveback LR. Clinical correlations of antibodies that bind, block, or modulate human acetylcholine receptors in myasthenia gravis. *Ann N Y Acad Sci.* (1987) 505:526–38. doi: 10.1111/j.1749-6632.1987.tb51321.x
37. Chan KH, Lachance DH, Harper CM, Lennon VA. Frequency of seronegativity in adult-acquired generalized myasthenia gravis. *Muscle Nerve.* (2007) 36:651–8. doi: 10.1002/mus.20854
38. Yang L, Maxwell S, Leite MI, Waters P, Clover L, Fan X, et al. Non-radioactive serological diagnosis of myasthenia gravis and clinical features of patients from Tianjin, China. *J Neurol Sci.* (2011) 301:71–6. doi: 10.1016/j.jns.2010.10.023
39. Ching KH, Burbelo PD, Kimball RM, Clawson LL, Corse AM, Iadarola MJ. Recombinant expression of the AChR-alpha1 subunit for the detection of conformation-dependent epitopes in Myasthenia Gravis. *Neuromuscul Disord.* (2011) 21:204–13. doi: 10.1016/j.nmd.2010.12.003
40. Leite MI, Jacob S, Viegas S, Cossins J, Clover L, Morgan BP, et al. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain.* (2008) 131(Pt 7):1940–52. doi: 10.1093/brain/awn092
41. Jacob S, Viegas S, Leite MI, Webster R, Cossins J, Kennett R, et al. Presence and pathogenic relevance of antibodies to clustered acetylcholine receptor in ocular and generalized myasthenia gravis. *Arch Neurol.* (2012) 69:994–1001. doi: 10.1001/archneurol.2012.437
42. Devic P, Petiot P, Simonet T, Stojkovic T, Delmont E, Franques J, et al. Antibodies to clustered acetylcholine receptor: expanding the phenotype. *Eur J Neurol.* (2014) 21:130–4. doi: 10.1111/ene.12270
43. Rodriguez Cruz PM, Al-Hajjar M, Huda S, Jacobson L, Woodhall M, Jayawant S, et al. Clinical features and diagnostic usefulness of antibodies to clustered acetylcholine receptors in the diagnosis of seronegative myasthenia gravis. *JAMA Neurol.* (2015) 72:642–9. doi: 10.1001/jamaneurol.2015.0203
44. Tsonis AI, Zisimopoulou P, Lazaridis K, Tzartos J, Matsigkou E, Zouvelou V, et al. MuSK autoantibodies in myasthenia gravis detected by cell based assay–A multinational study. *J Neuroimmunol.* (2015) 284:10–7. doi: 10.1016/j.jneuroim.2015.04.015
45. Vincent A, Huda S, Cao M, Cetin H, Koneczny I, Rodriguez Cruz PM, et al. Serological and experimental studies in different forms of myasthenia gravis. *Ann N Y Acad Sci.* (2018) 1413:143–53. doi: 10.1111/nyas.13592
46. Shi QG, Wang ZH, Ma XW, Zhang DQ, Yang CS, Shi FD, et al. Clinical significance of detection of antibodies to fetal and adult acetylcholine receptors in myasthenia gravis. *Neurosci Bull.* (2012) 28:469–74. doi: 10.1007/s12264-012-1256-0
47. Chang T, Leite MI, Senanayake S, Gunaratne PS, Gamage R, Riffsy MT, et al. Clinical and serological study of myasthenia gravis using both radioimmunoprecipitation and cell-based assays in a South Asian population. *J Neurol Sci.* (2014) 343:82–7. doi: 10.1016/j.jns.2014.05.037
48. Yan C, Li W, Song J, Feng X, Xi J, Lu J, et al. Cell-based versus enzyme-linked immunosorbent assay for the detection of acetylcholine receptor antibodies in chinese juvenile myasthenia gravis. *Pediatr Neurol.* (2019) 98:74–9. doi: 10.1016/j.pediatrneurol.2019.01.016
49. Riemersma S, Vincent A, Beeson D, Newland C, Hawke S, Vernet-der Garabedian B, et al. Association of arthrogryposis multiplex congenita with maternal antibodies inhibiting fetal acetylcholine receptor function. *J Clin Invest.* (1996) 98:2358–63. doi: 10.1172/JCI119048
50. Saxena A, Stevens J, Cetin H, Koneczny I, Webster R, Lazaridis K, et al. Characterization of an anti-fetal AChR monoclonal antibody

isolated from a myasthenia gravis patient. *Sci Rep.* (2017) 7:14426. doi: 10.1038/s41598-017-14350-8
51. Trakas N, Tzartos SJ. Immunostick ELISA for rapid and easy diagnosis of myasthenia gravis. *J Immunol Methods.* (2018) 460:107–12. doi: 10.1016/j.jim.2018.06.016
52. Bokoliya S, Patil S, Nagappa M, Taly A. A simple, rapid and non-radiolabeled immune assay to detect anti-AChR antibodies in myasthenia gravis. *Lab Med.* (2019) 50:229–35. doi: 10.1093/labmed/lmy038
53. Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med.* (2001) 7:365–8. doi: 10.1038/85520
54. Skriapa L, Zisimopoulou P, Trakas N, Grapsa E, Tzartos SJ. Expression of extracellular domains of muscle specific kinase (MuSK) and use as immunoadsorbents for the development of an antigen-specific therapy. *J Neuroimmunol.* (2014) 276:150–8. doi: 10.1016/j.jneuroim.2014.09.013
55. Scuderi F, Marino M, Colonna L, Mannella F, Evoli A, Provenzano C, et al. Anti-p110 autoantibodies identify a subtype of "seronegative" myasthenia gravis with prominent oculobulbar involvement. *Lab Invest.* (2002) 82:1139–46. doi: 10.1097/01.LAB.0000028144.48023.9B
56. Sanders DB, El-Salem K, Massey JM, McConville J, Vincent A. Clinical aspects of MuSK antibody positive seronegative MG. *Neurology.* (2003) 60:1978–80. doi: 10.1212/01.WNL.0000065882.63904.53
57. Niks EH, Kuks JB, Verschuuren JJ. Epidemiology of myasthenia gravis with anti-muscle specific kinase antibodies in The Netherlands. *J Neurol Neurosurg Psychiatry.* (2007) 78:417–8. doi: 10.1136/jnnp.2006.102517
58. Kostera-Pruszczyk A, Kaminska A, Dutkiewicz M, Emeryk-Szajewska B, Strugalska-Cynowska MH, Vincent A, et al. MuSK-positive myasthenia gravis is rare in the Polish population. *Eur J Neurol.* (2008) 15:720–4. doi: 10.1111/j.1468-1331.2008.02176.x
59. Tsiamalos P, Kordas G, Kokla A, Poulas K, Tzartos SJ. Epidemiological and immunological profile of muscle-specific kinase myasthenia gravis in Greece. *Eur J Neurol.* (2009) 16:925–30. doi: 10.1111/j.1468-1331.2009.02624.x
60. Suzuki S, Utsugisawa K, Nagane Y, Satoh T, Kuwana M, Suzuki N. Clinical and immunological differences between early and late-onset myasthenia gravis in Japan. *J Neuroimmunol.* (2011) 230:148–52. doi: 10.1016/j.jneuroim.2010.10.023
61. Ohta K, Shigemoto K, Kubo S, Maruyama N, Abe Y, Ueda N, et al. MuSK antibodies in AChR Ab-seropositive MG vs AChR Ab-seronegative MG. *Neurology.* (2004) 62:2132–3. doi: 10.1212/01.WNL.0000129274.12702.92
62. Zouvelou V, Papathanasiou A, Koros C, Rentzos M, Zambelis T, Stamboulis E. Pure ocular anti-musk myasthenia under no immunosuppressive treatment. *Muscle Nerve.* (2013) 48:464. doi: 10.1002/mus.23847
63. Gomez AM, Van Den Broeck J, Vrolix K, Janssen SP, Lemmens MA, Van Der Esch E, et al. Antibody effector mechanisms in myasthenia gravis-pathogenesis at the neuromuscular junction. *Autoimmunity.* (2010) 43:353–70. doi: 10.3109/08916930903555943
64. Koneczny I, Stevens JA, De Rosa A, Huda S, Huijbers MG, Saxena A, et al. IgG4 autoantibodies against muscle-specific kinase undergo Fab-arm exchange in myasthenia gravis patients. *J Autoimmun.* (2017) 77:104–15. doi: 10.1016/j.jaut.2016.11.005
65. Kawakami Y, Ito M, Hirayama M, Sahashi K, Ohkawara B, Masuda A, et al. Anti-MuSK autoantibodies block binding of collagen Q to MuSK. *Neurology.* (2011) 77:1819–26. doi: 10.1212/WNL.0b013e318237f660
66. Huijbers MG, Zhang W, Klooster R, Niks EH, Friese MB, Straasheijm KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc Natl Acad Sci U.S.A.* (2013) 110:20783–8. doi: 10.1073/pnas.1313944110
67. Koneczny I, Cossins J, Waters P, Beeson D, Vincent A. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1–3 can disperse preformed agrin-independent AChR clusters. *PLoS ONE.* (2013) 8:e80695. doi: 10.1371/journal.pone.0080695
68. Bartoccioni E, Scuderi F, Minicuci GM, Marino M, Ciaraffa F, Evoli A. Anti-MuSK antibodies: correlation with myasthenia gravis severity. *Neurology.* (2006) 67:505–7. doi: 10.1212/01.wnl.0000228225.23349.5d
69. Niks EH, van Leeuwen Y, Leite MI, Dekker FW, Wintzen AR, Wirtz PW, et al. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. *J Neuroimmunol.* (2008) 195:151–6. doi: 10.1016/j.jneuroim.2008.01.013
70. Matthews I, Chen S, Hewer R, McGrath V, Furmaniak J, Rees Smith B. Muscle-specific receptor tyrosine kinase autoantibodies–a new immunoprecipitation assay. *Clin Chim Acta.* (2004) 348:95–9. doi: 10.1016/j.cccn.2004.05.008
71. Trakas N, Zisimopoulou P, Tzartos SJ. Development of a highly sensitive diagnostic assay for muscle-specific tyrosine kinase (MuSK) autoantibodies in myasthenia gravis. *J Neuroimmunol.* (2011) 240–241:79–86. doi: 10.1016/j.jneuroim.2011.09.007
72. Han J, Zhang J, Li M, Zhang Y, Lv J, Zhao X, et al. A novel MuSK cell-based myasthenia gravis diagnostic assay. *J Neuroimmunol.* (2019) 337:577076. doi: 10.1016/j.jneuroim.2019.577076
73. Li M, Han J, Zhang Y, Lv J, Zhang J, Zhao X, et al. Clinical analysis of Chinese anti-low-density-lipoprotein-receptor-associated protein 4 antibodies in patients with myasthenia gravis. *Eur J Neurol.* (2019) 26:1296-e84. doi: 10.1111/ene.13979
74. Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, et al. Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell.* (2008) 135:334–42. doi: 10.1016/j.cell.2008.10.002
75. Higuchi O, Hamuro J, Motomura M, Yamanashi Y. Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. *Ann Neurol.* (2011) 69:418–22. doi: 10.1002/ana.22312
76. Pevzner A, Schoser B, Peters K, Cosma NC, Karakatsani A, Schalke B, et al. Anti-LRP4 autoantibodies in AChR- and MuSK-antibody-negative myasthenia gravis. *J Neurol.* (2012) 259:427–35. doi: 10.1007/s00415-011-6194-7
77. Zhang B, Tzartos JS, Belimezi M, Ragheb S, Bealmear B, Lewis RA, et al. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. *Arch Neurol.* (2012) 69:445–51. doi: 10.1001/archneurol.2011.2393
78. Shen C, Lu Y, Zhang B, Figueiredo D, Bean J, Jung J, et al. Antibodies against low-density lipoprotein receptor-related protein 4 induce myasthenia gravis. *J Clin Invest.* (2013) 123:5190–202. doi: 10.1172/JCI66039
79. Li Y, Zhang Y, Cai G, He D, Dai Q, Xu Z, et al. Anti-LRP4 autoantibodies in Chinese patients with myasthenia gravis. *Muscle Nerve.* (2017) 56:938–42. doi: 10.1002/mus.25591
80. Zisimopoulou P, Evangelakou P, Tzartos J, Lazaridis K, Zouvelou V, Mantegazza R, et al. A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis. *J Autoimmun.* (2014) 52:139–45. doi: 10.1016/j.jaut.2013.12.004
81. Cordts I, Bodart N, Hartmann K, Karagiorgou K, Tzartos JS, Mei L, et al. Screening for lipoprotein receptor-related protein 4-, agrin-, and titin-antibodies and exploring the autoimmune spectrum in myasthenia gravis. *J Neurol.* (2017) 264:1193–203. doi: 10.1007/s00415-017-8514-z
82. Tzartos JS, Zisimopoulou P, Rentzos M, Karandreas N, Zouvelou V, Evangelakou P, et al. LRP4 antibodies in serum and CSF from amyotrophic lateral sclerosis patients. *Ann Clin Transl Neurol.* (2014) 1:80–7. doi: 10.1002/acn3.26
83. Rivner MH, Liu S, Quarles B, Fleenor B, Shen C, Pan J, et al. Agrin and low-density lipoprotein-related receptor protein 4 antibodies in amyotrophic lateral sclerosis patients. *Muscle Nerve.* (2017) 55:430–2. doi: 10.1002/mus.25438
84. Williams CL, Lennon VA. Thymic B lymphocyte clones from patients with myasthenia gravis secrete monoclonal striational autoantibodies reacting with myosin, alpha actinin, or actin. *J Exp Med.* (1986) 164:1043–59. doi: 10.1084/jem.164.4.1043
85. Yamamoto T, Sato T, Sugita H. Antifilamin, antivinculin, and antitropomyosin antibodies in myasthenia gravis. *Neurology.* (1987) 37:1329–33. doi: 10.1212/WNL.37.8.1329
86. Aarli JA, Stefansson K, Marton LS, Wollmann RL. Patients with myasthenia gravis and thymoma have in their sera IgG autoantibodies against titin. *Clin Exp Immunol.* (1990) 82:284–8. doi: 10.1111/j.1365-2249.1990.tb05440.x
87. Bang ML, Centner T, Fornoff F, Geach AJ, Gotthardt M, McNabb M, et al. The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. *Circ Res.* (2001) 89:1065–72. doi: 10.1161/hh2301.100981

88. Gautel M, Lakey A, Barlow DP, Holmes Z, Scales S, Leonard K, et al. Titin antibodies in myasthenia gravis: identification of a major immunogenic region of titin. *Neurology.* (1993) 43:1581–5. doi: 10.1212/WNL.43.8.1581
89. Buckley C, Newsom-Davis J, Willcox N, Vincent A. Do titin and cytokine antibodies in MG patients predict thymoma or thymoma recurrence? *Neurology.* (2001) 57:1579–82. doi: 10.1212/WNL.57.9.1579
90. Yamamoto AM, Gajdos P, Eymard B, Tranchant C, Warter JM, Gomez L, et al. Anti-titin antibodies in myasthenia gravis: tight association with thymoma and heterogeneity of nonthymoma patients. *Arch Neurol.* (2001) 58:885–90. doi: 10.1001/archneur.58.6.885
91. Romi F, Skeie GO, Gilhus NE, Aarli JA. Striational antibodies in myasthenia gravis: reactivity and possible clinical significance. *Arch Neurol.* (2005) 62:442–6. doi: 10.1001/archneur.62.3.442
92. Aarli JA. Myasthenia gravis in the elderly: is it different? *Ann N Y Acad Sci.* (2008) 1132:238–43. doi: 10.1196/annals.1405.040
93. Szczudlik P, Szyluk B, Lipowska M, Ryniewicz B, Kubiszewska J, Dutkiewicz M, et al. Antititin antibody in early- and late-onset myasthenia gravis. *Acta Neurol Scand.* (2014) 130:229–33. doi: 10.1111/ane.12271
94. Baggi F, Andreetta F, Antozzi C, Simoncini O, Confalonieri P, Labeit S, et al. Anti-titin and antiryanodine receptor antibodies in myasthenia gravis patients with thymoma. *Ann N Y Acad Sci.* (1998) 841:538–41. doi: 10.1111/j.1749-6632.1998.tb10978.x
95. Romi F, Skeie GO, Aarli JA, Gilhus NE. Muscle autoantibodies in subgroups of myasthenia gravis patients. *J Neurol.* (2000) 247:369–75. doi: 10.1007/s004150050604
96. Chen XJ, Qiao J, Xiao BG, Lu CZ. The significance of titin antibodies in myasthenia gravis–correlation with thymoma and severity of myasthenia gravis. *J Neurol.* (2004) 251:1006–11. doi: 10.1007/s00415-004-0479-z
97. Choi Decroos E, Hobson-Webb LD, Juel VC, Massey JM, Sanders DB. Do acetylcholine receptor and striated muscle antibodies predict the presence of thymoma in patients with myasthenia gravis? *Muscle Nerve.* (2014) 49:30–4. doi: 10.1002/mus.23882
98. Somnier FE, Engel PJ. The occurrence of anti-titin antibodies and thymomas: a population survey of MG 1970–1999. *Neurology.* (2002) 59:92–8. doi: 10.1212/WNL.59.1.92
99. Stergiou C, Lazaridis K, Zouvelou V, Tzartos J, Mantegazza R, Antozzi C, et al. Titin antibodies in "seronegative" myasthenia gravis–A new role for an old antigen. *J Neuroimmunol.* (2016) 292:108–15. doi: 10.1016/j.jneuroim.2016.01.018
100. Kufukihara K, Watanabe Y, Inagaki T, Takamatsu K, Nakane S, Nakahara J, et al. Cytometric cell-based assays for anti-striational antibodies in myasthenia gravis with myositis and/or myocarditis. *Sci Rep.* (2019) 9:5284. doi: 10.1038/s41598-019-41730-z
101. Skeie GO, Mygland A, Treves S, Gilhus NE, Aarli JA, Zorzato F. Ryanodine receptor antibodies in myasthenia gravis: epitope mapping and effect on calcium release *in vitro*. *Muscle Nerve.* (2003) 27:81–9. doi: 10.1002/mus.10294
102. Mygland A, Tysnes OB, Matre R, Volpe P, Aarli JA, Gilhus NE. Ryanodine receptor autoantibodies in myasthenia gravis patients with a thymoma. *Ann Neurol.* (1992) 32:589–91. doi: 10.1002/ana.410320419
103. Takamori M, Motomura M, Kawaguchi N, Nemoto Y, Hattori T, Yoshikawa H, et al. Anti-ryanodine receptor antibodies and FK506 in myasthenia gravis. *Neurology.* (2004) 62:1894–6. doi: 10.1212/01.WNL.0000125254.99397.68
104. Skeie GO, Lunde PK, Sejersted OM, Mygland A, Aarli JA, Gilhus NE. Myasthenia gravis sera containing antiryanodine receptor antibodies inhibit binding of [3H]-ryanodine to sarcoplasmic reticulum. *Muscle Nerve.* (1998) 21:329–35. doi: 10.1002/(sici)1097-4598(199803)21:3<329::aid-mus6>3.0.co;2-c
105. Romi F, Aarli JA, Gilhus NE. Myasthenia gravis patients with ryanodine receptor antibodies have distinctive clinical features. *Eur J Neurol.* (2007) 14:617–20. doi: 10.1111/j.1468-1331.2007.01785.x
106. Cossins J, Belaya K, Zoltowska K, Koneczny I, Maxwell S, Jacobson L, et al. The search for new antigenic targets in myasthenia gravis. *Ann N Y Acad Sci.* (2012) 1275:123–8. doi: 10.1111/j.1749-6632.2012.06833.x
107. Gasperi C, Melms A, Schoser B, Zhang Y, Meltoranta J, Risson V, et al. Anti-agrin autoantibodies in myasthenia gravis. *Neurology.* (2014) 82:1976–83. doi: 10.1212/WNL.0000000000000478
108. Zhang B, Shen C, Bealmear B, Ragheb S, Xiong WC, Lewis RA, et al. Autoantibodies to agrin in myasthenia gravis patients. *PLoS ONE.* (2014) 9:e91816. doi: 10.1371/journal.pone.0091816
109. Yan M, Liu Z, Fei E, Chen W, Lai X, Luo B, et al. Induction of anti-agrin antibodies causes myasthenia gravis in mice. *Neuroscience.* (2018) 373:113–21. doi: 10.1016/j.neuroscience.2018.01.015
110. Suzuki S, Satoh T, Yasuoka H, Hamaguchi Y, Tanaka K, Kawakami Y, et al. Novel autoantibodies to a voltage-gated potassium channel Kv1.4 in a severe form of myasthenia gravis. *J Neuroimmunol.* (2005) 170:141–9. doi: 10.1016/j.jneuroim.2005.08.017
111. Suzuki S, Utsugisawa K, Yoshikawa H, Motomura M, Matsubara S, Yokoyama K, et al. Autoimmune targets of heart and skeletal muscles in myasthenia gravis. *Arch Neurol.* (2009) 66:1334–8. doi: 10.1001/archneurol.2009.229
112. Suzuki S, Baba A, Kaida K, Utsugisawa K, Kita Y, Tsugawa J, et al. Cardiac involvements in myasthenia gravis associated with anti-Kv1.4 antibodies. *Eur J Neurol.* (2014) 21:223–30. doi: 10.1111/ene.12234
113. Romi F, Suzuki S, Suzuki N, Petzold A, Plant GT, Gilhus NE. Anti-voltage-gated potassium channel Kv1.4 antibodies in myasthenia gravis. *J Neurol.* (2012) 259:1312–6. doi: 10.1007/s00415-011-6344-y
114. Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, et al. Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature.* (1995) 377:232–6. doi: 10.1038/377232a0
115. Agius MA, Zhu S, Kirvan CA, Schafer AL, Lin MY, Fairclough RH, et al. Rapsyn antibodies in myasthenia gravis. *Ann N Y Acad Sci.* (1998) 841:516–21. doi: 10.1111/j.1749-6632.1998.tb10972.x
116. Agius MA, Zhu S, Aarli JA. Antirapsyn antibodies occur commonly in patients with lupus. *Ann N Y Acad Sci.* (1998) 841:525–6. doi: 10.1111/j.1749-6632.1998.tb10974.x
117. Gallardo E, Martinez-Hernandez E, Titulaer MJ, Huijbers MG, Martinez MA, Ramos A, et al. Cortactin autoantibodies in myasthenia gravis. *Autoimmun Rev.* (2014) 13:1003–7. doi: 10.1016/j.autrev.2014.08.039
118. Cortes-Vicente E, Gallardo E, Martinez MA, Diaz-Manera J, Querol L, Rojas-Garcia R, et al. Clinical characteristics of patients with double-seronegative myasthenia gravis and antibodies to cortactin. *JAMA Neurol.* (2016) 73:1099–104. doi: 10.1001/jamaneurol.2016.2032
119. Illa I, Cortes-Vicente E, Martinez MA, Gallardo E. Diagnostic utility of cortactin antibodies in myasthenia gravis. *Ann N Y Acad Sci.* (2018) 1412:90–4. doi: 10.1111/nyas.13502
120. Labrador-Horrillo M, Martinez MA, Selva-O'Callaghan A, Trallero-Araguas E, Grau-Junyent JM, Vilardell-Tarres M, et al. Identification of a novel myositis-associated antibody directed against cortactin. *Autoimmun Rev.* (2014) 13:1008–12. doi: 10.1016/j.autrev.2014.08.038
121. Mappouras DG, Philippou G, Haralambous S, Tzartos SJ, Balafas A, Souvatzoglou A, et al. Antibodies to acetylcholinesterase cross-reacting with thyroglobulin in myasthenia gravis and Graves's disease. *Clin Exp Immunol.* (1995) 100:336–43. doi: 10.1111/j.1365-2249.1995.tb03674.x
122. Geen J, Howells RC, Ludgate M, Hullin DA, Hogg SI. The prevalence of anti-acetylcholinesterase antibodies in autoimmune disease. *Autoimmunity.* (2004) 37:579–85. doi: 10.1080/08916930400021360
123. Provenzano C, Marino M, Scuderi F, Evoli A, Bartoccioni E. Anti-acetylcholinesterase antibodies associate with ocular myasthenia gravis. *J Neuroimmunol.* (2010) 218:102–6. doi: 10.1016/j.jneuroim.2009.11.004
124. Cartaud A, Strochlic L, Guerra M, Blanchard B, Lambergeon M, Krejci E, et al. MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. *J Cell Biol.* (2004) 165:505–15. doi: 10.1083/jcb.200307164
125. Zoltowska Katarzyna M, Belaya K, Leite M, Patrick W, Vincent A, Beeson D. Collagen Q–a potential target for autoantibodies in myasthenia gravis. *J Neurol Sci.* (2015) 348:241–4. doi: 10.1016/j.jns.2014.12.015
126. Tu H, Pirskanen-Matell R, Heikkinen A, Oikarainen T, Risteli J, Pihlajaniemi T. Autoimmune antibodies to collagen XIII in myasthenia gravis patients. *Muscle Nerve.* (2018) 57:506–10. doi: 10.1002/mus.25969
127. De Bellis A, Sansone D, Coronella C, Conte M, Iorio S, Perrino S, et al. Serum antibodies to collagen XIII: a further good marker of active Graves' ophthalmopathy. *Clin Endocrinol.* (2005) 62:24–9. doi: 10.1111/j.1365-2265.2004.02167.x
128. Berger B, Stich O, Labeit S, Rauer S. Screening for anti-titin antibodies in patients with various paraneoplastic neurological syndromes. *J*

*Neuroimmunol.* (2016) 295–296:18–20. doi: 10.1016/j.jneuroim.2016.04.004
129. Evoli A, Tonali PA, Padua L, Monaco ML, Scuderi F, Batocchi AP, et al. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. *Brain.* (2003) 126(Pt 10):2304–11. doi: 10.1093/brain/awg223
130. Evoli A, Bianchi MR, Riso R, Minicuci GM, Batocchi AP, Servidei S, et al. Response to therapy in myasthenia gravis with anti-MuSK antibodies. *Ann N Y Acad Sci.* (2008) 1132:76–83. doi: 10.1196/annals.1405.012
131. Guptill JT, Sanders DB, Evoli A. Anti-MuSK antibody myasthenia gravis: clinical findings and response to treatment in two large cohorts. *Muscle Nerve.* (2011) 44:36–40. doi: 10.1002/mus.22006
132. Silvestri NJ, Wolfe GI. Treatment-refractory myasthenia gravis. *J Clin Neuromuscul Dis.* (2014) 15:167–78. doi: 10.1097/CND.0000000000000034
133. Iorio R, Damato V, Alboini PE, Evoli A. Efficacy and safety of rituximab for myasthenia gravis: a systematic review and meta-analysis. *J Neurol.* (2015) 262:1115–9. doi: 10.1007/s00415-014-7532-3
134. Leite MI, Strobel P, Jones M, Micklem K, Moritz R, Gold R, et al. Fewer thymic changes in MuSK antibody-positive than in MuSK antibody-negative MG. *Ann Neurol.* (2005) 57:444–8. doi: 10.1002/ana.20386
135. Barnett C, Tabasinejad R, Bril V. Current pharmacotherapeutic options for myasthenia gravis. *Expert Opin Pharmacother.* (2019) 20:2295–303. doi: 10.1080/14656566.2019.1682548
136. Lagoumintzis G, Zisimopoulou P, Kordas G, Lazaridis K, Poulas K, Tzartos SJ. Recent approaches to the development of antigen-specific immunotherapies for myasthenia gravis. *Autoimmunity.* (2010) 43:436–45. doi: 10.3109/08916930903518099
137. Lazaridis K, Zisimopoulou P, Lagoumintzis G, Skriapa L, Trakas N, Evangelakou P, et al. Antigen-specific apheresis of autoantibodies in myasthenia gravis. *Ann N Y Acad Sci.* (2012) 1275:7–12. doi: 10.1111/j.1749-6632.2012.06788.x
138. Lazaridis K, Evaggelakou P, Bentenidi E, Sideri A, Grapsa E, Tzartos SJ. Specific adsorbents for myasthenia gravis autoantibodies using mutants of the muscle nicotinic acetylcholine receptor extracellular domains. *J Neuroimmunol.* (2015) 278:19–25. doi: 10.1016/j.jneuroim.2014.12.001
139. Lazaridis K, Dalianoudis I, Baltatzidi V, Tzartos SJ. Specific removal of autoantibodies by extracorporeal immunoadsorption ameliorates experimental autoimmune myasthenia gravis. *J Neuroimmunol.* (2017) 312:24–30. doi: 10.1016/j.jneuroim.2017.09.001
140. Lazaridis K, Baltatzidou V, Tektonidis N, Tzartos SJ. Antigen-specific immunoadsorption of MuSK autoantibodies as a treatment of MuSK-induced experimental autoimmune myasthenia gravis. *J Neuroimmunol.* (2020) 339:577136. doi: 10.1016/j.jneuroim.2019.577136
141. Okumura S, McIntosh K, Drachman DB. Oral administration of acetylcholine receptor: effects on experimental myasthenia gravis. *Ann Neurol.* (1994) 36:704–13. doi: 10.1002/ana.410360504
142. Ma CG, Zhang GX, Xiao BG, Link J, Olsson T, Link H. Suppression of experimental autoimmune myasthenia gravis by nasal administration of acetylcholine receptor. *J Neuroimmunol.* (1995) 58:51–60. doi: 10.1016/0165-5728(94)00187-S
143. Barchan D, Souroujon MC, Im SH, Antozzi C, Fuchs S. Antigen-specific modulation of experimental myasthenia gravis: nasal tolerization with recombinant fragments of the human acetylcholine receptor alpha-subunit. *Proc Natl Acad Sci U.S.A.* (1999) 96:8086–91. doi: 10.1073/pnas.96.14.8086
144. Karachunski PI, Ostlie NS, Okita DK, Conti-Fine BM. Prevention of experimental myasthenia gravis by nasal administration of synthetic acetylcholine receptor T epitope sequences. *J Clin Invest.* (1997) 100:3027–35. doi: 10.1172/JCI119857
145. Baggi F, Andreetta F, Caspani E, Milani M, Longhi R, Mantegazza R, et al. Oral administration of an immunodominant T-cell epitope downregulates Th1/Th2 cytokines and prevents experimental myasthenia gravis. *J Clin Invest.* (1999) 104:1287–95. doi: 10.1172/JCI7121
146. Wu B, Deng C, Goluszko E, Christadoss P. Tolerance to a dominant T cell epitope in the acetylcholine receptor molecule induces epitope spread and suppresses murine myasthenia gravis. *J Immunol.* (1997) 159:3016–23.
147. Luo J, Lindstrom J. AChR-specific immunosuppressive therapy of myasthenia gravis. *Biochem Pharmacol.* (2015) 97:609–19. doi: 10.1016/j.bcp.2015.07.011
148. Lindstrom J, Luo J, Kuryatov A. Myasthenia gravis and the tops and bottoms of AChRs: antigenic structure of the MIR and specific immunosuppression of EAMG using AChR cytoplasmic domains. *Ann N Y Acad Sci.* (2008) 1132:29–41. doi: 10.1196/annals.1405.007
149. Luo J, Lindstrom J. Antigen-specific immunotherapeutic vaccine for experimental autoimmune myasthenia gravis. *J Immunol.* (2014) 193:5044–55. doi: 10.4049/jimmunol.1401392
150. Lindstrom J, Peng X, Kuryatov A, Lee E, Anand R, Gerzanich V, et al. Molecular and antigenic structure of nicotinic acetylcholine receptors. *Ann N Y Acad Sci.* (1998) 841:71–86. doi: 10.1111/j.1749-6632.1998.tb10910.x
151. Yi HJ, Chae CS, So JS, Tzartos SJ, Souroujon MC, Fuchs S, et al. Suppression of experimental myasthenia gravis by a B-cell epitope-free recombinant acetylcholine receptor. *Mol Immunol.* (2008) 46:192–201. doi: 10.1016/j.molimm.2008.08.264
152. Luo J, Kuryatov A, Lindstrom JM. Specific immunotherapy of experimental myasthenia gravis by a novel mechanism. *Ann Neurol.* (2010) 67:441–51. doi: 10.1002/ana.21901
153. Lazaridis K, Zisimopoulou P, Giastas P, Bitzopoulou K, Evangelakou P, Sideri A, et al. Expression of human AChR extracellular domain mutants with improved characteristics. *Int J Biol Macromol.* (2014) 63:210–7. doi: 10.1016/j.ijbiomac.2013.11.003
154. Leite MI, Waters P, Vincent A. Diagnostic use of autoantibodies in myasthenia gravis. *Autoimmunity.* (2010) 43:371–9. doi: 10.3109/08916930903541208
155. Vincent A, Waters P, Leite MI, Jacobson L, Koneczny I, Cossins J, et al. Antibodies identified by cell-based assays in myasthenia gravis and associated diseases. *Ann N Y Acad Sci.* (2012) 1274:92–8. doi: 10.1111/j.1749-6632.2012.06789.x
156. Steinman L. The road not taken: antigen-specific therapy and neuroinflammatory disease. *JAMA Neurol.* (2013) 70:1100–1. doi: 10.1001/jamaneurol.2013.3553

**Conflict of Interest:** ST has shares in the research and diagnostic laboratory Tzartos NeuroDiagnostics.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 February 2020
doi: 10.3389/fimmu.2020.00240

# New Approaches to Targeting B Cells for Myasthenia Gravis Therapy

*Ruksana Huda**

*Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, United States*

Current therapies for myasthenia gravis (MG) are limited, and many investigations have recently focused on target-specific therapies. B cell-targeting monoclonal antibody (mAb) therapies for MG are increasingly attractive due to their specificity and efficacy. The targeted B cell biomarkers are mainly the cluster of differentiation (CD) proteins that mediate maturation, differentiation, or survival of pathogenic B cells. Additional B cell-directed therapies include non-specific peptide inhibitors that preferentially target specific B cell subsets. The primary goals of such therapies are to intercept autoantibodies and prevent the generation of an inflammatory response that contributes to the pathogenesis of MG. Treatment of patients with MG using B cell-directed mAbs, antibody fragments, or selective inhibitors have exhibited moderate to high efficacy in early studies, and some of these therapies appear to be highly promising for further drug development. Numerous other biologics targeting various B cell surface molecules have been approved for the treatment of other conditions or are either in clinical trials or preclinical development stages. These approaches remain to be tested in patients with MG or animal models of the disease. This review article provides an overview of B cell-targeted treatments for MG, including those already available and those still in preclinical and clinical development. We also discuss the potential benefits as well as the shortcomings of these approaches to development of new therapies for MG and future directions in the field.

**Keywords: myasthenia gravis, experimental autoimmune myasthenia gravis, B cell, AChR, MuSK, autoantibody**

OPEN ACCESS

***Edited by:***
*Sonia Berrih-Aknin, Institut National de la Santé et de la Recherche Médicale (INSERM), France*

***Reviewed by:***
*Pilar Martinez, Maastricht University, Netherlands*
*Guenter Steiner, Medical University of Vienna, Austria*

***Correspondence:**
*Ruksana Huda*
*rhuda@utmb.edu*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *06 November 2019*
***Accepted:*** *29 January 2020*
***Published:*** *21 February 2020*

***Citation:***
*Huda R (2020) New Approaches to Targeting B Cells for Myasthenia Gravis Therapy. Front. Immunol. 11:240. doi: 10.3389/fimmu.2020.00240*

## INTRODUCTION

Myasthenia gravis (MG) is a chronic autoimmune neuromuscular disorder. Patients with MG who are seropositive for autoantibodies to the acetylcholine receptor (AChR), muscle-specific tyrosine kinase (MuSK), or low-density lipoprotein receptor-related protein 4 (LRP4) present with voluntary muscle weakness due to dysfunctional neuromuscular junctions and impaired neuromuscular transmission (1, 2). Traditional therapies for MG including thymectomy, intravenous immunoglobulin (IVIg), plasmapheresis, and corticosteroid therapy can induce remission, but do not cure the disease. Furthermore, 10–20% of patients remain refractory to immunosuppressive therapies (3). Hence, there is an immense need for new and effective treatments for MG, particularly refractory disease.

B cell-directed monoclonal antibody (mAb) therapies show great promise, and many are currently under development. These approaches primarily intend to eliminate or reduce the numbers of intact plasma B cells, or precursors of plasma cells, by blocking specific cell-surface biomarkers or cluster of differentiation (CD) antigens (4, 5). B cells have also been targeted indirectly, through inhibition of crucial molecules expressed by T-helper cells or other immune cells, or by inhibition of cytokines and chemokines that mediate affinity-maturation of B cells

into plasma cells. Important membrane or signaling proteins that are exclusively or abundantly expressed in B cells or that contribute to their growth, survival, or overactivity in the context of autoantibody production and MG pathogenicity are typically regarded as B cell-specific therapeutic targets or biomarkers. To date, novel interventions designed to target such biomarkers have mostly comprised monoclonal antibody-based treatment approaches. Antibody fragment-based therapies have also been investigated, while peptide- or RNA-based therapies are less common, and many new potential therapies are rapidly emerging.

Both preclinical animal models and clinical studies in patients have provided abundant information on B cell-targeting therapies. A rodent model of MG (experimental autoimmune MG, EAMG), induced by immunization with Torpedo AChR, typically mimics clinical aspects of generalized MG (6, 7). Although in their early stages, the results from many preclinical studies are of considerable importance for future translational research and clinical application of new therapies. This review describes recent advances in targeted therapeutic approaches for MG, with a specific focus on B cell-targeted treatments for this disease.

## B CELLS AS A TARGET FOR MG THERAPY

Both B cells and T cells are central players in the adaptive immune system. The signaling mechanisms that regulate B cell differentiation and activation processes are incompletely understood. Therefore, current B cell-directed therapies focus primarily on targeting intermediary (e.g., mature B cell subsets or plasmablasts) or terminally differentiated (e.g., plasma cells or memory B cells) B cell subsets with pathogenic implications.

### B Cell Biology and Subsets

B cell development begins in the bone marrow, the primary lymphoid organ, with the expression of B cell receptors (BCRs) in progenitor (Pro)-B cells (8, 9). Pro-B cells generate Pre-B cells, with complete light chain rearrangement and IgM expression. These Pre-B cells then leave the bone marrow to enter the peripheral circulation as transitional or mature B cell subsets. With the help of $CD4^+$ T cells, mature B cells are activated to undergo somatic hypermutation and clonal selection, which generates follicular B cells in the secondary lymphoid organs, preferentially the spleen. These cells form germinal centers (GCs) and plasma cells that produce high-affinity class-switched antibodies, and memory and surrounding marginal zone B cells (**Figure 1**). Long-lived plasma cells stably maintain serum antibody levels, whereas memory B cells are responsible for recall responses upon antigen re-exposure (10). Most memory B cells and some long-lived plasma cells migrate to the bone marrow and replenish circulatory antibodies when needed. Although B cells reside predominantly in secondary lymphoid tissues (spleen and lymph nodes), during an infection or disease condition their numbers increase in peripheral blood and non-lymphoid tissues (11, 12). With the assistance of tissue-resident memory T cells, tissue-resident B cells may induce rapid plasmablast responses.

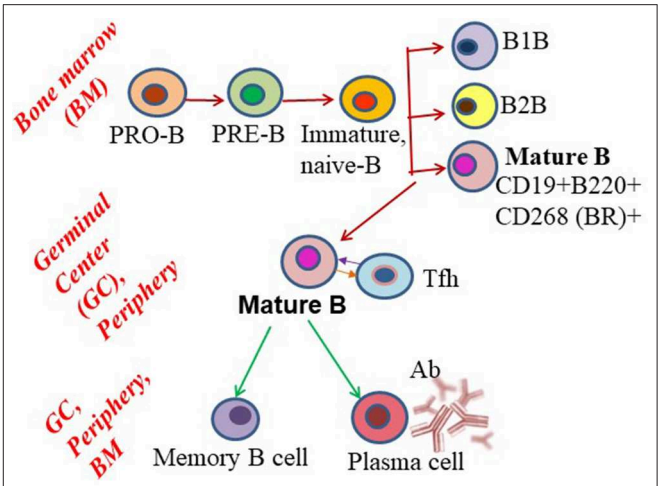



**FIGURE 1 |** Schematic showing B cell subset differentiation. Pro-B cells progress through a series of developmental steps to generate mature B cells, which further differentiate into plasma and memory B cells.

### Rationale for MG Therapies Targeting B Cells

MG is caused by autoantibody produced exclusively from autoantigen-specific plasma B cells (13). In approximately 80% of MG patients, the autoantigen is AChR, and the pathogenic autoantibodies are mainly IgG1 and IgG3 isotype anti-AChR autoantibodies (IgG2b in the EAMG mouse model). Anti-AChR antibodies cause generalized MG (affecting body muscles) and ocular MG (afflicting extraocular or eye muscles), which occur in ∼85 and 50% of patients, respectively. Around 40% of patients seronegative for anti-AChR antibody, present with IgG4 isotype (IgG1 in mouse) antibodies against MuSK. An IgG2a autoantibody against the agrin receptor, LRP4, has also been detected in patients with MG seronegative for both anti-AChR and anti-MuSK antibodies (14). The mechanisms by which autoantibodies cause muscle pathology in MG have been well-described. Most anti-AChR autoantibodies recognize the main immunogenic region present in the α subunit of AChR, which is a four subunit protein. Binding of anti-AChR autoantibodies with AChR in the membrane reduces the availability of the functional receptor to ACh, either by blocking the receptor or leading to its internalization. Subsequent activation of complement cascades by the autoantibody leading to the formation of a "membrane attack complex", also lyses myocytes (15, 16). Both MuSK and LRP autoantibodies can disperse postsynaptic AChR clusters and thereby cause AChR deficiency and muscle fatigue (17). Based on adequate evidence that autoantibodies cause MG development and progression through depletion of molecules critical for muscle function, as well as contributing to persistent inflammation, the majority of recent B cell-targeted therapies have focused on depleting B cells, thus reducing or removing the source of autoantigen-specific autoantibodies in patients with MG.

In addition to their main pathogenic role in autoantibody generation, B cells also serve as antigen-presenting cells to

directly bind antigen on the BCR and present intracellularly processed antigenic peptides on their surface major histocompatibility complex (MHC) class II molecules (18). By upregulating costimulatory molecules, B cells then activate T cells to regulate their proinflammatory effector functions through secretion of a variety of cytokines, including tumor necrosis factor (TNF)-α, lymphotoxin, and granulocyte macrophage-colony-stimulating factor (19). In contrast, regulatory B cells (B-regs) secrete the anti-inflammatory cytokine interleukin 10 (IL10) contributing to B cell tolerance. Given their pathogenic roles associated with autoantibody and inflammatory mediator production, B cells are considered a preferred target for therapeutic intervention in MG.

## CD Biomarkers for B Cell-Directed Therapy Approaches

The CD antigens are immune cell biomarkers designated at Human Leukocyte Differentiation Antigens Workshops, which are held worldwide. B cells constitutively express a variety of CD antigens on their surfaces, which define distinct B cell subsets, in association with one or more specific biological functions such as survival, adhesion, activation, or inhibition. Due to their differential expression or activation in disease states, CD molecules serve as valuable cell surface signatures for B cell-targeted therapies in clinical trials.

To date, at least 58 CD molecules are established as expressed by B cells; these belong to the Ig superfamily (Ig-SF), tumor necrosis factor receptor superfamily (TNFR-SF), and cytokine receptor family. The Ig-SF includes five sub-families of CD antigens: Fc receptor-like (FCR-L), FCR, signaling lymphocytic activation molecule (SLAM), triggering receptors expressed on myeloid cells (TREM), and nectin. The following are among B cell-restricted CD antigens exclusively expressed (bolded) on B cells, and expressed either as a receptor or a ligand on B cells: CD10, **CD19**, **CD20**, **CD21**, **CD22**, CD23, CD24, CD27, CD37 to CD39, CD40, CD72 to CD78, **CD79a**, **CD79b**, CD80 to CD86, CD138, CD139, **CD179a**, **CD179b**, CD180, CD252, CD254, CD267 to CD269, CD275, CD307e, CD315 to CD317, CD307a to CD307d, and CD351 to CD363 (20, 21). Some therapeutically relevant B cell subtypes associated with exclusive expression of specific CD surface markers include plasma cells, which express CD269 and CD138 (Syndecan-1); mature B cells, expressing CD19, CD268, and CD79b; and memory B cells, with CD27. Many other B cell-specific CD antigens have been targeted (**Figure 2**), and those yet to be explored or targeted have potential for development as new diagnostic markers for MG therapy in the near future.

## Major Mechanisms Associated With B Cell-Targeted mAb Therapy

The mechanisms by which CD antigen-specific mAbs mediate B cell depletion can be direct apoptosis of B cells, but often antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP), while complement-dependent cytotoxicity (CDC) or cellular toxicity (CDCC) are also used (22) (**Figures 3A–D**). In ADCC, mAb binding to the B cell epitope is immediately followed by crosslinking of the fragment crystallizable (Fc) region of the mAb with the Fc receptor (FcγR) of effector cells (usually macrophages). The effector cells then polarize and release cytotoxic granules by perforin- or granzyme-mediated apoptotic pathways to destroy target B cells. During ADCP, once a mAb is bound to the target B cell, Fc-FcγR crosslinking activates effector cells, and the target cell is phagocytosed by the effector cell for intracellular destruction. In CDC/CDCC, binding of the Fc portion of B cell-bound mAb with C1q (a complement component) initiates activation of the complement cascade. C3b and C4b act as opsonins and subsequently form membrane-attack complexes (MACs) on target cells, to perforate the cell for lysis. Engineering the Fc arm of a mAb (e.g., glycoengineering and site mutagenesis) may further increase its effector function and serum stability (23).

# CLINICAL APPROACHES TO B CELL-TARGETED THERAPY FOR MG

The first therapeutic mAb for targeted therapy in patients was a mouse anti-CD3 mAb, muromonab, used to prevent tissue rejection (24). Subsequently, mAbs have been engineered to incorporate both human and mouse sequences (humanized), followed by production of fully human recombinant mAbs. Chimeric (xi) antibodies consist of human amino acid sequences in the constant region, while humanized (zu) mAbs contain human sequences in the variable region. mAbs with partial chimeric and humanized sequences (xizu) or fully human (u) sequences have also been produced. The abbreviation "-ci (r)" is used to describe mAbs that target a circulatory system component, while li(m) denotes targeting of the immune system (e.g., -limumab) (25) (**Figure 4**). Many mAbs have been produced against different epitopes of the same CD molecule; however, their therapeutic potential has only been tested in one or a few specific diseases. Bi- or multi-specific mAbs that recognize two or multiple epitopes of the same antigen have also been developed. Single-chain variable fragment (scFv) molecules, consisting of fusions of variable regions of heavy and light chains connected by a short peptide linker, have also been used. Currently, the only additional non-mAb biologicals applied for targeted therapy are peptide inhibitors and recently generated antibody (Ab)-mimetics, which are ligand-specific, small synthetic proteins.

## Biologicals That Directly Target B Cells in MG

### mAbs

#### *CD20-targeting mAbs*

Rituximab or RTX (also known as Rituxan, Rixathon, or Truxima [Genentech, San Francisco, CA, USA]) is a chimeric murine-human IgG1*k* mAb that targets CD20, a 33-kDa protein expressed on pro-B cells and all mature B cells, but not long-lived plasma or plasmablast cells. CD20 has an important role in the growth and differentiation of B cells into plasma cells, and rituximab can efficiently deplete CD20-positive B cells in MG

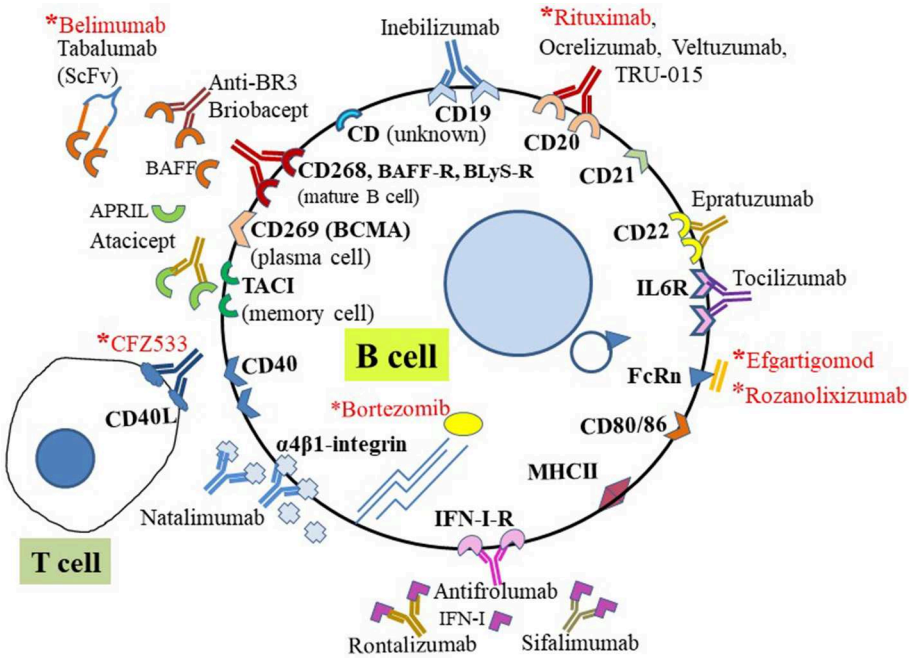



**FIGURE 2 |** B cell-targeting therapies using CD surface biomarkers. Schematic representation of representative CD antigens expressed on the human B cell surface and targeted for B cell-specific therapy in autoimmune diseases. Those with asterisks (red) have been targeted for potential treatment of MG and are either approved for treatment or under investigation. For direct targeting, biologics (e.g., mAb or mAb fragments) directly bind cell surface CD molecules or receptors. Indirect treatments involve targeting soluble ligands of receptors.

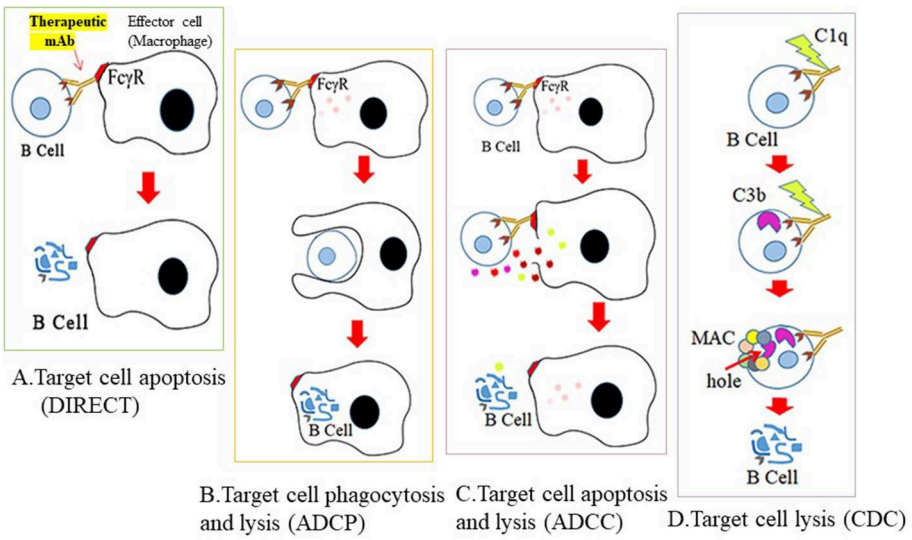



**FIGURE 3 |** Mechanisms for therapeutic depletion of B cells. Therapeutic antibodies mediate their cytotoxic effects by four possible mechanisms. Cross-linking of mAb to B cell surface antigen **(A)** blocks ligand binding of essential receptors that mediate B cell survival (direct apoptosis), **(B)** triggers engagement of effector cells through recognition of its Fc sequence, and subsequent phagocytosis by effector cells (ADCP), **(C)** lysis by granzymes secreted from effector cells (ADCC), or **(D)** activates the complement cascade and lysis by C3 deposits and MAC formation on the cell surface.

patients; however, it is ineffective in reducing pathogenic AChR-Ab levels (26). Long-lived plasma cells are the major producers of autoAb and lack CD20, hence rituximab targets only short-lived plasma cells and $CD20^{+}$, IL10-producing B-regs, or B10 cells, and reduction of autoAb is generally short term and insufficient, resulting in only transient clinical improvement (27). Thus, rituximab-treated AChR-MG and MuSK-MG patients often have disease relapse or recurrence after an initial phase of disease

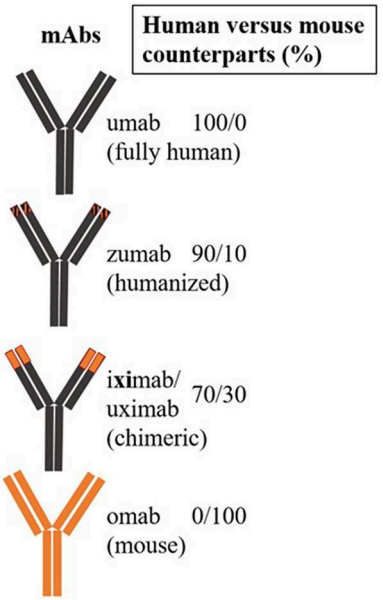



**FIGURE 4 |** Diagram showing mAb characterization and naming for therapeutic use.

remission (28). Nevertheless, some studies have reported the efficacy of rituximab for treatment of MG, particularly MuSK-MG (29, 30). RTX was approved by USA FDA for treating refractory RA through intravenous infusion (31). It is also an off-label prescription for the treatment of refractory SLE, and has shown 51% complete remission, and 34% partial remission in SLE and Lupus nephritis (LN) patients (32).

### *CD40-targeting mAbs*

Iscalimab or CFZ533 (Novartis Pharmaceuticals, Basel, Switzerland) is a fully human, Fc-silenced, IgG1 mAb that blocks the CD40 signaling pathway, thus preventing activation, but not causing depletion, of B cells and other CD40-positive cells. CD40 is expressed on B cells, T cells, and antigen-presenting cells, and its ligand, CD154, is primarily expressed on activated T cells (33). The CD40-CD154 interaction is important for isotype switching, GC formation, memory B cell generation, and Ab production (34). CFZ533 was evaluated as an add-on therapy for patients with generalized MG. A multi-center, randomized, double-blind, placebo-controlled clinical trial that measured quantitative MG muscle function scores has been completed, and the results are pending on Clinical Trials.gov.

### *FcRn-targeting mAbs*

Beyond CDs, fragment crystallizable neonatal receptor (FcRn), an MHC class I-related receptor, was recently recognized as an important target in MG. This receptor is present on the cell surface and intracellular vesicles in many cells, including B cells, but not T cells. FcRn targeting has gained momentum in current therapies that aim to reduce pathogenic autoantibodies, as the receptor can inhibit cellular IgG degradation pathways that recycle IgG to maintain or elevate serum IgG levels (35). The receptor is also known to be involved in antigen presentation of peptides from the IgG immune complexes. Inhibition of FcRn with mAb or a mAb-fragment has shown promising results in reducing serum levels of pathogenic autoantibody in some autoimmune diseases, including MG; several trials are ongoing with the aim of establishing FcRn antagonists as a potent therapy for MG.

Efgartigimod (ARGX-113; Argenx, Breda, the Netherlands) is an FcRn antagonist investigational antibody fragment undergoing phase 3 ADAPT clinical trial for MG treatment. The therapeutic potential of ARGX-113 against immune thrombocytopenia and skin blistering diseases is also being evaluated. ARGX-113 is an Fc fragment of a CD70-specific recombinant Ab on a human IgG1 background (FR70-hIgG1) carrying mutations at residues specific for high-affinity binding to FcRn in B cells. The molecule blocks binding of circulating IgG to FcRn, thereby preventing IgG recycling and accelerating the removal of pathogenic IgG from the circulation and other cells. A single intravenous dose of ARGX-113 inhibited FcRn and caused a rapid and significant decrease in serum levels of IgG1, IgG2, and IgG3, but not IgD, IgE, IgM, or serum albumin, in patients with MG, relative to placebo (36, 37). In another phase 2 MG study involving 15 centers, three doses of ARGX-113 treatment in 1 month met both primary and secondary endpoints of tolerability, safety, and efficacy. This treatment rapidly decreased total IgG, anti-AChR Ab, and improved disease in 15% of patients (38).

Rozanolixizumab (UCB7665) is another lead candidate, humanized FcRn mAb. In a phase 2 trial completed in 2018, subcutaneous infusion of rozanolixizumab in patients with generalized MG significantly reduced anti-AChR autoantibody by at least 68% from baseline. Further development and recruitment of patients with MG for the phase 3 trial was initiated in 2019. Rozanolixizumab is also being evaluated for use in the treatment of immune thrombocytopenia (39).

Nipocalimab (M281), manufactured by Momenta Pharmaceuticals (Cambridge, MA, USA), is a fully human, recombinant anti-FcRn, glycosylated, IgG1 mAb. M281 received U.S. FDA-approved Fast Track designation for the treatment of warm autoimmune hemolytic anemia (phase 2/3) in the USA. It is also being evaluated in a phase 2 clinical trial (VIVACITY) which is a randomized, double-blinded, placebo-controlled, multi-dose trial including 60 patients with generalized MG; and the results are expected by mid-2020.

Ab-mimetics are ligand-specific small peptides of 3–20 kDa. They are analogous to the Fab arms of antibodies that lack Fc and are neither glycosylated nor immunogenic (40). ABY-039 (Alexion, New Haven, CT, USA) is a bivalent Ab-mimetic with a prolonged half-life form that exhibits high-affinity binding with FcRn. This therapeutic is currently under consideration for clinical trials.

## Biologicals That Indirectly Target B Cells in MG

### mAbs

#### *B cell-activating factor (BAFF)-targeting mAb*

Belimumab (Human Genome Sciences Inc., Rockville, MD, USA; GlaxoSmithKline, Brentford, UK) is a human IgG1λ

recombinant mAb that neutralizes the biologically active soluble form of BAFF, also known as B lymphocyte-stimulating factor, or BLyS, zTNF4, TNFSF13B, THANK, and TALL-1. Both membrane-bound and soluble forms of BAFF are produced by non-B cells; for example, monocytic and dendritic cells. BAFF binds with three different receptors: (1) Blys receptor 3 or BAFF receptor, which is predominantly expressed on mature B cells; (2) B cell maturation antigen (BCMA), which is exclusively found on plasma and memory plasma B cells; and (3) transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), which is present on marginal zone and class-switched memory B cells. Based on preclinical experiments demonstrating that BAFF overexpression under autoimmune conditions induces autoreactive B cells that correlate with increased autoantibody levels, belimumab was developed to inhibit binding of BAFF to its receptor. BAFF also has a role in MG development and progression (41). Although belimumab treatment in patients with SLE had moderate efficacy in a multicenter phase 3 trials, treatment in an FDA-approved randomized MG study did not produce significant effects in patients with either AChR-MG or MuSK-MG (42).

### Inhibitors

***Proteasome-targeting inhibitors***

Bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, MA, USA) is an FDA-approved proteasome inhibitor for treatment of cancer that has also exhibited clinical efficacy in MG (43). Further, bortezomib has can induce clinical improvement in SLE and has shown promising results in the treatment of MuSK-MG (43, 44). In the EAMG rat model, bortezomib reduced anti-AChR-antibody levels, prevented motor endplate damage, and induced clinical improvement (45). The inhibitor has also been shown to deplete plasma cells and specific autoantibody production in primary thymic cell cultures from patients with early-onset MG (46). Bortezomib allows cellular accumulation of misfolded or unfolded damaged protein, or unprocessed protein, that cannot be degraded or recycled or form a processed protein via proteasome pathway. Excessive build-up of such non-functional proteins leads to cell death (47). As plasma cells are actively engaged in producing autoantibodies in MG, significant accumulation of damaged and unprocessed proteins occurs, due to their rapid transcription and translation activities. Bortezomib can also hinder nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation through inhibition of IκB proteolysis, thereby suppressing transcription of NFκB-regulated genes (e.g., IL6, BAFF-R, etc.) (48). This drug has been assessed in a phase IIa trial on patients with therapy-refractory MG with significant disease activity, and the study result is currently awaited (49).

## B Cell-Targeting MG Therapies That Act via Blockade of Cytokines and Chemokines

Proinflammatory cytokines and chemokines (ILs) have major roles in MG pathogenesis. Inflammatory cytokines prime and activate dendritic cells, antigen-specific T-helper cells, and B cells, and induce pathogenic differentiation and development of plasma cells. Therefore, drugs designed to inhibit cytokine/chemokine activity also represent valid potential treatment strategies.

Tocilizumab (TCZ; RoActemra® or Actemra®; Roche, Basel, Switzerland), also known as atlizumab, is a recombinant humanized mAb against the IL6 receptor (IL6-R). Of various cytokines that mediate Th1 and Th2 responses, IL6 plays a prominent damaging role in MG (50). Mice with an acquired or inborn deficiency of IL6 are resistant to MG, and anti-IL6 antibody reduces autoantibody levels and suppresses disease in a rat model of EAMG (50, 51). Although monocytes/macrophages are the main producers of IL6, it is also generated by numerous other cells, including muscle, epithelial, and B cells themselves. IL6 binds to IL6-R, CD126, or soluble IL6-R, and the ligand-receptor complex binds to CD136 (GP136), which dimerizes and subsequently activates intracellular kinases. Tocilizumab binds both cell-surface-bound and soluble IL6-R and prevents the proinflammatory effects of IL6. A published case report described two patients with MG refractory to rituximab treatment who showed clinical improvement after tocilizumab treatment, without any effect on autoantibody titer (52). Tocilizumab treatment has also demonstrated clinical effectiveness against RA, juvenile idiopathic arthritis, Castleman's disease, and Crohn's disease.

TNF is also produced at low levels by B cells. Conflicting results have been reported regarding the use of the TNF-α-inhibiting molecule, Enbrel® (etanercept, Benepali, Erelzi; Amgen, Thousand Oaks, CA, USA), and anti-TNF-α mAb treatment in MG and preclinical models. While etanercept (a fusion molecule containing the ligand-binding domain of human TNF receptor 2 and IgG1 Fc) was beneficial for patients with low plasma levels of IL6 and interferon (IFN)γ (53, 54), this TNF-α antagonist decoy receptor originally developed for treatment of RA, reportedly exacerbated MG in a patient with RA (55) and also reactivated tuberculosis in some patients (56, 57).

## B Cell-Targeting Potential Biologicals in Trial for Non-MG Autoimmune Diseases

This section describes some B cell-targeted potential therapeutics that have not yet been clinically tested for use in patients with MG. These drugs have shown promising results in early stage clinical studies for non-MG autoimmune diseases and therefore are potential pipeline drugs for future testing in MG.

### Anti-CD19 mAb

Inebilizumab (MEDI-551) is a humanized high-affinity anti-CD19, IgG1κ mAb. Although plasma cells lack CD19 expression, this mAb induces effective ADCC to deplete almost all other B cells, including precursor-plasma cells. In phase 3, double-masked, randomized, placebo-controlled "N-Momentum" trial, inebilizumab demonstrated increased efficacy for the treatment of neuromyelitis optica spectrum disorder (NMOSD). In April 2019, the company Viela Bio received FDA Breakthrough Therapy Designation (BTD) approval for inebilizumab. BTD approval permits expedited development and fast regulatory review of drugs for life-threatening conditions, and those that achieve a minimum of one clinically significant endpoint. Inebilizumab has also received Orphan Drug Designation by both

the FDA and the European Medicines Agency for treatment of NMO or NMOSD. This mAb has not yet been investigated for MG treatment.

### Anti-CD20 mAb

Ocrelizumab (Ocrevus; Genentech), another CD20-binding humanized mAb, is FDA-approved for treatment of relapsing-remitting multiple sclerosis (RRMS) and primary progressive multiple sclerosis. Ublituximab (TG-1101; TG Therapeutics, New York, NY, USA) is a glycoengineered, B cell-depleting effective anti-CD20 mAb that has entered a phase 3 trial for treatment of MS. Veltuzumab (Immunomedics, Morris Plains, NJ, USA), a fully human anti-CD20 mAb, is currently under development for treatment of non-Hodgkin lymphoma and autoimmune diseases. TRU-015 (Trubion Pharmaceuticals Inc., Seattle, WA, USA: Pfizer Inc., New York, NY, USA), a fully human, anti-CD20 IgG fusion protein, is currently being evaluated for RA therapy. Anti-CD20 mAbs, such as: obinutuzumab (Gazyva; GlycArt Biotechnology AG, Schlieren, Switzerland; Roche), ofatumumab (Arzerra®), and tositumomab have been used in combination with chemo- or radiotherapy for the treatment of cancer.

### Anti-IL6 mAb

Antibodies such as sarilumab, sirukumab, and siltuximab were developed to target IL6 for the treatment of RA or other diseases (58); however, these drugs have not yet been considered for use in the treatment of MG.

### Anti-IFN mAb

Rontalizumab was developed by Genentech for treatment of SLE. It is a humanized IgG1 anti-IFNα mAb that neutralizes all IFNα subtypes and inhibits signaling through the type I IFN receptor (IFNAR). Primary and secondary endpoints were not met in the phase 2 trial. In a separate study, SLE patients with low IFN signature metrics who were treated with rontalizumab showed improvements in disease activity, reduced flares, and low steroid requirements (59). Sifalimumab, also a human anti-IFNα mAb, was developed by Medimmune (Gaithersburg, MD, USA). Recently, its trial was terminated by the company and replaced with a competing product, anifrolumab, for phase 3 trials. There are contradicting reports of the beneficial and adverse effects of IFN treatment in isolated case studies of patients with MG or EAMG experiment (60–62). As yet, IFN-I has not been targeted for MG clinical trial.

### Anti-α4β1 Integrin mAb

Natalizumab (Biogen, Cambridge, MA, USA) is a recombinant humanized mAb that targets $\alpha_4\beta_1$-integrin expressed by B cells. Natalizumab prevents binding of B cells to the endothelial adhesion molecule, VCAM, and consequently inhibits transmigration of B cells from the blood into tissues. Clinical trials revealed significant improvement and clinical efficacy in patients with RRMS treated with natalizumab.

### BAFF and TACI Inhibitors

Anthera Pharmaceuticals (Hayward, CA, USA) developed another BAFF specific inhibitor, blisibimod, with high avidity against both soluble and membrane residing BAFF. In phase 2 clinical trial, blisibimod generated a response in SLE patients with disease severity (4). Further, in phase 3 trials of responder patients, the secondary endpoint was not met, although this treatment was associated with successful steroid-sparing and reductions in SLE autoantibodies and B cells. Atacicept (developed by ZymoGenetics, Seattle, WA, USA; handled by Merck Serono, Darmstadt, Germany) is a recombinant fusion protein that blocks the activation of TACI by APRIL and BAFF.

## PRECLINICAL STUDIES OF POTENTIAL B CELL INHIBITION USING THE EAMG MODEL

Many promising results of the use of B cell-targeted therapies for MG in mice models have been reported. These preclinical studies demonstrate the potential to target B cells for future translation in the clinic for MG therapy. The following section describes some preclinical studies that targeted B cells alone or in combination with other immune cells involved in MG pathogenesis.

### Proteasome Inhibitors

ONX-0914, a selective inhibitor of the immunoproteasome, has been reported to ameliorate EAMG severity by reducing the frequency of T follicular helper cells, antigen-presenting cells, and Th17 cells, as well as decreasing the affinity of B cell-generated autoantibodies (63).

### Chemokine Antagonists

Although MG is a B cell-mediated disease, B cell/T cell interaction plays a critical role in MG pathology. New therapies may also consider targeting factors involved in these specific interactions, particularly proteins belonging to the CC and CXC family of chemokine ligands and receptors, which are produced by peripheral blood mononuclear cells, lymph node cells, macrophages, and thymic GCs. Based on *in silico* analyses, potential therapeutic chemokine targets include: CXCR2, CXCR3, CXCL1, CXCL3, CCL, CCL19, and CCL20 (64–67).

### MicroRNA Inhibitors

MicroRNAs (miRNAs) are important immune regulators of numerous soluble inflammatory mediators and are potential targets for future intervention in MG. Differential levels of miRNAs in activated B cells (e.g., miR-146a) or serum have also been reported in an EAMG mouse model (68), and inhibition of these molecules reduced B cell activation and AChR-specific antibody levels. miR-150-5p and miR-21-5p are found at higher levels in patients seropositive for AChR, and their levels decrease following immunosuppressive therapy and thymectomy. Increased levels of the Let-7 family of miRNAs in MuSK-positive MG are also of great interest (69).

### Humanized scFv Against a DQB1 Allele Associated With Susceptibility to MG

In a preclinical study, a humanized scFv was developed from the mouse mAb, LG11, which targets MG-susceptible-specific

human leukocyte antigen (HLA) alleles. The scFv was shown to block the proliferation of T cells cultured from peripheral blood lymphocytes from patients with MG carrying DQB1*0601, which is associated with susceptibility to MG (70).

### Recombinant AChR Fragment

Induction of tolerance by adoptive transfer of autologous regulatory T cells (T-regs) or mucosal delivery of AChR is effective for treatment of EAMG (71); however, the usefulness of these treatments has yet to be fully evaluated clinically. Consonni et al. recently reported that repeated intranasal administration of microgram quantities of a fusion protein that carries the immunodominant peptide from AChR, mCTA1-T146, suppressed both induction of EAMG, as well as ongoing disease in mice. Treated mice showed increased preservation of muscle AChR and low levels of anti-AChR serum antibodies. Tolerance was induced by increased T-reg cell activation and upregulated expression of *Tgfβ*, *Il10*, *Il27*, and *Foxp3* mRNAs in the spleens and lymph nodes of mice (72). Similar induction of tolerance in EAMG has been described in response to nasal or oral delivery of recombinant AChR fragment, presumably by skewing Th1 to Th2/Th3 immune responses (73).

### BAFF Receptor-Specific mAb-siRNA Conjugates

In a different approach to targeting specific B cells, a fusion mAb-siRNA conjugate was constructed using a small <7-kDa protein (protamine), that covalently binds with B cell-targeting mAbs through hetero-bifunctional linkers and forms stable electrostatic bonds with B cell-specific siRNAs. The mAb in the conjugate binds to the B cell receptor and, upon internalization by receptor-mediated endocytosis, releases siRNA from the complex into the cell for degradation of B cell-specific pathogenic mRNAs. Treatment of EAMG mice with these conjugates can markedly reduce B cell frequencies (74). In an ongoing study, conjugates consisting of innate immune-resistant, modified siRNAs are being evaluated for therapeutic efficacy.

## PERSPECTIVES: ADVANTAGES, LIMITATIONS, AND FUTURE CHALLENGES FOR B CELL-TARGETING THERAPIES FOR MG

There is an immediate but unmet need for effective MG therapy, particularly for patients with refractory disease. The high specificity, less off-target effects, and long-lasting, robust effects of B cell-specific mAb therapy make it attractive and especially useful for inhibiting proteins that do not have binding pockets available for an inhibitor. With the advent of humanized and fully human mAbs, mAb therapy is currently used as a first-line or standard treatment among targeted therapy approaches for many autoimmune diseases. The introduction of novel engineered mAbs is further evidence of the important progress occurring in the field.

Although mAbs can produce long-term cell-specific effects, stand-alone mAb therapy has some limitations, including inadequate understanding of its *in vivo* mechanism of action, adverse effects, and non-sensitivity to therapy (e.g., unaltered autoantibody levels or clinical pathology) despite significant depletion of target cells, which have been observed in many patients with MG following various B cell-directed mAb therapies. Currently, a major challenge for mAb therapy for autoimmune disease is inefficient reduction of pathogenic antibody, which is usually the primary goal of therapy. Tissue-trafficking of pathogenic plasma cells or precursor plasma cells to distant sites following targeted therapy is likely one cause of resistance to complete elimination of those cells, autoantibody reduction, and disease activity. Another highly probable cause is therapy-induced expression of molecules such as type I IFNs, which stimulate antigen presentation and affinity maturation of antibody-producing cells (74). High-dose mAbs may impart some therapeutic benefits to patients, but they also cause severe side effects, and patients are at risk of contracting infections and may generate innate-immune or interferonogenic effects (74). Hence, determination of the optimal dose for each therapeutic mAb is critical.

An approach using a combination of potent mAbs for targeted therapy may be effective. Targeting more than one subset of B cells, multiple targets, or specific B cells alongside B cell-interacting T cells and B cell-activating soluble mediators in the microenvironment, may result in synergistic reduction of autoantibody levels and therapeutic benefits. Treatment with mAb alone (or mAb-based therapy) is often inconvenient for both preclinical and clinical assessment due to their prolonged manufacturing times and high-cost relative to small molecule peptide inhibitors or therapeutic nucleic acids. However, most inhibitors have non-specific modes of action, risking induction of peptide-specific humoral responses or loss of activity over time. Nucleic acid therapy using modified RNA should be considered to prevent interferonogenic innate immune responses or immunogenicity of the therapeutic itself. Combining or conjugating more than one therapeutic molecule may also greatly enhance the therapeutic potential of B cell-specific therapy.

Changes in specific saccharide or sugar residues (fucosylation and galactosylation) can significantly modulate mAb effector function. Enzymatic removal of fucose and addition of galactose moieties to IgG1 mAbs can markedly increase their ADCC activity (75). The mechanism involves preferential binding of Fc to activating-FcγRIIIa (relative to inhibitory-FcγR) on effector cells, as determined by *in vitro* natural killer cell-based assays. Site-selective glycoengineering of both the Fc and Fab domains of a chimeric anti-epidermal growth factor receptor (EGFR) mAb has recently been shown to lead to increased ADCC activity (76). Future strategies for targeted B cell therapies may further exploit these technological advances by producing engineered mAbs that can potentially enhance B cell killing function and therapeutic efficacy.

Development of promising therapies largely depends on success in preclinical studies. The EAMG mouse model is valuable for therapeutic evaluation and redesign of promising B cell-targeted MG therapies. The classical EAMG model,

generated by Torpedo-AChR immunizations, is preferable to systems using passive autoantibody transfer and thymic engraftment models (77–80) due to its chronic nature and mimicry of human MG. However, disadvantages of this model are the delay and lack of homogeneity in the incidence and clinical grades of immunized mice, despite comparable levels of high-affinity anti-AChR antibody. Hence, in individual studies researchers may choose to immunize an excess of animals to bolster statistical power. Another drawback of the EAMG model is the rapid progression of symptoms following disease onset, leading to mortality in some mice and reducing the number of experimental animals and durability of long-term assessment of therapeutic agents. The traditional tedious and inefficient method of immunogen purification from Torpedo tissue is also inconvenient and can be improved by utilizing new technologies and biomolecules such as UltraLink Biosupport (ThrmoFisher, CA, USA), a high-performance resin for the neurotoxin coupling reaction. Improving this animal model for more effective exploitation in preclinical therapy development will be essential for successful intervention in the clinic and effective MG therapy. Regarding therapeutic evaluation in clinical settings, recruitment of patients with similar disease profiles and their subsequent retention, as well as the fluctuating nature of the disease, are often challenging for proper evaluation of therapy. Other factors that potentially affect the successful outcome of clinical trials include study length, cost burden, and regulatory aspects.

It is important to be vigilant for occurrence of adverse effects of immunotherapy for cancer in patients with MG or underlying mild or latent MG (81–83). For example, treatment of patients with MG with ipilimumab (anti-CTLA4 mAb) for melanoma and lung cancer had fatal consequences (84). Further, combination therapy using mAb or inhibitors against checkpoint proteins (PD-1, PD-L1, and anti-CTLA-4) to treat cancers can induce or exacerbate MG, or even cause patient death (85, 86). It is likely that the robust immune activation and inflammatory response triggered by checkpoint protein-specific mAbs, although critical for cancer therapy, is detrimental to patients with MG, due to rapid development of myasthenic crisis; however, patients with MG and associated conditions other than cancer, such as thyroiditis due to anti-thyroid antibodies, lupus with anti-nuclear antibodies, and neuro-myelitis spectrum disorder with anti-aquaporin-4 antibodies (5), do not show adverse reactions following B cell-directed therapy.

## CONCLUSIONS

Developing strategies to find or develop an effective therapy for MG and many other debilitating and potentially life-threatening autoimmune diseases is an important research priority. Many potent candidate B cell-targeted therapies for MG are less effective or unsuccessful at the preclinical and clinical phases of development. These failures are primarily caused by weak immunosuppressive responses, non-specific immunogenicity, or safety concerns. Furthermore, targeted therapies currently licensed for use are effective but have drawbacks. Therefore, identifying and developing safe and effective means to improve the efficacy of these crucial therapies are urgently required. More preclinical studies of MG are needed to validate both new therapeutics and those that have already been proven effective for related neuromuscular autoimmune diseases. Additionally, identification of new and validated target(s), repurposing other targeted therapies, administration of combination therapies directed at multiple targets, and targeting antigen-specific B cells rather than a pan-B cell approach, may help to turn these targeted-therapy approaches into effective methods for ameliorating MG.

Overall, recent advances in targeted-therapy approaches have contributed significantly to our knowledge that has subsequently led to a multitude of new therapeutic modulations and emerging therapy approaches. For example, the development of highly potent, non-immunogenic, engineered mAbs and synthetic alternatives to mAbs, such as Ab-mimetics (e.g., monobodies and nanobodies), and even small RNA therapeutics, is encouraging and offers hope. These approaches may soon lead to the production of additional next-generation targeted therapeutics and long-awaited effective interventions against MG.

## AUTHOR CONTRIBUTIONS

The author reviewed the literature, contributed solely to the writing, and approved the manuscript for publication.

## FUNDING

RH's research has been supported by grants from the Association Francaise contre les Myopathies (AFM), France; the Myasthenia Gravis Foundation of America (MGFA); and Conquer Myasthenia Gravis, USA.

## ACKNOWLEDGMENTS

The author thanks Linsey A. Yeager and Maxim Ivannikov for editing the manuscript, and acknowledges Kirk Ford, Media Specialist at UTMB, for assistance with Figures in the manuscript.

## REFERENCES

1. Koneczny I, Herbst R. Myasthenia gravis: pathogenic effects of autoantibodies on neuromuscular architecture. *Cells*. (2019) 8:E671. doi: 10.3390/cells8070671
2. Gilhus NE, Tzartos S, Evoli A, Palace J, Burns TM, Verschuuren JJGM. Myasthenia gravis *Nat Rev Dis Primers*. (2019) 5:30. doi: 10.1038/s41572-019-0079-y
3. Schneider-Gold C, Hagenacker T, Melzer N, Ruck T. Understanding the burden of refractory myasthenia gravis. *Ther Adv Neurol*

*Disord*. (2019) 12:1756286419832242. doi: 10.1177/1756286419832242
4. Behin A, Le Panse R. New pathways and therapeutic targets in autoimmune myasthenia gravis. *J Neuromuscul Dis*. (2018) 5:265–77. doi: 10.3233/JND-170294
5. Dalakas MC. Immunotherapy in myasthenia gravis in the era of biologics. *Nat Rev Neurol*. (2019) 15:113–24. doi: 10.1038/s41582-018-0110-z
6. Christadoss P, Poussin M, Deng C. Animal models of myasthenia gravis. *Clin Immunol*. (2000) 94:75–87 doi: 10.1006/clim.1999.4807
7. Fuchs S, Aricha R, Reuveni D, Souroujon MC. Experimental autoimmune myasthenia gravis (EAMG): from immunochemical characterization to therapeutic approaches. *J Autoimmun*. (2014) 54:51–9. doi: 10.1016/j.jaut.2014.06.003
8. Tobón GJ, Izquierdo JH, Cañas CA. B lymphocytes: development, tolerance, and their role in autoimmunity-focus on systemic lupus erythematosus. *Autoimmune Dis*. (2013) 2013:827254. doi: 10.1155/2013/827254
9. Hofmann K, Clauder AK, Manz RA. Targeting B cells and plasma cells in autoimmune diseases. *Front Immunol*. (2018) 9:835. doi: 10.3389/fimmu.2018.00835
10. O'Connor BP, Cascalho M, Noelle RJ. Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population. *J Exp Med*. (2002) 195:737–45. doi: 10.1084/jem.20011626
11. Masopust D, Soerens AG. Tissue-resident T cells and other resident leukocytes. *Annu Rev Immunol*. (2019) 37:521–46. doi: 10.1146/annurev-immunol-042617-053214
12. Allie SR, Bradley JE, Mudunuru U, Schultz MD, Graf BA, Lund FE, et al. The establishment of resident memory B cells in the lung requires local antigen encounter. *Nat Immunol*. (2019) 20:97–108. doi: 10.1038/s41590-018-0260-6
13. Meriggioli MN, Sanders DB. Muscle autoantibodies in myasthenia gravis: beyond diagnosis? *Expert Rev Clin Immunol*. (2012) 8:427–38. doi: 10.1586/eci.12.34
14. Beck G, Yabumoto T, Baba K, Sasaki T, Higuchi O, Matsuo H, et al. Double seronegative myasthenia gravis with Anti-LRP4 antibodies presenting with dropped head and acute respiratory insufficiency. *Intern Med*. (2016) 55:3361–63. doi: 10.2169/internalmedicine.55.7030
15. Kusner LL, Kaminski HJ. The role of complement in experimental autoimmune myasthenia gravis. *Ann NY Acad Sci*. (2012) 1274:127–32. doi: 10.1111/j.1749-6632.2012.06783.x
16. Huda R, Tüzün E, Christadoss P. Targeting complement system to treat myasthenia gravis. *Rev Neurosci*. (2014) 4:575–83. doi: 10.1515/revneuro-2014-0021
17. Phillips WD, Vincent A. Pathogenesis of myasthenia gravis: update on disease types, models, and mechanisms. *F1000Res*. (2016) 5:F1000. doi: 10.12688/f1000research.8206.1
18. Kohler S, Keil TO, Swierzy M, Hoffmann S, Schaffert H, Ismail M, et al. Disturbed B cell subpopulations and increased plasma cells in myasthenia gravis patients. *J Neuroimmunol*. (2013) 264:114–9. doi: 10.1016/j.jneuroim.2013.09.006
19. Rivera A, Chen CC, Ron N, Dougherty JP, Ron Y. Role of B cells as antigen-presenting cells *in vivo* revisited: antigen-specific B cells are essential for T cell expansion in lymph nodes and for systemic T cell responses to low antigen concentrations. *Int Immunol*. (2001) 13:1583–93 doi: 10.1093/intimm/13.12.1583
20. Engel P, Boumsell L, Balderas R, Bensussan A, Gattei V, Horejsi V, et al. CD nomenclature 2015: human leukocyte differentiation antigen workshops as a driving force in immunology. *J Immunol*. (2015) 195:4555–63. doi: 10.4049/jimmunol.1502033
21. Matesanz-Isabel J, Sintes J, Llinàs L, de Salort J, Lázaro A, Engel P. New B-cell CD molecules. *Immunol Lett*. (2011) 134:104–12. doi: 10.1016/j.imlet.2010.09.019
22. Derer S, Kellner C, Berger S, Valerius T, Peipp M. Fc engineering: design, expression, and functional characterization of antibody variants with improved effector function. *Methods Mol Biol*. (2012) 907:519–36. doi: 10.1007/978-1-61779-974-7_30
23. Lei C, Gong R, Ying T. Editorial: antibody fc engineering: towards better therapeutics. *Front Immunol*. (2018) 9:2450. doi: 10.3389/fimmu.2018.02450
24. Singh S, Kumar NK, Dwiwedi P, Charan J, Kaur R, Sidhu P, et al. Monoclonal antibodies: a review. *Curr Clin Pharmacol*. (2018) 13:85–99. doi: 10.2174/1574884712666170809124728
25. Parren PWHI, Carter PJ, Plückthun A. Changes to international nonproprietary names for antibody therapeutics 2017 and beyond: of mice, men and more. *MAbs*. (2017) 9:898–906. doi: 10.1080/19420862.2017.1341029
26. Anderson D, Phan C, Johnston WS, Siddiqi ZA. Rituximab in refractory myasthenia gravis: a prospective, open-label study with long-term follow-up. *Ann Clin Transl Neurol*. (2016) 3:552–5. doi: 10.1002/acn3.314
27. Yi JS, Guptill JT, Stathopoulos P, Nowak RJ, O'Connor KC. B cells in the pathophysiology of myasthenia gravis. *Muscle Nerve*. (2018) 57:172–184. doi: 10.1002/mus.25973
28. Stathopoulos P, Kumar A, Heiden JAV, Pascual-Goñi E, Nowak RJ, O'Connor KC. Mechanisms underlying B cell immune dysregulation and autoantibody production in MuSK myasthenia gravis. *Ann NY Acad Sci*. (2018) 1412:154–65. doi: 10.1111/nyas.13535
29. Jing S, Lu J, Song J, Luo S, Zhou L, Quan C, et al. Effect of low-dose rituximab treatment on T- and B-cell lymphocyte imbalance in refractory myasthenia gravis. *J Neuroimmunol*. (2019) 332:216–23. doi: 10.1016/j.jneuroim.2019.05.004
30. Topakian R, Zimprich F, Iglseder S, Embacher N, Guger M, Stieglbauer K, et al. High efficacy of rituximab for myasthenia gravis: a comprehensive nationwide study in Austria. *J Neurol*. (2019) 266:699–706. doi: 10.1007/s00415-019-09191-6
31. Alshaiki F, Obaid E, Almuallim A, Taha R, El-Haddad H, Almoallim H. Outcomes of rituximab therapy in refractory lupus: a meta-analysis. *Eur J Rheumatol*. (2018) 5:118–26. doi: 10.5152/eurjrheum.2018.17096
32. Mok CC. Rituximab for the treatment of rheumatoid arthritis: an update. *Drug Des Devel Ther*. (2013) 8:87–100. doi: 10.2147/DDDT.S41645
33. Karnell JL, Rieder SA, Ettinger R, Kolbeck R. Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond. *Adv Drug Deliv Rev*. (2018) 13:S0169–409 X(18)30308–9. doi: 10.1016/j.addr.2018.12.005
34. Ristov J, Espie P, Ulrich P, Sickert D, Flandre T, Dimitrova M, et al. Characterization of the *in vitro* and *in vivo* properties of CFZ533, a blocking and non-depleting anti-CD40 monoclonal antibody. *Am J Transplant*. (2018) 18:2895–904. doi: 10.1111/ajt.14872
35. Kuo TT, Aveson VG. Neonatal Fc receptor and IgG-based therapeutics. *MAbs*. (2011) 3:422–30. doi: 10.4161/mabs.3.5.16983
36. Guidon AC, Juel VC. Efgartigimod: a novel antibody depletion therapy in myasthenia gravis. *Neurology*. (2019) 92:1079–80. doi: 10.1212/WNL.0000000000007605
37. Ulrichts P, Guglietta A, Dreier T, van Bragt T, Hanssens V, Hofman E, et al. Neonatal Fc receptor antagonist efgartigimod safely and sustainably reduces IgGs in humans. *J Clin Invest*. (2018) 128:4372–86. doi: 10.1172/JCI97911
38. Howard JF Jr, Bril V, Burns TM, Mantegazza R, Bilinska M, Szczudlik A, et al. Efgartigimod MG study group. randomized phase 2 study of FcRn antagonist efgartigimod in generalized myasthenia gravis. *Neurology*. (2019) 92: e2661–73. doi: 10.1212/WNL.0000000000007600
39. Zuercher AW, Spirig R, Baz Morelli A, Rowe T, Käsermann F. Next-generation Fc receptor-targeting biologics for autoimmune diseases. *Autoimmun Rev*. (2019) 18:102366. doi: 10.1016/j.autrev.2019.102366
40. Ta AN, McNaughton BR. Antibody and antibody mimetic immunotherapeutics. *Future Med Chem*. (2017) 9:1301–04. doi: 10.4155/fmc-2017-0057
41. Berrih-Aknin S, Ragheb S, Le Panse R, Lisak RP. Ectopic germinal centers, BAFF and anti-B-cell therapy in myasthenia gravis. *Autoimmun Rev*. (2013) 12:885–93. doi: 10.1016/j.autrev.2013.03.011
42. Hewett K, Sanders DB, Grove RA, Broderick CL, Rudo TJ, Bassiri A, et al. Randomized study of adjunctive belimumab in participants with generalized myasthenia gravis. *Neurology*. (2018) 90:e1425–34. doi: 10.1212/WNL.0000000000005323
43. Kohler S, Märschenz S, Grittner U, Alexander T, Hiepe F, Meisel A. Bortezomib in antibody-mediated autoimmune diseases (TAVAB): study protocol for a unicentric, non-randomised, non-placebo controlled trial. *BMJ Open*. (2019) 9:e024523. doi: 10.1136/bmjopen-2018-024523
44. Schneider-Gold C, Reinacher-Schick A, Ellrichmann G, Gold R. Bortezomib in severe MuSK-antibody positive myasthenia gravis: first clinical experience. *Ther Adv Neurol Disord*. (2017) 10:339–41. doi: 10.1177/1756285617721093
45. Gomez AM, Vrolix K, Martínez-Martínez P, Molenaar PC, Phernambucq M, van der Esch E, et al. Proteasome inhibition with bortezomib depletes plasma

cells and autoantibodies in experimental autoimmune myasthenia gravis. *J Immunol*. (2011) 186:2503–13. doi: 10.4049/jimmunol.1002539
46. Gomez AM, Willcox N, Vrolix K, Hummel J, Nogales-Gadea G, Saxena A, et al. Proteasome inhibition with bortezomib depletes plasma cells and specific autoantibody production in primary thymic cell cultures from early-onset myasthenia gravis patients. *J Immunol*. (2014) 193:1055–63. doi: 10.4049/jimmunol.1301555
47. Thibaudeau TA, Smith DM. A practical review of proteasome pharmacology. *Pharmacol Rev*. (2019) 71:170–97. doi: 10.1124/pr.117.015370
48. Karin M. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene*. (1999) 18:6867–74. doi: 10.1038/sj.onc.1203219
49. Tüzün E, Huda R, Christadoss P. Complement and cytokine based therapeutic strategies in myasthenia gravis. *J Autoimmun*. (2011) 37:136–43. doi: 10.1016/j.jaut.2011.05.006
50. Deng C, Goluszko E, Tüzün E, Yang H, Christadoss P. Resistance to experimental autoimmune myasthenia gravis in IL-6-deficient mice is associated with reduced germinal center formation and C3 production. *J Immunol*. (2002) 2:1077–83. doi: 10.4049/jimmunol.169.2.1077
51. Aricha R, Mizrachi K, Fuchs S, Souroujon MC. Blocking of IL-6 suppresses experimental autoimmune myasthenia gravis. *J Autoimmun*. (2011) 36:135–41. doi: 10.1016/j.jaut.2010.12.001
52. Jonsson DI, Pirskanen R, Piehl F. Beneficial effect of tocilizumab in myasthenia gravis refractory to rituximab. *Neuromuscul Disord*. (2017) 27:565–68. doi: 10.1016/j.nmd.2017.03.007
53. Tüzün E, Meriggioli MN, Rowin J, Yang H, Christadoss P. Myasthenia gravis patients with low plasma IL-6 and IFN-gamma benefit from etanercept treatment. *J Autoimmun*. (2005) 24:261–8. doi: 10.1016/j.jaut.2005.01.013
54. Duan RS, Wang HB, Yang JS, Scallon B, Link H, Xiao BG. Anti-TNF-α antibodies suppress the development of experimental autoimmune myasthenia gravis. *J Autoimmun*. (2002) 19:169–74. doi: 10.1006/jaut.2002.0618
55. Fee DB, Kasarskis EJ. Myasthenia gravis associated with etanercept therapy. *Muscle Nerve*. (2009) 39:866–70. doi: 10.1002/mus.21280
56. Miossec P. Reactivation of tuberculosis during treatment with inhibitors of TNF. *Rev Prat*. (2018) 68:537–40.
57. Lin PL, Myers A, Smith L, Bigbee C, Bigbee M, Fuhrman C et al. Tumor necrosis factor neutralization results in disseminated disease in acute and latent *Mycobacterium tuberculosis* infection with normal granuloma structure in a cynomolgus macaque model. *Arthritis Rheum*. (2010) 62:340–50. doi: 10.1002/art.27271
58. Raimondo MG, Biggioggero M, Crotti C, Becciolini A, Favalli EG. Profile of sarilumab and its potential in the treatment of rheumatoid arthritis. *Drug Des Devel Ther*. (2017) 11:1593–603. doi: 10.2147/DDDT.S100302
59. Kalunian KC, Merrill JT, Maciuca R, McBride JM, Townsend MJ, Wei X et al. A Phase II study of the efficacy and safety of rontalizumab (rhuMAb interferon-α) in patients with systemic lupus erythematosus (ROSE). *Ann Rheum Dis*. (2016) 75:196–202. doi: 10.1136/annrheumdis-2014-206090
60. Deng C, Goluszko E, Baron S, Wu B, Christadoss P. IFN-alpha therapy is effective in suppressing the clinical experimental myasthenia gravis. *J Immunol*. (1996) 157:5675–82
61. Congeni JP, Kirkpatrick RB. Pegylated interferon induced myasthenia crisis–a case report. *J Clin Neuromuscul Dis*. (2013) 14:123–5. doi: 10.1097/CND.0b013e318285257f
62. Baik SJ, Kim TH, Kim HI, Rhie JY. Myasthenia crisis induced by pegylated-interferon in patient with chronic hepatitis c: a case report. *Medicine*. (2016) 95:e3782. doi: 10.1097/MD.0000000000003782
63. Liu RT, Zhang P, Yang CL, Pang Y, Zhang M, Zhang N, et al. ONX-0914, a selective inhibitor of immunoproteasome, ameliorates experimental autoimmune myasthenia gravis by modulating humoral response. *J Neuroimmunol*. (2017) 311:71–8. doi: 10.1016/j.jneuroim.2017.08.005
64. Molin CJ, Westerberg E, Punga AR. Profile of upregulated inflammatory proteins in sera of myasthenia gravis patients. *Sci Rep*. (2017) 7:39716. doi: 10.1038/srep39716
65. Feferman T, Maiti PK, Berrih-Aknin S, Bismuth J, Bidault J, Fuchs S, et al. Overexpression of IFN-induced protein 10 and its receptor CXCR3 in myasthenia gravis. *J Immunol*. (2005) 174:5324–31. doi: 10.4049/jimmunol.174.9.5324
66. Feferman T, Aricha R, Mizrachi K, Geron E, Alon R, Souroujon MC, et al. Suppression of experimental autoimmune myasthenia gravis by inhibiting the signaling between IFN-gamma inducible protein 10 (IP-10) and its receptor CXCR3. *J Neuroimmunol*. (2009) 209:87–95. doi: 10.1016/j.jneuroim.2009.01.021
67. Weiss JM, Robinet M, Aricha R, Cufi P, Villeret B, Lantner F et al. Novel CXCL13 transgenic mouse: inflammation drives pathogenic effect of CXCL13 in experimental myasthenia gravis. *Oncotarget*. (2016) 7:7550–62. doi: 10.18632/oncotarget.6885
68. Zhang J, Jia G, Liu Q, Hu J, Yan M, Yang B, et al. Silencing miR-146a influences B cells and ameliorates experimental autoimmune myasthenia gravis. *Immunology*. (2015) 144:56–67. doi: 10.1111/imm.12347
69. Punga AR, Punga T. Circulating microRNAs as potential biomarkers in myasthenia gravis patients. *Ann NY Acad Sci*. (2018) 1412:33–40. doi: 10.1111/nyas.13510
70. Ayyar BV, Atassi MZ. Development of humanized scFv antibody fragment(s) that targets and blocks specific HLA alleles linked to myasthenia gravis. *Appl Microbiol Biotechnol*. (2017) 101:8165–79. doi: 10.1007/s00253-017-8557-1
71. Aricha R, Reuveni D, Fuchs S, Souroujon MC. Suppression of experimental autoimmune myasthenia gravis by autologous T regulatory cells. *J Autoimmun*. (2016) 67:57–64. doi: 10.1016/j.jaut.2015.09.005
72. Consonni A, Sharma S, Schön K, Lebrero-Fernández C, Rinaldi E, Lycke NY, et al. A novel approach to reinstating tolerance in experimental autoimmune myasthenia gravis using a targeted fusion protein, mCTA1-T146. *Front Immunol*. (2017) 8:1133. doi: 10.3389/fimmu.2017.01133
73. Im SH, Barchan D, Fuchs S, Souroujon MC. Mechanism of nasal tolerance induced by a recombinant fragment of acetylcholine receptor for treatment of experimental myasthenia gravis. *J Neuroimmunol*. (2000) 111:161–8. doi: 10.1016/S0165-5728(00)00395-7
74. Ibtehaj N, Huda R. High-dose BAFF receptor specific mAb-siRNA conjugate generates Fas-expressing B cells in lymph nodes and high-affinity serum autoantibody in a myasthenia mouse model. *Clin Immunol*. (2017) 176:122–30. doi: 10.1016/j.clim.2017.01.005
75. Thomann M, Reckermann K, Reusch D, Prasser J, Tejada ML. Fc-galactosylation modulates antibody-dependent cellular cytotoxicity of therapeutic antibodies. *Mol Immunol*. (2016) 73:69–75. doi: 10.1016/j.molimm.2016.03.002
76. Giddens JP, Lomino JV, DiLillo DJ, Ravetch JV, Wang LX. Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody. *Proc Natl Acad Sci USA*. (2018) 115:12023–7. doi: 10.1073/pnas.1812833115
77. Losen M, Martinez-Martinez P, Molenaar PC, Lazaridis K, Tzartos S, Brenner T, et al. Standardization of the experimental autoimmune myasthenia gravis (EAMG) model by immunization of rats with torpedo californica acetylcholine receptors–recommendations for methods and experimental designs. *Exp Neurol*. (2015) 270:18–28. doi: 10.1016/j.expneurol.2015.03.010
78. Kusner LL, Losen M, Vincent A, Lindstrom J, Tzartos S, Lazaridis K, et al. Guidelines for pre-clinical assessment of the acetylcholine receptor–specific passive transfer myasthenia gravis model-Recommendations for methods and experimental designs. *Exp Neurol*. (2015) 270:3–10. doi: 10.1016/j.expneurol.2015.02.025
79. Phillips WD, Christadoss P, Losen M, Punga AR, Shigemoto K, Verschuuren J, et al. Guidelines for pre-clinical animal and cellular models of MuSK-myasthenia gravis. *Exp Neurol*. (2015) 270:29–40. doi: 10.1016/j.expneurol.2014.12.013
80. Mantegazza R, Cordiglieri C, Consonni A, Baggi F. Animal models of myasthenia gravis: utility and limitations. *Int J Gen Med*. (2016) 9:53–64. doi: 10.2147/IJGM.S88552
81. Moreira A, Loquai C, Pföhler C, Kähler KC, Knauss S, Heppt MV, et al. Myositis and neuromuscular side-effects induced by immune checkpoint inhibitors. *Eur J Cancer*. (2019) 106:12–23. doi: 10.1016/j.ejca.2018.09.033
82. Pauken KE, Dougan M, Rose NR, Lichtman AH, Sharpe AH. Adverse events following cancer immunotherapy: obstacles and opportunities. *Trends Immunol*. (2019) 40:511–23. doi: 10.1016/j.it.2019.04.002
83. Johnson DB, Manouchehri A, Haugh AM, Quach HT, Balko JM, Lebrun-Vignes B, et al. Neurologic toxicity associated with immune checkpoint inhibitors: a pharmacovigilance study. *J Immunother Cancer*. (2019) 7:134. doi: 10.1186/s40425-019-0617-x

84. Makarious D, Horwood K, Coward JIG. Myasthenia gravis: an emerging toxicity of immune checkpoint inhibitors. *Eur J Cancer*. (2017) 82:128–36. doi: 10.1016/j.ejca.2017.05.041
85. Cooper DS, Meriggioli MN, Bonomi PD, Malik R. Severe exacerbation of myasthenia gravis associated with checkpoint inhibitor immunotherapy. *J Neuromuscul Dis*. (2017) 4:169–73. doi: 10.3233/JND-170219
86. Becquart O, Lacotte J, Malissart P, Nadal J, Lesage C, Guillot B, et al. Myasthenia gravis induced by immune checkpoint inhibitors. *J Immunother*. (2019) 42:309–12. doi: 10.1097/CJI.0000000000000278

**Conflict of Interest:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 21 February 2020
doi: 10.3389/fimmu.2020.00151

# Estrogen Receptor, Inflammatory, and FOXO Transcription Factors Regulate Expression of Myasthenia Gravis-Associated Circulating microRNAs

*Alyson A. Fiorillo[1,2], Christopher R. Heier[1,2], Yu-Fang Huang[3], Christopher B. Tully[1], Tanel Punga[4] and Anna Rostedt Punga[3]**

[1] Center for Genetic Medicine Research, Children's Research Institute, Washington, DC, United States, [2] Genomics & Precision Medicine, The George Washington University, Washington, DC, United States, [3] Department of Neuroscience, Clinical Neurophysiology, Uppsala University, Uppsala, Sweden, [4] Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

OPEN ACCESS

**Edited by:**
*Kutty Selva Nandakumar, Southern Medical University, China*

**Reviewed by:**
*Pia Bernasconi, Fondazione IRCCS Istituto Neurologico Carlo Besta, Italy*
*Alexander Marx, University of Heidelberg, Germany*
*Janine Adele Lamb, University of Manchester, United Kingdom*

***Correspondence:**
*Anna Rostedt Punga*
*anna.rostedt.punga@neuro.uu.se*

**Specialty section:**
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

**Received:** *09 November 2019*
**Accepted:** *20 January 2020*
**Published:** *21 February 2020*

**Citation:**
*Fiorillo AA, Heier CR, Huang Y-F, Tully CB, Punga T and Punga AR (2020) Estrogen Receptor, Inflammatory, and FOXO Transcription Factors Regulate Expression of Myasthenia Gravis-Associated Circulating microRNAs. Front. Immunol. 11:151. doi: 10.3389/fimmu.2020.00151*

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate important intracellular biological processes. In myasthenia gravis (MG), a disease-specific pattern of elevated circulating miRNAs has been found, and proposed as potential biomarkers. These elevated miRNAs include miR-150-5p, miR-21-5p, and miR-30e-5p in acetylcholine receptor antibody seropositive (AChR+) MG and miR-151a-3p, miR-423-5p, let-7a-5p, and let-7f-5p in muscle-specific tyrosine kinase antibody seropositive (MuSK+) MG. In this study, we examined the regulation of each of these miRNAs using chromatin immunoprecipitation sequencing (ChIP-seq) data from the Encyclopedia of DNA Elements (ENCODE) to gain insight into the transcription factor pathways that drive their expression in MG. Our aim was to look at the transcription factors that regulate miRNAs and then validate some of those *in vivo* with cell lines that have sufficient expression of these transcription factors This analysis revealed several transcription factor families that regulate MG-specific miRNAs including the Forkhead box or the FOXO proteins (FoxA1, FoxA2, FoxM1, FoxP2), AP-1, interferon regulatory factors (IRF1, IRF3, IRF4), and signal transducer and activator of transcription proteins (Stat1, Stat3, Stat5a). We also found binding sites for nuclear factor of activated T-cells (NFATC1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), early growth response factor (EGR1), and the estrogen receptor 1 (ESR1). AChR+ MG miRNAs showed a stronger overall regulation by the FOXO transcription factors, and of this group, miR-21-5p, let-7a, and let 7f were found to possess ESR1 binding sites. Using a murine macrophage cell line, we found activation of NF-κB -mediated inflammation by LPS induced expression of miR-21-5p, miR-30e-5p, miR-423-5p, let-7a, and let-7f. Pre-treatment of cells with the anti-inflammatory drugs prednisone or deflazacort attenuated induction of inflammation-induced miRNAs. Interestingly, the activation of inflammation induced packaging of the AChR+-specific miRNAs miR-21-5p and miR-30e-5p into exosomes, suggesting a possible mechanism for the elevation of these miRNAs in MG

patient serum. In conclusion, our study summarizes the regulatory transcription factors that drive expression of AChR+ and MuSK+ MG-associated miRNAs. Our findings of elevated miR-21-5p and miR-30e-5p expression in immune cells upon inflammatory stimulation and the suppressive effect of corticosteroids strengthens the putative role of these miRNAs in the MG autoimmune response.

**Keywords: microRNA, myasthenia gravis, estradiol, miR-21-5p, NF-κB, FOXO**

## INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate important intracellular biological processes. The field of circulating miRNAs in autoimmune disorders, especially as potential biomarkers have expanded in the recent years, including myasthenia gravis (MG) (1). In MG there is a shortage of biomarkers to predict the clinical course and this is particularly troublesome due to the fluctuating nature of skeletal muscle fatigue with periods of unpredictable worsening (2). MG is a heterogeneous disease that still can be subdivided into two main subgroups based on antibody target: patients with antibodies against the acetylcholine receptors (AChR+ MG; ∼85%) and those with antibodies against the muscle-specific tyrosine kinase (MuSK+ MG; ∼7%) (3). These two antibody subtypes differ, for example, in the involvement of the thymus which is considered to play a major role in AChR+ MG, especially in terms of thymus inflammation (hyperplasia) but also in regards to treatment response (4).

Recent reports have defined different signatures of elevated serum miRNAs in AChR+ and MuSK+ MG (1). In AChR+ MG, the miRNAs miR-150-5p, and miR-21-5p were upregulated compared to healthy controls and these levels were also lower in patients treated with immunosuppression as compared to immunosuppression naïve patients (5, 6). In addition to miR-150-5p and miR-21-5p, levels of miR-30e-5p were found to be upregulated in late onset AChR+ MG patients and expression of these miRNAs correlated with disease course (7). Interestingly, miR-30e-5p levels were found to be higher in patients with ocular AChR+ MG who later develop generalized MG as compared to those that remain ocular. Given this, expression of miR-30e-5p has the potential to predict clinical disease course (8). On the other hand, the miRNA profile in sera from MuSK+ MG patients revealed elevated miR-151a-3p, let-7a-5p, let-7f-5p, and miR-423-5p levels (9).

miRNAs modulate gene expression by targeting multiple mRNAs encoding proteins involved in a variety of signaling pathways and cellular processes (10). In a similar manner, miRNAs could influence and alter the autoimmunity signaling pathway involved in autoimmune disorders such as MG. Several pathways including the Nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) pathway are inappropriately regulated in several inflammatory disorders including chronic muscle inflammation (11). Toll-like receptor signaling activates NF-κB-driven inflammation. Elevated levels of Toll-like receptors (TLRs) 2, 3, 4, 5, 8, and 9 have been detected in MG peripheral blood mononuclear cells (PBMCs) (12). Moreover, TLR4, TLR7, and TLR9 have been implicated in amplifying autoimmunity in the MG thymus (13, 14).

In MG, T regulatory cells (Tregs) from the thymus of MG patients are profoundly defective in their suppressive activity (15) and there is also an imbalance in peripheral blood Tregs (16). Therefore, genes involved in these two pathways, Treg differentiation and NF-κB signaling, are anticipated to be associated with MG predisposition. Nevertheless, it is not clear which transcription factors regulate the MG-associated miRNA expression in AChR+ vs. MuSK+ MG. In this study, we examined the regulatory transcription factors that drive expression of extracellular miRNAs found in the serum of patients with AChR+ and MuSK+ MG. Our overall objective in this analysis was to outline transcription factors that regulate these miRNAs and then validate a subset of these (i.e., NF-κB and Estrogen Receptor, ESR1) *in vivo* with cell lines that have sufficient expression of these transcription factors [RAW macrophage cells for NF-κB (17) and T-cells for ESR (18)].

## MATERIALS AND METHODS

### Bioinformatics

We examined the surrounding regulatory region of each miRNA gene to gain insight into the mechanisms of response to treatment as previously reported (19). Briefly, we examined the binding of transcription factors that are most relevant in MG (20–22) using chromatin immunoprecipitation sequencing (ChIP-seq) data. ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) was queried for physical binding to DNA loci encoding the human miRNA target of interest (23, 24). Both the independent promoter/enhancer of the miRNA was queried, and for miRNAs that were encoded within introns of a gene, the promoter and enhancer of that gene was additionally queried. The proximal promoter was considered the region directly upstream of the miRNA or gene, within 2 kb (25) while the enhancer was considered the region within 10 kb of the miRNA or gene (26). In addition, we examined the following histone modifications which are enriched at regulatory elements such as promoters or enhancers: histone H3K4 trimethylation (found near promoters), H3K4 monomethylation (found near regulatory elements), and H3K27 acetylation (found near active regulatory elements). For each of these analyses, we used UC Santa Cruz (UCSC) Genome Browser Release 4 (https://genome.ucsc.edu/index.html) with alignment to the GRCh37/hg19 genome build. Each ChIP-seq dataset was analyzed using the ENCODE Regulation Super-Track listed under the Regulation menu. Transcription factors were assayed using the Txn Factor

ChIP Track. In regions bound by each transcription factor, DNA motifs recognized by that transcription factor were identified through the Factorbook repository within this track. Consensus motif sequence logo pictograms for each transcription factor were also visualized through Factorbook. Histone modifications were examined using the Layered H3K4Me1, Layered H3K4Me3, and Layered H3K27Ac Tracks. Raw data images for visualization of gene loci and ChIP-seq data were obtained using the PDF/PS function in the View menu of the genome browser. Binding of transcription factors was queried in ChIP-seq datasets produced using all 9 cell line tracks to identify all possible transcription factor binding. These include: GM12878, a lymphoblast cell line; H1-hESC, an embryonic stem cell line; HeLa-S3, a cervical cancer cell line; HepG2, a liver cancer cell line; HSMM, a human skeletal muscle myoblast cell line; HUVEC, a human umbilical vein endothelial cell line; K562, a human immortalized myelogenous leukemia cell line; NHEK, Human Epidermal Keratinocytes; and NHLF; a human lung fibroblast cell line. Histone modifications were also queried in ChIP-seq datasets using all nine available cell line tracks.

## Circulating miR-21-5p Analysis in Patients and Healthy Controls

Detailed patient characteristics have been described previously (5). In brief, stored serum samples were collected in tubes without additives from 54 healthy blood donors (27 females, mean age: 45 ± 16 years; 27 male, mean age: 47 ± 15 years) without any medications and from 73 MG patients (42 females; mean age: 57 ± 15 years), at Uppsala University Hospital. The MG group consisted of 27 female MG patients without any immunosuppression (mean age: 55 ± 13 years), 10 male MG patient without any immunosuppression (mean age: 62 ± 18 years), 15 female MG patients with immunosuppression (mean age: 53 ± 15 years), and 21 male MG patients with immunosuppression (mean age: 62 ± 14 years). In the MG patient cohort without immunosuppression, 76% were AChR+ and 24% were AChR/MuSK antibody seronegative (AChR/MuSK-) and among those with immunosuppression 70% were AChR+ and 30% were AChR-/MuSK-. Mean MGC score was 6.1 (range: 0–34) in the group without immunosuppression and 4.6 (range: 0–18) in the group with immunosuppressive treatment (5). The study was approved by the local Ethical Review Board in Uppsala (Dnr 2010/446) and all patients signed informed consent. RNA isolation, cDNA synthesis and qPCR were performed as previously described (5, 6). In brief, total RNA was isolated from 200 µl serum by using miRCURY™ RNA Isolation Kit—Biofluids (Exiqon#300112) and 2 µl of isolated RNA sample was applied for cDNA synthesis in a 10 µl reaction mix by using Universal cDNA Synthesis Kit II (Exiqon #20330). The RT-qPCR analysis was performed with ExiLENT SYBR® Green master mix (Exiqon #203421) and cDNA reactions were diluted 100X. The following quality controls were included on Pick-&-Mix microRNA PCR panel plates: the interpolate calibration (UniSp3), RNA extraction control (UniSp2 and UniSp4), cDNA synthesis control (UniSp6), and hemolysis test (miR- 23a-3p–miR-451a) (Exiqon). The ΔCT value of hemolysis markers [ΔCT (hemolysis) = CT(miR-23a-3p) – CT(miR-451a)], was used to detect hemolysis and samples with a ΔCT >7 were excluded. Reference miRNAs were miR-93-5p, miR-191-5p, miR-423-4p, and miR-103a and quantification of relative miRNA expression was performed with the comparative CT method.

## ELISA

Serum samples from 20 female AChR+ MG patients (mean age: 54 ± 12 years) without immunosuppressive medication that were also analyzed for miR-21-5p levels, and 20 age matched female healthy controls (mean age: 51 ± 14 years) were analyzed. Enzyme-linked immunosorbent assay (ELISA) was performed for 17-β-estradiol using Human Estradiol kit (KAQ0621, Invitrogen), following the manufacturer's instructions. All samples were assayed in duplicate at an absorbance of 450 nm, and the detection range was 13–935 pg/ml. Inter-assay CV was 6.1%. Calculating and analysis of data was conducted by Spark® Multimode Microplate Reader (Tecan Trading AG).

## miR-21-5p Expression in T Cells Treated With β-Estradiol and 4-Hydroxytamoxifen

Human T cell line KE-37 (DSMZ, ACC 46) was used for β-estradiol (E2758, Sigma) and 4-hydroxytamoxifen (H7904, Sigma) treatments, since human T cells express estrogen receptor (18).

Cells were plated in 35 mm dishes with RPMI1640 media (R7509, Sigma) with 10% charcoal stripped FBS (TA3382101, Thermo Fisher Scientific) at a density of 1,000,000 cells/dish. Each condition was performed in triplicate.

For β-estradiol and 4-hydroxytamoxifen co-treated group, cells were treated with 0.1 nM β-estradiol and $10^{-}$µM 4-hydroxytamoxifen for 24 h. For dose-response and time-course group, cells were treated for 24, 48, and 72 h, respectively, with ß-estradiol at 0, 5, and 50 nM. The expression of miR-21-5p was examined and analyzed by qRT-PCR, using reference genes miR-103a and miR-191-4p for normalization. miRNA isolation was performed using miRNeasy Mini Kit (217004, Qiagen) and cDNA was synthesized. PCR amplification system included cDNA templates (1:50 dilution), ExiLENT SYBR® Green Master Mix (201420-01, Exiqon) and miRNA-specific primers (Qiagen). PCR amplification was conducted in presence of Rox Reference Dye by Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies). Log conversion of the miRNA expression data was done in order to obtain data more similar to a normal distribution for the statistical tests.

## miRNA-Induction in Macrophage Cells

The murine macrophage cell line RAW 264.7, a standard model system for assessment of LPS-induced inflammatory response (17), was cultured in DMEM with 10% FBS. Cells were seeded in a 6-well plate at a density of 500,000 cells/well. Each condition was performed in triplicate. Cells were pretreated with 10 µM of the indicated drug or a DMSO vehicle-only control. Inflammatory signaling was induced using LPS (Thermo Fisher Scientific) or a PBS-only vehicle control at a dilution of 1:1,000 as previously reported (27). After 48 h exosomes were collected from the media

using ExoQuick (SBI), and RNA was subsequently extracted using TRIzol. Cells were also lysed for RNA using TRIzol at 24 or 48 h post-LPS induction. Expression of exosome-specific miRNAs was quantified by TaqMan Assay and results were normalized to the Geometric Mean of miR-17 and miR-93 as these miRNAs are reported to remain unchanged in exosomes (28). Expression of cell-specific miRNAs was also quantified by TaqMan Assay and values were normalized to sno202. The Taqman IDs for all miRNAs queried is as follows: snoRNA202, 001090; miR-93, 001090; miR-17, 002308; miR-151-3p, 001190; miR-21-5p, 000397; let-7a, 000377; let-7f, 000382, miR-150−5p, 000473; miR-30e-5p, 002223; miR-423-5p, 002340.

## Statistical Analysis

D Ágostino & Pearson normality test revealed that qPCR data were normally distributed (p>0.05) whereas ELISA data were not ($p < 0.05$). Comparison of miR-21-5p serum data between male and female controls and MG patients was done using one-way ANOVA, followed by unpaired *T*-test comparing female controls and MG patients as well as male controls and MG patients. Comparison of 17-β-estradiol levels between female MG patients and healthy controls was done by Wilcoxon matched-pairs signed rank test. A $p < 0.05$ was considered significant. Spearman Rank correlation was used to determine any correlation between estradiol and miR-21-5p levels as well as between miR-21-5p and age.

# RESULTS

## Transcription Factors Regulating AChR+ vs. MuSK+ MG-Associated miRNAs

The previously identified MG-associated miRNAs in serum included miR-150-5p, miR-21-5p and miR-30e-5p (AChR+ MG) as well as miR-151, miR-423, let-7f, and let-7a (MuSK+ MG) (1). Our bioinformatics analysis of the ChIP-seq data from ENCODE revealed several transcription factor binding sites on the aforementioned miRNA genes (**Figure 1**). For this analysis, we utilized data from all available cell lines deposited into ENCODE, to get an overview of the regulation of each miRNA. We used an arbitrary cutoff of 2 kb for the proximal promoter region and 10 kb for the proximal enhancer region. To increase confidence that the regions analyzed were bona fide regulatory elements we also overlayed ChIP-seq data showing verified histone modifications indicative of active promoters (H3K4 trimethylation) and enhancers/regulatory regions (H3K4 monomethylation, H3K27 acetylation (**Figure 1**).

At least nine transcription factors were found to have binding sites in the defined regulatory regions of 7 MG-associated miRNAs. The identified transcription factors included the Forkhead box proteins (FoxA1, FoxP2), the interferon regulatory factors (IRF1, IRF4), signal transducer and activator of transcription proteins (STAT1), AP-1 transcription factors (FOS, Jun, JunB) and NF-κB (denoted as RELA in ENCODE) (**Figure 1**). When we looked at the totality of transcription factor binding sites on the 7 miRNA regulator regions queried, we found that FOS, JunD, FoxA1, and RELA had the highest occupancy on the MG-associated miRNAs ($N = 30$, 28, 27, and 23, respectively). Intriguingly, only miR-21-5p, let-7a, and let-7f were found to possess estrogen receptor 1 (ESR1) binding sites. We also found nuclear factor of activated T-cells (NFATC1) and early growth response factor (EGR1) binding sites on the majority of the miRNA genes queried (**Figure 1**).

Due to the different signature of circulating miRNAs in AChR+ MG vs. MuSK+ MG, we next in silico determined the set of transcription factors that more specifically regulate the expression of these subtype specific miRNAs. AChR+ MG miRNAs showed a stronger overall regulation by the FOXO transcription factors, considering that miR-21-5p, miR-150-5p, and miR-30e-5p together possessed 52 binding sites for FOXO transcription factors (**Figure 2A**). On the contrary, MuSK+ miRNAs possessed less binding sites for the FOXO family (nine binding sites, **Figure 2A**) and instead had the most binding sites for the FOS, Jun, JunD, and Stat3 proteins. Further, in-depth analysis of each miRNA regulatory region revealed binding sites for the FOXO transcription factors within the miR-21 and the miR-30e loci (**Figures 2B–D**).

## Higher miR-21-5p Levels in Female Immunosuppression Naïve MG Patients

We next focused on the relationship of estrogen and miR-21-5p, as our bioinformatics analysis demonstrated heavy regulation of this miRNA by ESR1, showing six binding sites within its promoter/enhancer region (**Figures 1**, **3A,B**). Based on this, we tested the hypothesis that miR-21-5p levels would be markedly different between male and female MG patients. To do this, we re-analyzed miR-21-5p levels from archival data of AChR+ MG patients and healthy controls, this time grouping them by gender (5). There was no difference between serum miR-21-5p levels of healthy female controls ($N = 27$) and healthy male controls ($N = 27$; $p = 0.33$) or between female ($N = 27$) or male ($N = 10$) MG patients with immunosuppression ($p = 0.90$). However, female MG patients without immunosuppression had significantly higher values than male MG patients without immunosuppression ($p = 0.015$; **Figure 4A**). miR-21-5p levels were also higher in female MG patients without immunosuppression compared to healthy female controls ($p = 0.0001$; **Figure 4B**) as well as female MG patients with immunosuppression ($p = 0.019$, **Figure 4B**). One-way ANOVA did not reveal any difference in miR-21-5p levels between the groups of AChR+ and AChR/MuSK- patients (without and with immunosuppression, respectively; data not shown; $p = 0.21$).

We next explored whether age could play a role for miR-21-5p levels. However, correlation analysis did not reveal any relationship between age and miR-21-5p levels in healthy female controls ($R = 0.25$; $p = 0.21$) nor in male healthy controls ($R = -0.04$; $p = 0.85$). Neither was there any correlation between miR-21-5p levels and age in female MG patients without immunosuppression ($R = 0.17$; $p = 0.39$). A significant age correlation was seen however for miR-21-5p in male MG patients without immunosuppression ($R = 0.75$; $p = 0.017$), i.e., increasing miR-21-5p levels with increasing age.

**A**

| Transcription factor | miRNAS | | | | | |
|---|---|---|---|---|---|---|
| | miR-21-5p | miR-150-5p | miR-30e-5p | miR-151-3p | miR-423-5p | let7a-1-5p, let-7f-1-5p |
| ESR1 | 6 | | | | | 1 |
| EGR1 | 5 | 3 | 8 | 3 | | 4 |
| FOS | 10 | | 4 | 10 | 1 | 5 |
| JUN | 10 | 1 | 1 | 6 | 1 | 2 |
| JUNB | 4 | | | | | |
| JUND | 13 | 4 | 4 | 4 | 2 | 1 |
| FOXA1 | 18 | 1 | 5 | 1 | 2 | |
| FOXA2 | 8 | 1 | 3 | | | |
| FOXM1 | 9 | 1 | 2 | 1 | 1 | |
| FOXP2 | 1 | | 3 | 2 | 1 | 1 |
| IRF1 | 5 | 2 | 1 | 1 | 2 | 2 |
| IRF3 | | | | | | 2 |
| IRF4 | 5 | 1 | 2 | 1 | 2 | 1 |
| NFATC1 | 5 | 2 | 1 | 1 | 1 | |
| RELA (NF-kappaB) | 13 | 3 | 4 | 1 | 1 | 1 |
| STAT1 | 7 | 1 | 2 | 2 | 2 | 1 |
| STAT2 | 3 | | 1 | | | 1 |
| STAT3 | 14 | 1 | | 4 | 3 | 2 |
| STAT5A | 5 | 1 | 2 | 1 | 1 | |

**B**

| Group | Transcription factors | Disease relevance |
|---|---|---|
| Forhead box proteins | FoxA1, FoxA2, FoxM1, FoxP2 | Cancer, Aging , Autoimmunity |
| AP-1 transcription factors | FOS, Jun, JunB, JunD | Elevated in Rheumatoid arthritis and cancer |
| Interferon regulatory factor | IRF1, IRF3, IRF4 | Autoimmune disorders; aging & autoimmunity |
| Signal transducer & activator of transcription (STAT) | Stat1, Stat3, Stat5a | Autoimmune disease, Activated by cytokines |
| NF-kappaB | NF-kappaB | Inflammatory diseases, Stimulated by TNF-α and IL-1 |
| NFAT (Nuclear factor of activated T-cells) | NFATC1 | Autoimmune disease/ Cyclosporin A blocks activity |
| Early Growth Response Factor | EGR1 | Regulates genes involved in genes synaptic homeostasis and plasticity |
| Estrogen Receptor | ESR1 | Elevated in female MG patients; regulated by estrogen |

**FIGURE 1 |** Overview transcription factors that regulate MG-associated miRNAs. **(A)** Using the UCSC Genome browser, each of the seven miRNAs identified from our previous reports (1, 5) were queried for transcription factor regulation. **(A)** Shows an overview of all transcription factor binding sites within the proximal promoter (∼2 kb) of the miRNA, the proximal promoter of the gene surrounding the miRNA locus (if applicable), the enhancer region (within ∼10 kb) of the miRNA, and the enhancer region of the gene surrounding the miRNA locus (if applicable). **(B)** Overview of the disease relevance and function for each major transcription factor group listed in **(A)**.

Based on the link between ESR1 and miR-21-5p, we assumed that serum estradiol levels could be correlated to miR-21-5p levels. Therefore, we next assessed estrogen levels in female controls vs. female MG patients. While there was no significant difference between 17-β-estradiol levels in female MG patients compared to their age-and sex matched controls, there was a trend toward increased serum estradiol in female MG patients ($p = 0.07$; **Figure 4C**). Nevertheless, there was no clear correlation between miR-21-5p and 17-β -estradiol levels when taking the entire female cohort, i.e., immunosuppression naïve MG patients and healthy controls, into consideration ($p = 0.75$; Spearman $R = -0.05$; **Figure 4D**). In summary, the observed higher miR-21-5p levels in female MG patients without immunosuppression compared to male patients does not appear to be mediated by estradiol levels alone.

Finally, in order to determine whether estradiol would induce expression of miR-21-5p in a human T cell line, these cells were treated with 17-β-estradiol at different concentrations and duration. Treatment with 17-β-estradiol for 24, 48, and 72 h did not result in a significant increase in miR-21-5p levels (data not shown). Further, treatment with the estrogen receptor inhibitor tamoxifen did not induce significant reduction of miR-21-5p levels (**Supplementary Figure 1**).

## NF-κB Pathway Up-Regulates Expression of MG-Associated miRNAs

Based on the finding that AChR+ MG-associated miRNAs have a large number of putative NF-κB binding sites within the regulatory domains (**Figure 5A**), the effect on the expression of these miRNAs was assayed through induction of inflammatory signaling in a murine macrophage cell line. Upon inflammatory induction by LPS, which activates NF-κB through TLRs, such as TLR4, levels of miR-21-5p, miR-30e-5p, miR-151a-3p, miR-423-5p, let-7a-5p,

**A**

| miRNA | Number of putative FOXO binding sites |
|---|---|
| **miRNAs dysregulated in AchR+ MG** | |
| miR-21-5p | 36 |
| miR-150-5p | 3 |
| miR-30e-5p | 13 |
| **miRNAs elevated in MuSK+ MG** | |
| miR-151-3p | 4 |
| miR-423-5p | 4 |
| let7a-1-5p, let-7f-1-5p | 1 |

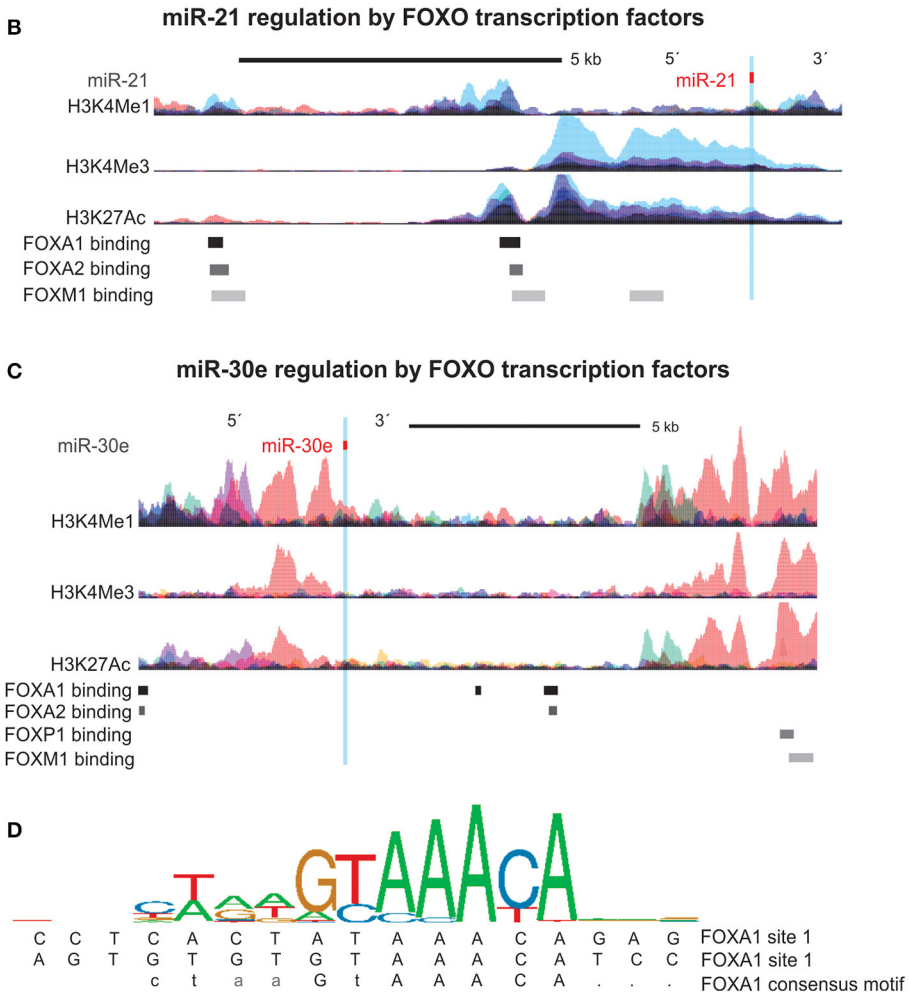



**FIGURE 2 |** Promoter analysis of miRNAs indicates regulation by FOXO transcription factors. FOXO Transcription factor binding sites and histone (H3) modifications that mark regulatory regions were examined using ChIP-seq data from ENCODE. DNA binding motifs for each transcription factor were identified through the Factorbook repository. **(A)** Table of all FOXO binding site found in regulatory regions of miRNAs dysregulated in both AchR+ and MuSK+ MG, demonstrating a high number of binding sites within miRNAs specific to the AchR+ subtype. **(B,C)** Schematic illustrating binding sites for FOXO transcription factors within the **(B)** miR-21 locus (seven of 26 binding sites shown) and **(C)** miR-30e locus (seven of 13 binding sites shown). Corresponding epigenetic modification maps are provided showing the location of histone modifications associated with active promoters (H3K4me3) and poised/active enhancers (H3K4me1 and H3K27Ac) in the immediate vicinity of both loci. **(D)** Sequence logo pictogram of base frequency at FOXA1 binding sites, with the consensus FOXA1 motif provided immediately below. Also provided are two representative FOXA1 binding site sequences near miR-30e, listed in order from the 5′ to 3′ direction.

and let-7f-5p were significantly elevated (**Figure 5B**). In addition, packaging of miR-21-5p and miR-30e-5p into exosomes was induced by inflammation (**Figure 5C**). Cell-specific expression of miRNAs was then assessed after anti-inflammatory treatment. Macrophages pretreated with the immunosuppressive treatment prednisolone and deflazacort attenuated the induction of the same miRNAs, miR-21 and miR-30e (**Figures 5D,E**).

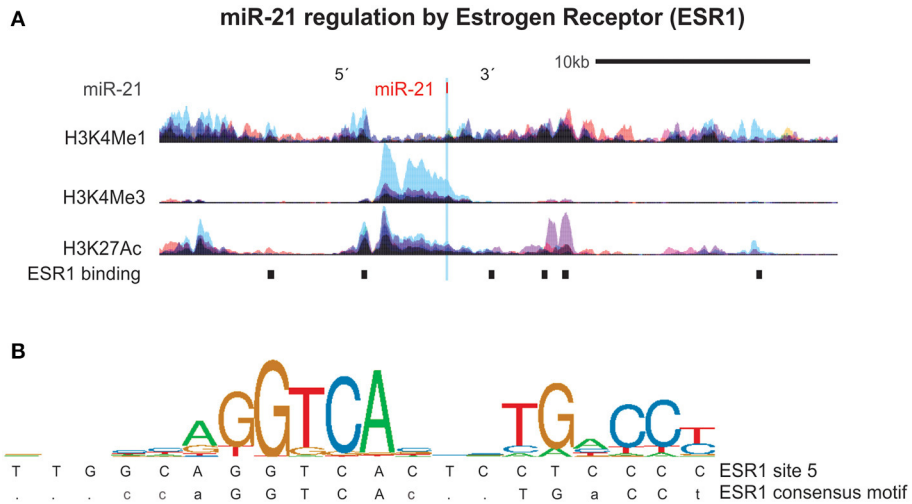



**FIGURE 3 |** miR-21 is regulated by the Estrogen Receptor 1 (ESR1). **(A)** Estrogen receptor 1 (ESR1) binding sites and histone (H3) modifications that mark regulatory regions were examined using ChIP-seq data from ENCODE as detailed in **Figure 2**. Shown are the six ESR1 binding sites in the region surrounding the miR-21 DNA locus. **(B)** Sequence logo pictogram of base frequency at ESR1 binding sites, with the consensus ESR1 motif provided immediately below. Also provided is a representative ESR1 binding site sequences near miR-30e, listed in order from the 5′ to 3′ direction.

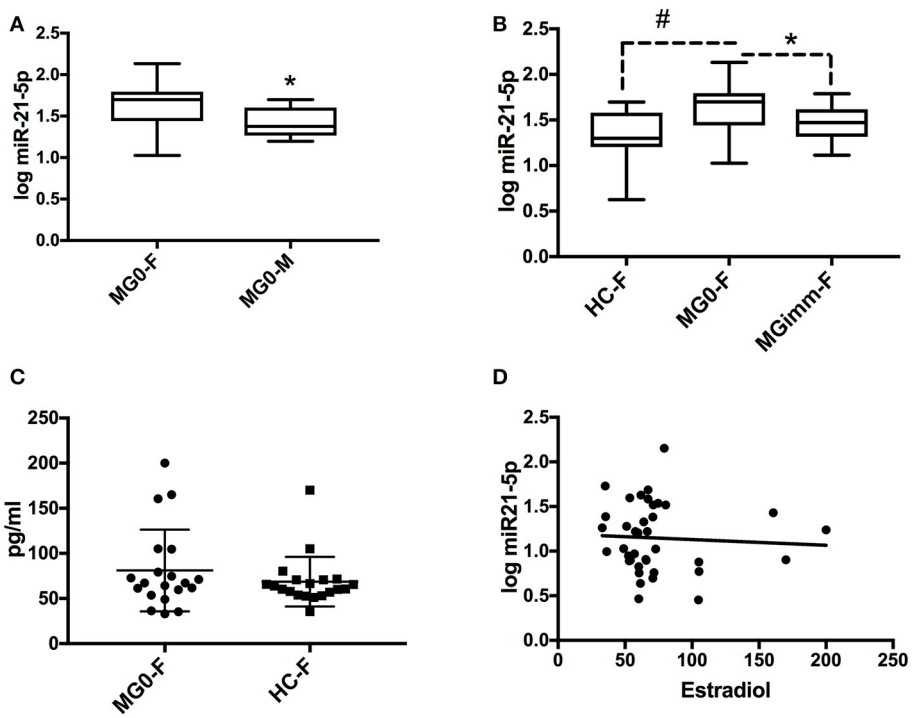



**FIGURE 4 |** miR-21-5p levels are highest in female MG patients without immunosuppression. **(A)** miR-21-5p levels in female (MG0-F; $N = 27$) and male MG (MG0-M; $N = 10$) patients without immunosuppression. **(B)** miR-21-5p levels in female healthy controls (HC-F; $N = 27$), MG0-F and MG patients with immunosuppression (MGimm-F; $N = 15$). Data in panels **(A,B)** are shown in logarithmic (log) scale and the boxplot refers to mean ± SD and whiskers state min to max values. **(C)** Serum 17-ß-estradiol in female MG patients ($N = 20$) vs. female age matched healthy controls ($N = 20$). **(D)** Correlation between 17-ß-estradiol and miR-21-5p among female healthy controls ($N = 20$) and MG patients without immunosuppression ($N = 20$). Spearman $R = -0.053$; $p = 0.75$. $^{*}p < 0.05$; $^{\#}p < 0.0001$.

**A**

| miRNA | Number of putative NF-kappaB binding sites |
|---|---|
| **miRNAs dysregulated in AchR+ MG** | |
| miR-21-5p | 13 |
| miR-150-5p | 3 |
| miR-30e-5p | 4 |
| **miRNAs elevated in MuSK+ MG** | |
| miR-151-3p | 1 |
| miR-423-5p | 1 |
| let7a-1-5p, let-7f-1-5p | 1 |

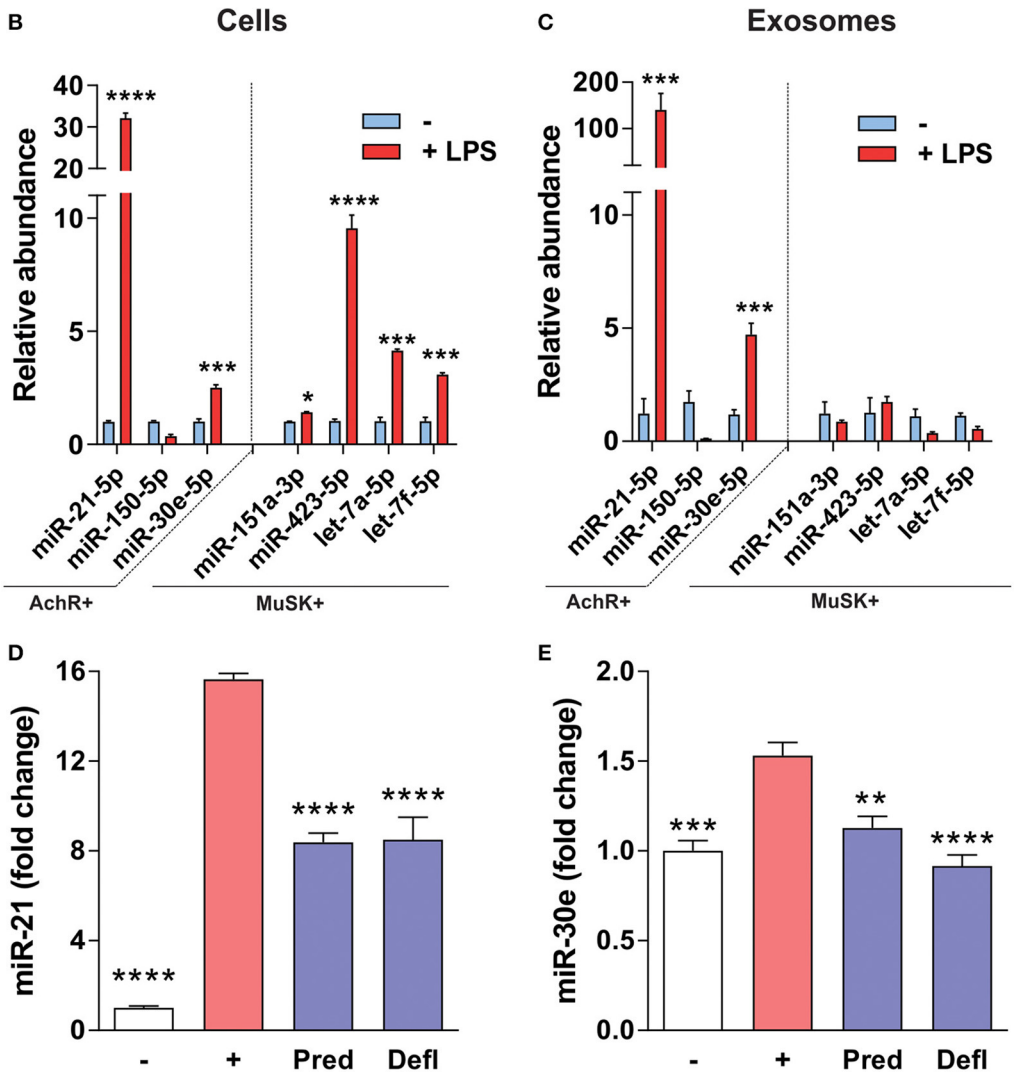



**FIGURE 5 |** NF-κB-mediated regulation of MG miRNAs. **(A)** Table shows the number of putative NF-κB binding sites within the regulatory regions of each miRNA listed. **(B,C)** Inflammatory signaling was induced in RAW 264.7 for 48 h using LPS. Expression of miRNAs was assayed by qRT-PCR. For each figure LPS treatment was compared to the respective no treatment control. **(B)** In a murine macrophage cell line, LPS induces miR-21-5p, miR-30e-5p, miR-151a-3p, miR-423-5p, let-7a-5p, and let-7f-5p **(C)** LPS induces packaging of miR-21-5p and miR-30e, but not other MG-associated miRNAs, into exosomes ($n = 4$, student's two-tailed *t*-test, ****$p < 0.0001$; ***$p < 0.001$; *$p < 0.05$). **(D,E)** RAW 264.7 macrophages were pretreated with the indicated drug at 10 μM and inflammatory signaling was induced for 24 h using LPS. Expression of miRNAs within cells was assayed by qRT-PCR for **(D)** miR-21-5p and **(E)** miR-30e-5p, respectively ($n = 4$ ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$, ANOVA with *post hoc* vs.; [–] = no LPS control, [+] = LPS plus vehicle, Defl, deflazacort; Pred, prednisolone.

## DISCUSSION

Research on circulating miRNAs in autoimmune disorders, including MG, has developed drastically in recent years. In this study, we examined the transcription factors that regulate MG-associated miRNAs in order to understand what drives the dysregulated serum miRNA profile observed in MG patients. Moving forward, understanding these mechanisms will have important implications toward immunosuppressive and anti-inflammatory drug development in MG. Pathway-based analyses that combine information across multiple genes into a limited number of molecular networks have been found to be a powerful approach that can also be used for miRNAs. The majority of identified transcription factors for MG-associated miRNAs are involved in autoimmunity-related pathways, especially the FOXO proteins, AP-1 transcription factors, NF-κB, IRF, and STAT family and NFAT. In line with this, the AChR+ MG-associated miRNAs might undergo stronger overall regulation by the FOXO transcription factors, considering the 52 binding sites in miR-21-5p, miR-150-5p, and miR-30e-5p for FOXO transcription factors. In AChR+ MG, T regulatory cells (Tregs) have reduced suppressive activity and one of the potential causes of this is decreased expression of FOXP3 in the MG thymus and peripheral blood (15, 29). The Treg specific transcription factor, Fork head/winged-helix transcription factor (FOXP3), has been shown to regulate both the development and the function of Tregs (30). FOXP3 expression is partly controlled by the phosphorylation of signal transducer and activator of transcription 5 (STAT5) induced by the IL-2 transduction pathway and low FOXP3 levels in Tregs may result from decreased STAT5 phosphorylation after IL2-signaling (31). The subtype of MuSK+ MG and AChR+ MG differ in several ways, mainly in that MuSK antibodies are of IgG4 subclass (32, 33), whereas the AChR antibodies are of IgG1 subclass. Further in MuSK+ MG, thymus pathology is normal compared to AChR+ MG (34), where thymus hyperplasia is considered to play a major role. In line with this, the circulating miRNA profile differs between these antibody subtypes of MG and thus it could be anticipated that the transcription factor profile is also different, as encountered here.

The proinflammatory NF-κB signaling pathway is related to inflammatory diseases and stimulated by TNF-α and IL-1. NF-κB had 23 binding sites across all MG-associated miRNAs, the majority being located in the AChR+ MG-associated miRNA genes. This is in support of a large study on almost 1,200 European MG patients with early onset AChR+ MG, where several risk genes were found related to the NF-κB signaling pathway (35). Here, the genetic associations to MG outside the HLA complex indicate VAV1, a key signal transducer crucial for T and B cell activation and BAFF, a cytokine important in the proliferation and differentiation of B cells. Combined VAV1 and BAFF haplotypes conferred a greater risk in combination and in addition to CD86, and these share the same signaling pathway, NF-κB (35). It was therefore not entirely surprising to find that NF-κB regulates transcription of the miRNAs found to be upregulated in AChR+ MG. Together, these data implicate that patients with early onset AChR+ MG may very well be predisposed to dysregulated proinflammatory signaling. miR-21-5p is also regulated by NF-κB, which would, in part, explain why MG patients on immunosuppressive drug therapy, i.e., mainly prednisone, have reduced levels of miR-21 compared to patients without immunosuppression (5). Nevertheless, other immunomodulatory medications such as azathioprine with a slower onset treatment effect and more long-acting properties, potentially have different effects on miR-21-5p and miR-150-5p as compared to the more rapid onset treatment effect of prednisone (36).

After querying ChIP-seq data to understand how MG-associated miRNAs are regulated, we functionally tested NF-κB-specfic miRNA regulation in a murine macrophage cell line and ESR1-specific miRNA regulation in a human T cell line. Our results have important implications about how the overexpression of MG-associated miRNAs may occur, but they also have their limitations. For instance, administration of LPS along with prednisone/deflazacort pre-treatment in murine macrophage cells serves to confirm that NF-κB can indeed induce expression of MG-associated miRNAs (miR-21-5p, miR-30e-5p, miR-151a-3p, miR-423-5p, let-7a-5p, and let-7f-5p) within cells and that blocking NF-κB can attenuate expression of a subset of these miRNAs (miR-21-5p and miR-30e-5p). However, in future studies it will be important to determine how these miRNAs respond to inflammation in individual patients that may have different levels and activation of NF-κB/NF-κB signaling. We also detected miR-21-5p and miR-30e in the exosomes of murine macrophages in response to LPS administration. This observation suggests that in MG patient serum, miR-21-5p and miR-30e may have originated from inflammatory cells that shed miRNA-containing exosomes in response to elevated inflammation. In future experiments it will be important to perform these experiments in patient-derived cell lines to determine (1) the totality of cell/tissues types that may be responsible for releasing miRNAs into the serum via exosomes and (2) other transcription factors might drive miRNA packaging into exosomes.

We analyzed miR-21-5p expression in a human T-cell line after treatment with estradiol, but did not, however, see significant induction. It is possible that estradiol was not sufficient to induce miR-21-5p expression because other transcription factors that co-regulate this miRNA (such as NF-κB) confounding the data. This is a likely scenario given that in female MG patients, we only observed significant elevation of serum miR-21-5p in when patients on immunopressive drugs were removed from analysis; this suggests heavy regulation of miR-21-5p by NF-κB. It is also possible that already high baseline ESR1 activation in this cell line prevented any additional activation of miR-21-5p upon estradiol administration.

To build our bioinformatics and cell line results, in future studies it will be important to determine how each of the identified transcription factors is expressed and how these collectively coordinate miRNA transcription in MG. While the cell culture experiments performed here were to determine if, in our hands, specific miRNAs are regulated by NF-κB and ESR1, these experiments do not yield the full picture of how these MG-associated miRNAs become dysregulated in MG.

While the data presented here allow us to postulate on which transcription factors are responsible for driving the expression of AChR+ and MuSK+ MG-associated miRNAs, what would be quite interesting in future studies is to derive macrophage and T-cells from AChR+ and MuSK+ patients and perform similar treatments, as mentioned above. Further, we could perform RNA-seq in comparison to healthy controls to look at potential upregulation of specific transcription factors. In follow up studies, such experiments will help us to determine which transcription factors would be the best therapeutic targets for AChR+ and MuSK+ MG.

There are distinct sex-associated differences in the expression of tissue-specific antigens that are controlled by the autoimmune regulator, a key factor for central tolerance in the autoimmune response. A recent study showed that in females, estrogen induces epigenetic changes in the autoimmune regulator (AIRE) gene, causing reduced AIRE expression below a threshold that increases female susceptibility to autoimmune diseases and in particular MG (37). In addition, worsening in female MG patients have been described in particular phases of the normal menstrual cycle, commonly (up to 50%) with symptoms being at their worst just before the next menstrual flow (38), when both progesterone and estrogen levels are the lowest. Previous studies indicate that 17ß-estradiol participate in the tolerization process by decreasing the expression of α-AChR and HLA-DR proteins and increases the expression of type I interferon and related molecules in thymic epithelial cells (37). With all the aforementioned effects of 17-β-estradiol on different aspects in MG in mind, the finding that ESR1 could regulate miR-21-5p, let-7a, and let-7f through binding sites is intriguing. This is in line with the higher levels of miR-21-5p in female immunosuppressive naïve MG patients compared to their male counterparts. However, we did not find a direct correlation between serum miR-21-5p and estradiol levels in female healthy controls or female MG patients without immunosuppression, indicating that other factors may influence the measurable circulating levels. Further, since 17-β-estradiol in women fluctuates during different phases of the menstrual cycle (39) and we did not sample the women during only one phase so this makes the interpretation of 17-β-estradiol levels more difficult.

In conclusion, the findings of our study highlight the connection between the MG-associated miRNAs and signaling pathways that govern inflammatory events and risk factors known to be important at least in AChR+ MG. This supports the important role of having biomarkers, such as miRNAs, that also are connected to the autoimmune events underlying the disease.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Review Board of Uppsala University (Dnr 2010/446). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AF, CH, TP, and AP contributed conception and design of the study. AF, CH, Y-FH, and CT performed experiments. AF and AP performed statistical analysis. AP and AF wrote the first draft of the manuscript. CH, Y-FH, and TP wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

## FUNDING

This study was supported by the Swedish Research Council (grant #2016-02184 to AP and TP, #2014-02048 and 2014-07603 to AP) and Göran Gustafsson foundation for medical research (to AP).

## ACKNOWLEDGMENTS

The authors are grateful to the MG patients and healthy controls who contributed with serum samples.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00151/full#supplementary-material

**Supplementary Figure 1 |** Human T-cell line KE-37 was treated for 24, 48, and 72 h, respectively, with ß-estradiol at 0 nM (control group), 5 and 50 nM. The relative expression of miR-21-5p (%) compared to the control group is displayed on the y-axis.

## REFERENCES

1. Punga AR, Punga T. Circulating microRNAs as potential biomarkers in myasthenia gravis patients. *Ann N Y Acad Sci.* (2018) 1412:33–40. doi: 10.1111/nyas.13510
2. Kaminski HJ, Kusner LL, Wolfe GI, Aban I, Minisman G, Conwit R, et al. Biomarker development for myasthenia gravis. *Ann N Y Acad Sci.* (2012) 1275:101–6. doi: 10.1111/j.1749-6632.2012.06787.x
3. Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurol.* (2015) 14:1023–36. doi: 10.1016/S1474-4422(15)00145-3
4. Cron MA, Maillard S, Villegas J, Truffault F, Sudres M, Dragin N, et al. Thymus involvement in early-onset myasthenia gravis. *Ann N Y Acad Sci.* (2018) 1412:137–45. doi: 10.1111/nyas.13519
5. Punga AR, Andersson M, Alimohammadi M, Punga T. Disease specific signature of circulating miR-150-5p and miR-21-5p in myasthenia gravis patients. *J Neurol Sci.* (2015) 356:90–6. doi: 10.1016/j.jns.2015.06.019
6. Punga T, Le Panse R, Andersson M, Truffault F, Berrih-Aknin S, Punga AR. Circulating miRNAs in myasthenia gravis: miR-150-5p as a new potential biomarker. *Ann Clin Transl Neurol.* (2014) 1:49–58. doi: 10.1002/acn3.24
7. Sabre L, Maddison P, Sadalage G, Ambrose PA, Punga AR. Circulating microRNA miR-21-5p, miR-150-5p and miR-30e-5p correlate with clinical

status in late onset myasthenia gravis. *J Neuroimmunol.* (2018) 321:164–70. doi: 10.1016/j.jneuroim.2018.05.003
8. Sabre L, Maddison P, Wong SH, Sadalage G, Ambrose PA, Plant GT. miR-30e-5p as predictor of generalization in ocular myasthenia gravis. *Ann Clin Transl Neurol.* (2019) 6:243–51. doi: 10.1002/acn3.692.
9. Punga T, Bartoccioni E, Lewandowska M, Damato V, Evoli A, Punga AR. Disease specific enrichment of circulating let-7 family microRNA in MuSK+ myasthenia gravis. *J Neuroimmunol.* (2016) 292:21–6. doi: 10.1016/j.jneuroim.2016.01.003
10. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol.* (2018) 9:402. doi: 10.3389/fendo.2018.00402
11. Monici MC, Aguennouz M, Mazzeo A, Messina C, Vita G. Activation of nuclear factor-kappaB in inflammatory myopathies and Duchenne muscular dystrophy. *Neurology.* (2003) 60:993–7. doi: 10.1212/01.WNL.0000049913.27181.51
12. Robinet M, Maillard S, Cron MA, Berrih-Aknin S, Le Panse R. Review on toll-like receptor activation in myasthenia gravis: application to the development of new experimental models. *Clin Rev Allergy Immunol.* (2017) 52:133–47. doi: 10.1007/s12016-016-8549-4
13. Cavalcante P, Barzago C, Baggi F, Antozzi C, Maggi L, Mantegazza R, et al. Toll-like receptors 7 and 9 in myasthenia gravis thymus: amplifiers of autoimmunity? *Ann N Y Acad Sci.* (2018) 1413:11–24. doi: 10.1111/nyas.13534
14. Cordiglieri C, Marolda R, Franzi S, Cappelletti C, Giardina C, Motta T, et al. Innate immunity in myasthenia gravis thymus: pathogenic effects of Toll-like receptor 4 signaling on autoimmunity. *J Autoimmun.* (2014) 52:74–89. doi: 10.1016/j.jaut.2013.12.013
15. Balandina A, Lecart S, Dartevelle P, Saoudi A, Berrih-Aknin S. Functional defect of regulatory CD4(+)CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis. *Blood.* (2005) 105:735–41. doi: 10.1182/blood-2003-11-3900
16. Wen Y, Yang B, Lu J, Zhang J, Yang H, Li J. Imbalance of circulating CD4+CXCR5+FOXP3+ Tfr-like cells and CD4+CXCR5+FOXP3- Tfh-like cells in myasthenia gravis. *Neurosci Lett.* (2016) 630:176–82. doi: 10.1016/j.neulet.2016.07.049
17. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity.* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
18. Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunol Lett.* (2005) 97:107–13. doi: 10.1016/j.imlet.2004.10.007
19. Fiorillo AA, Tully CB, Damsker JM, Nagaraju K, Hoffman EP, Heier CR. Muscle miRNAome shows suppression of chronic inflammatory miRNAs with both prednisone and vamorolone. *Physiol Genomics.* (2018) 50:735–45. doi: 10.1152/physiolgenomics.00134.2017
20. Zhang Y, Jia X, Xia Y, Li H, Chen F, Zhu J, et al. Altered expression of transcription factors IRF4 and IRF8 in peripheral blood B cells is associated with clinical severity and circulating plasma cells frequency in patients with myasthenia gravis. *Autoimmunity.* (2018) 51:126–34. doi: 10.1080/08916934.2018.1454913
21. Nancy P, Berrih-Aknin S. Differential estrogen receptor expression in autoimmune myasthenia gravis. *Endocrinology.* (2005) 146:2345–53. doi: 10.1210/en.2004-1003
22. Peng SL. Transcription factors in autoimmune diseases. *Front Biosci.* (2008) 13:4218–40. doi: 10.2741/3001
23. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. *Genome Res.* (2002) 12:996–1006. doi: 10.1101/gr.229102
24. Mathelier A, Fornes O, Arenillas DJ, Chen CY, Denay G, Lee J, et al. JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* (2016) 44:D110–5. doi: 10.1093/nar/gkv1176
25. Zhang MQ. Identification of human gene core promoters *in silico*. *Genome Res.* (1998) 8:319–26. doi: 10.1101/gr.8.3.319
26. MacIsaac KD, Lo KA, Gordon W, Motola S, Mazor T, Fraenkel E. A quantitative model of transcriptional regulation reveals the influence of binding location on expression. *PLoS Comput Biol.* (2010) 6:e1000773. doi: 10.1371/journal.pcbi.1000773
27. Heier CR, Yu Q, Fiorillo AA, Tully CB, Tucker A, Mazala DA, et al. Vamorolone targets dual nuclear receptors to treat inflammation and dystrophic cardiomyopathy. *Life Sci Alliance.* (2019) 2:e201800186. doi: 10.26508/lsa.201800186
28. Schwarzenbach H, da Silva AM, Calin G, Pantel K. Data normalization strategies for MicroRNA quantification. *Clin Chem.* (2015) 61:1333–42. doi: 10.1373/clinchem.2015.239459
29. Thiruppathi M, Rowin J, Ganesh B, Sheng JR, Prabhakar BS, Meriggioli MN. Impaired regulatory function in circulating CD4(+)CD25(high)CD127(low/-) T cells in patients with myasthenia gravis. *Clin Immunol.* (2012) 145:209–23. doi: 10.1016/j.clim.2012.09.012
30. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol.* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
31. Alahgholi-Hajibehzad M, Oflazer P, Aysal F, Durmus H, Gulsen-Parman Y, Marx A, et al. Regulatory function of CD4+CD25++ T cells in patients with myasthenia gravis is associated with phenotypic changes and STAT5 signaling: 1,25-Dihydroxyvitamin D3 modulates the suppressor activity. *J Neuroimmunol.* (2015) 281:51–60. doi: 10.1016/j.jneuroim.2015.03.008
32. Huijbers MG, Zhang W, Klooster R, Niks EH, Friese MB, Straasheijm KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc Natl Acad Sci USA.* (2013) 110:20783–8. doi: 10.1073/pnas.1313944110
33. Koneczny I, Cossins J, Waters P, Beeson D, Vincent A. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1-3 can disperse preformed agrin-independent AChR clusters. *PLoS ONE.* (2013) 8:e80695. doi: 10.1371/journal.pone.0080695
34. Marx A, Pfister F, Schalke B, Saruhan-Direskeneli G, Melms A, Strobel P. The different roles of the thymus in the pathogenesis of the various myasthenia gravis subtypes. *Autoimmun Rev.* (2013) 12:875–84. doi: 10.1016/j.autrev.2013.03.007
35. Avidan N, Le Panse R, Harbo HF, Bernasconi P, Poulas K, Ginzburg E, et al. VAV1 and BAFF, via NFkappaB pathway, are genetic risk factors for myasthenia gravis. *Ann Clin Transl Neurol.* (2014) 1:329–39. doi: 10.1002/acn3.51
36. Molin CJ, Sabre L, Weis CA, Punga T, Punga AR. Thymectomy lowers the myasthenia gravis biomarker miR-150-5p. *Neurol Neuroimmunol Neuroinflamm.* (2018) 5:e450. doi: 10.1212/NXI.0000000000000450
37. Dragin N, Bismuth J, Cizeron-Clairac G, Biferi MG, Berthault C, Serraf A, et al. Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. *J Clin Invest.* (2016) 126:1525–37. doi: 10.1172/JCI81894
38. Osserman KE, Genkins G. Studies in myasthenia gravis: review of a twenty-year experience in over 1200 patients. *Mt Sinai J Med.* (1971) 38:497–537.
39. Thorneycroft IH, Mishell DR Jr, Stone SC, Kharma KM, Nakamura RM. The relation of serum 17-hydroxyprogesterone and estradiol-17-beta levels during the human menstrual cycle. *Am J Obstet Gynecol.* (1971) 111:947–51. doi: 10.1016/0002-9378(71)90951-3

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 04 March 2020
doi: 10.3389/fimmu.2020.00213

# Circulating miRNAs as Potential Biomarkers in Myasthenia Gravis: Tools for Personalized Medicine

*Liis Sabre[1,2†], Tanel Punga[3†] and Anna Rostedt Punga[2*]*

[1] Department of Neurology and Neurosurgery, University of Tartu, Tartu, Estonia, [2] Department of Neuroscience, Clinical Neurophysiology, Uppsala University, Uppsala, Sweden, [3] Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

OPEN ACCESS

**Edited by:**
*Attila Mócsai,*
*Semmelweis University, Hungary*

**Reviewed by:**
*Lin Mei,*
*Case Western Reserve University, United States*
*Angela Vincent,*
*University of Oxford, United Kingdom*
*Paraskevi Zisimopoulou,*
*Pasteur Hellenic Institute, Greece*

***Correspondence:**
*Anna Rostedt Punga*
*anna.rostedt.punga@neuro.uu.se*

*†These authors share first authorship*

**Specialty section:**
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

**Received:** *08 November 2019*
**Accepted:** *27 January 2020*
**Published:** *04 March 2020*

**Citation:**
*Sabre L, Punga T and Punga AR (2020) Circulating miRNAs as Potential Biomarkers in Myasthenia Gravis: Tools for Personalized Medicine. Front. Immunol. 11:213. doi: 10.3389/fimmu.2020.00213*

Myasthenia gravis (MG) is an autoimmune disease caused by antibodies which attack receptors at the neuromuscular junction. One of the main difficulties in predicting the clinical course of MG is the heterogeneity of the disease, where disease progression differs greatly depending on the subgroup that the patient is classified into. MG subgroups are classified according to: age of onset [early-onset MG (EOMG; onset $\leq$ 50 years) *versus* late-onset MG (LOMG; onset $>$ 50 years]; the presence of a thymoma (thymoma-associated MG); antibody subtype [acetylcholine receptor antibody seropositive (AChR+) and muscle-specific tyrosine kinase antibody seropositive (MuSK+)]; as well as clinical subtypes (ocular *versus* generalized MG). The diagnostic tests for MG, such as antibody titers, neurophysiological tests, and objective clinical fatigue score, do not necessarily reflect disease progression. Hence, there is a great need for reliable objective biomarkers in MG to follow the disease course as well as the individualized response to therapy toward personalized medicine. In this regard, circulating microRNAs (miRNAs) have emerged as promising potential biomarkers due to their accessibility in body fluids and unique profiles in different diseases, including autoimmune disorders. Several studies on circulating miRNAs in MG subtypes have revealed specific miRNA profiles in patients' sera. In generalized AChR+ EOMG, miR-150-5p and miR-21-5p are the most elevated miRNAs, with lower levels observed upon treatment with immunosuppression and thymectomy. In AChR+ generalized LOMG, the miR-150-5p, miR-21-5p, and miR-30e-5p levels are elevated and decrease in accordance with the clinical response after immunosuppression. In ocular MG, higher levels of miR-30e-5p discriminate patients who will later generalize from those remaining ocular. In contrast, in MuSK+ MG, the levels of the let-7 miRNA family members are elevated. Studies of circulating miRNA profiles in Lrp4 or agrin antibody-seropositive MG are still lacking. This review summarizes the present knowledge of circulating miRNAs in different subgroups of MG.

**Keywords: circulating microRNA, biomarker, myasthenia gravis, miR-150-5p, miR-21-5p, miR-30e-5p**

## INTRODUCTION

Myasthenia gravis (MG) is a chronic autoimmune neuromuscular disorder with a prevalence of approximately 40–180 per million (1, 2). Antibodies in MG are directed against neuromuscular junction antigens; in the majority of patients, to the nicotinic acetylcholine receptor (AChR) in ∼85% and to muscle-specific tyrosine kinase (MuSK) in ∼7% of patients. More recently discovered antibody targets include the low-density lipoprotein receptor-related protein 4 (Lrp4) (3, 4) in ∼18% of AChR/MuSK antibody-seronegative patients (5) and agrin predominantly in patients with either MuSK, AChR, or Lrp4 antibodies (6, 7). MG patients suffer from fluctuating skeletal muscle fatigue and weakness. The etiology of the disease is unknown, although the thymus is considered to play a central role in the disease process as it is essential for T cell differentiation and the establishment of central tolerance (8, 9). Valid diagnostic measures for MG include antibody analysis, electrophysiological measures of impaired neuromuscular transmission, and objective clinical evaluation of skeletal muscle fatigue, such as the quantitative MG (QMG) score or MG composite (MGC) score.

One of the main difficulties in predicting the clinical course of MG is the heterogeneity of the disease, where disease progression differs greatly depending on the subgroup that the patient is classified into. The major described MG subgroups include: age of onset [early-onset MG (EOMG; onset $\leq$ 50 years) *versus* late-onset MG (LOMG; onset $>$ 50 years]; the presence of a thymoma (thymoma-associated MG, TAMG); and antibody subtype [acetylcholine receptor antibody-seropositive (AChR+) *versus* muscle-specific tyrosine kinase antibody seropositive (MuSK+)] (1). In addition to the subgroups of antibody subtype, age at onset, and thymus appearance, MG in all age groups (both EOMG and LOMG) can be further subdivided according to its clinical manifestations and the muscle groups involved, mainly ocular MG (OMG) and generalized MG (GMG) (10). Besides the highly variable pattern of the initial clinical presentation of MG, skeletal muscle fatigue fluctuates over days and even hours. Antibodies to AChR, MuSK, Lrp4, or agrin also have a useful role as diagnostic biomarkers for the confirmation of MG and classifying the disease subgroup. However, their titer does not necessarily correlate with the disease severity or response to treatment (11). There is therefore a need for reliable biomarkers of disease progression as well as pharmacodynamic biomarkers that better guide therapeutic response in MG (12, 13).

According to the Food and Drug Administration (FDA) and the National Institutes of Health (NIH) Biomarker Working Group, a biomarker is defined as an easily measured indicator of a normal or abnormal physiological process or response to intervention (14). Ideally, a valid biomarker in MG should easily differentiate MG patients from healthy individuals and also be able to differentiate MG subgroups, including EOMG *versus* LOMG, AChR+ *versus* MuSK+ MG, thymoma-associated MG, as well as OMG *versus* GMG.

## INTRACELLULAR MICRORNAS

MicroRNAs (miRNAs) are short, endogenous non-coding RNA molecules originally discovered in roundworm *Caenorhabditis elegans* in 1993 (15, 16). With the advent of high-throughput sequencing technologies, hundreds of miRNAs have been identified in worms, flies, plants, and mammals, including humans (17). Although the number of identified human miRNAs continuously increases, only a small fraction of them has been characterized in detail. A recent study estimated that there are about 2,300 true human mature miRNAs, 1,115 of which are currently annotated in the miRbase database[1] (V22) (18). The biosynthesis of miRNA involves cellular proteins Drosha and Dicer, which process a long primary miRNA (pri-miRNA) into ∼21–25 nucleotide double-stranded miRNA duplexes (19). Thereafter, only one RNA strand, so-called mature miRNA, is incorporated into a functional RNA-induced silencing complex (RISC) by binding to the Argonaute (Ago) proteins. Every miRNA duplex can generate two mature miRNAs: 5′-strand miRNA (known also as miRNA-5p) and 3′-strand miRNA (known also as miRNA-3p). Which of these strands is incorporated into RISC depends on the thermodynamic properties of individual miRNA duplexes (20). Remarkably, as little as 7-bp complementarities between miRNA and mRNA is enough to block targeted mRNA translation into protein (21). Considering the huge variety of mature miRNAs, it is obvious that their interactions with mRNAs regulate the key cellular processes, such as differentiation, proliferation, and apoptosis (22, 23). Not surprisingly, alterations in miRNA expression and RISC incorporation are dysregulated in many disorders, including cancer and cardiovascular and autoimmune diseases (24–26).

## EXTRACELLULAR CIRCULATING MIRNAS

### Characteristics of Circulating miRNAs

In addition to their intracellular accumulation, mature miRNAs are also detectable outside of the cells, in the extracellular space. These miRNAs, so-called circulating miRNAs, can be found in human body fluids, including blood plasma and serum, urine, saliva, semen, tears, breast milk, amniotic fluid, cerebrospinal fluid, and peritoneal and pleural fluids (27–31). The composition and the concentration of circulating miRNAs vary in different body fluids, with some distinct miRNA species dominant in specific biofluids (32).

Although miRNAs are detectable in different biofluids, the majority of circulating miRNA studies have been conducted in human serum and plasma samples due to easy access and the well-established miRNA isolation/analysis methods in this biological material. A particular feature of circulating miRNAs is that they are very stable and endure harsh treatments, such as low/high pH, high RNase concentrations, extended storage, and multiple freeze–thaw cycles (27, 30, 33).

[1] www.mirbase.org

miRNAs are secreted through various types of membrane-enclosed extracellular vesicles (EVs), such as microvesicles and exosomes. The membrane encapsulation protects miRNAs from degradation and facilitates the uptake of extracellular miRNAs by the recipient cells (31, 34). The uptake of miRNAs can vary depending on the type of EV and the recipient cell origin (35). For example, tumor-derived exosomes are incorporated into organ-specific cells with the help of specific sets of exosomal integrins (36). The EVs contain specific subsets of miRNAs, which differ from the donor cell miRNA profile. An elegant study by Skog and co-workers (34) revealed that exosomal miRNA profiles differ between glioblastoma patients and healthy controls, implying that miRNAs are selectively loaded into EVs. In contrast to the well-established miRNA biogenesis, the molecular mechanisms behind miRNA loading into EVs are still poorly understood. Available studies suggest that RNA-binding proteins (e.g., Ago2 and hnRNPA2B) might provide specificity for miRNA loading into EVs (37, 38). Also, the metabolic state of a cell and its origin can influence miRNA loading into EVs (39).

### Circulating miRNAs as Potential Biomarkers

Their stability and ability to be transported in the extracellular fluids have made circulating miRNAs promising therapeutic and diagnostic tools. From the therapeutic point of view, circulating miRNAs can be considered as paracrine and endocrine signaling molecules with the ability to change gene expression on nearby and distant target cells in the body, respectively (40). Furthermore, the correlation between circulating miRNA amount and the disease outcome has put these tiny molecules in the spotlight as potential biomarkers for disease diagnosis and monitoring (39). Circulating miRNAs fulfill the requirements for a biomarker as they are specific, very stable, easily accessible in a minimally invasive manner, and their detection is cost-effective. Truly, multiple studies have shown that changes in circulating miRNA amounts can be correlated to a variety of diseases, including cancer, neurodegenerative disorders, and obesity (39). Based on that, specific changes in circulating miRNA levels can be assigned to particular diseases. For example, several studies have established circulating miRNA profiles in biofluids from obese patients. Increased levels of miR-140-5p, miR-142-3p, and miR-222, accompanied by reduced accumulations of miR-532-5p, miR-125b, miR-130b, miR-221, miR-15a, miR-423-5p, and miR-520c-3p, are reported in obese patients' plasma (41). In addition, a liver-specific miRNA, miR-122, is found elevated in the sera of obese patients (42). These two studies not only illustrate the potential of specific circulating miRNAs as the biomarkers of obesity but also show the complexity of the circulating miRNAs in different biofluids. The use of circulating miRNAs as biomarkers is not limited to disease diagnosis and monitoring. In particular, the quantitative detection of circulating miRNAs can also be used to monitor the health of the individuals during their physical exercises and dietary regimes (43, 44).

The number of studies showing circulating miRNAs as potential biomarkers are constantly rising. However, there are still some concerns about their application as biomarkers. This involves sample collection, miRNA isolation, miRNA detection, and data analysis. The application of different sample collection and miRNA isolation protocols can lead to the under- and overrepresentation of different miRNA species. This can have serious consequences considering that the evaluation of disease status relies on a miRNA profile and not on one single miRNA (45). Quantitative reverse transcription PCR (qRT-PCR) is very often used as the standard method to evaluate miRNA expression profiles. This method is robust, easy to perform, and fast. However, normalization of the qRT-PCR data between different circulating miRNA samples is challenging due to the lack of a universal "housekeeping gene" in the EVs. Different normalization methods have been used, which, however, makes the interpretation of results puzzling and can lead to contradicting results between different studies (46). Hence, development of standardized methods and protocols, both for circulating miRNA sample processing and analysis, should reduce the present challenges and boost the practical usage of circulating miRNAs as biomarkers (47).

## EXTRACELLULAR CIRCULATING MIRNAS AS POTENTIAL BIOMARKERS IN MG SUBGROUPS

### Acetylcholine Receptor Antibody-Seropositive Early-Onset MG

The first study on circulating miRNAs assessed miRNAs in the serum samples of AChR+ female generalized EOMG patients without immunosuppressive treatment (48). Extracellular miR-150-5p and miR-21-5p levels were elevated, whereas the miR-27a-3p level was reduced in MG patient sera compared to healthy controls (48). This study also indicated that miR-150-5p specifically decreased upon thymectomy, in line with clinical improvement. A follow-up study in a more heterogeneous clinical cohort of both male and female AChR+ and AChR- MG patients in 2015 also compared the miR-150-5p and miR-21-5p levels with healthy controls and patients with other autoimmune diseases, such as psoriasis and Addison's disease (**Figure 1**). The levels of these two miRNAs were significantly reduced in the sera from MG patients on immunosuppressive treatment (49). This study was followed by a longitudinal study on extracellular miR-150-5p and miR-21-5p before and after thymectomy, assaying sera from 80 patients participating in the prospective international randomized trial of thymectomy in MG (MGTX Trial) (50). Longitudinal analysis of miR-150-5p and miR-21-5p indicated that the miR-150-5p levels decreased significantly 2 years after thymectomy, whereas no significant reduction was found in the prednisone group (51). The reason for the miR-150-5p levels not decreasing in the prednisone group could in part relate to the known sensitivity of miR-150 to exposure to corticosteroids, which causes levels to increase (52). These encouraging results raise the possibility of using extracellular miR-150-5p as a possible sensitive serum biomarker especially for AChR+ MG, although different effects that certain immunosuppression may have on the miR-150-5p levels should

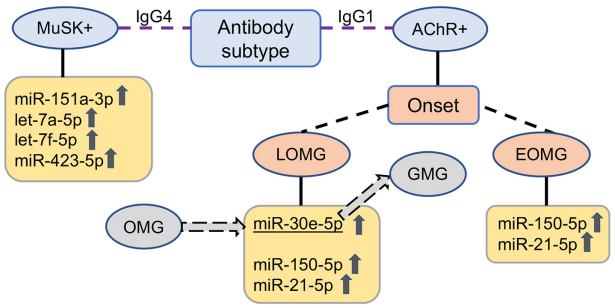



**FIGURE 1 |** Upregulated circulating miRNAs in the serum of myasthenia gravis (MG) patients according to antibody subtype. In acetylcholine receptor antibody-seropositive (AChR +) early-onset MG (EOMG) patients, the immuno-miRs miR-150-5p and miR-21-5p are elevated; in late-onset MG (LOMG) patients, miR-30e-5p is additionally elevated. miR-30e-5p is also a potential predictor of progression from ocular to generalized MG. In muscle-specific tyrosine kinase antibody-seropositive (MuSK +) MG patients with IgG4 antibodies, another upregulated circulating miRNA profile, miR-151a-3p, let-7a-5p, let-7f-5p, and miR-423-5p, is found. *OMG* ocular MG, *GMG* generalized MG, *IgG1* immunoglobulin G subclass 1, *IgG4* immunoglobulin G subclass 4.

be considered (51). Intriguingly, the miR-150-5p and miR-21-5p levels decreased after physical exercise intervention in MG patients (53).

The aforementioned circulating miRNAs are not the only reported alterations in AChR+ MG patients' biofluids. Another profiling of circulating miRNAs in different AChR+ MG patient (EOMG, LOMG, and TAMG) sera revealed that at least seven miRNAs were downregulated (miR-15b, miR-122, miR-140-3p, miR-185, miR-192, miR-20b, and miR-885-5p) compared with healthy controls (54). Nevertheless, differences in this profile of miRNAs were not found between treated and untreated MG patients (54).

## Late-Onset MG

Late-Onset MG is a specific subtype of MG where patients most often have thymus atrophy, in contrast to EOMG where thymus hyperplasia is much more frequent (55), although the majority of LOMG patients also are AChR+. LOMG is also special in that it affects relatively more male patients. In order to establish a signature of extracellular miRNA in LOMG, one separate study addressed this MG group. Compared to healthy controls, five miRNAs were found strongly elevated in LOMG patients with no immunosuppressive treatment: miR-106b-3p, miR-30e-5p, miR-223-5p, miR-140-5p, and miR-19b-3p (56). To assess the prospective influence of these miRNAs in immunosuppressive-naive generalized LOMG patients with immunosuppression, these miRNAs were longitudinally analyzed in 73 LOMG patients using sera collected for 2 years after the MG onset (56). Since 96% of these LOMG patients were AChR+, the previously found elevated miRNAs miR-21-5p and miR-150-5p (57) were also analyzed. This study found a steady decline in clinical MGC score at and after 1-year follow-up, which correlated with reduced levels of miR-150-5p, miR-21-5p, and miR-30e-5p (56; **Figure 1**), related to immunosuppression initiation after onset. Intriguingly, patients with generalized LOMG had higher levels of miR-150-5p and miR-21-5p than those with ocular LOMG (56). None of these miRNAs correlated with the AChR antibody levels, hence supporting the previous studies (48, 49, 51). Interestingly, the data from another cohort study on circulating miRNA in LOMG did not overlap regarding the results from the study by Nogales-Gadea (54). The discrepancies between these two studies (54, 56) could be explained by the differences in the LOMG patient cohort numbers as well as their immunosuppressive treatment status.

## Ocular MG

Ocular MG is defined as clinical MG symptoms and signs restricted to the extraocular muscles, manifesting as ptosis and/or diplopia. Retrospective studies report that up to 80% of MG patients with initially purely ocular symptoms develop secondary generalized MG (GMG), most within 2 years from disease onset (58, 59). Although there are no predictive factors for the risk of conversion from OMG to GMG, AChR+ patients are likely to be at higher risk of conversion than AChR antibody-seronegative patients (60). Since the aforementioned miRNA study in LOMG (56) revealed lower levels of some miRNAs in patients with OMG compared to GMG, a recent study aimed at determining whether circulating miRNAs could be used as potential predictors of disease generalization in MG (61). For this purpose, 83 OMG patients (82 immunosuppressive-naive) were assayed within 3 months of OMG diagnosis and at a follow-up visit. In this study, only 13 patients developed GMG. Two miRNAs were found to be significantly higher in the groups of patients who developed GMG compared to OMG: miR-30e-5p and miR-150-5p. Of these two miRNAs, miR-30e-5p had 96% sensitivity for differentiating OMG and GMG in all patients and 100% in LOMG patients (61; **Figure 1**). Considering that treatment with corticosteroids could modify the progression of OMG to GMG (62), and that half of the OMG patients generalize within 1 year (63), predictive biomarkers would be useful to individually tailor the immunosuppressive treatment in OMG. This could, for example, imply initiating immunosuppressive treatment at an earlier stage if the miR-30e-5p levels are higher. Hence, the study by Sabre and co-workers indicated miR-30e-5p as a potential predictor of generalization in patients with OMG symptoms (61). Another study in this field reported the downregulation of miR-20b in generalized and AChR+ OMG patients, and miR-20b expression in generalized MG was much lower than that found in OMG (64). Furthermore, the miR-20b levels increased after treatment with corticosteroids in this particular study (64).

## Muscle-Specific Tyrosine Kinase Antibody-Seropositive MG

MuSK+ MG is considered a more homogenous disease subtype that differs from AChR+ MG in pathogenesis, clinical picture, neurophysiological manifestations, and response to treatment (65). Therefore, it is not surprising that MuSK+ MG and AChR+ MG are associated with different circulating miRNA

profiles. The elevated miRNAs in sera from MuSK+ MG patients instead include miR-151a-3p, let-7a-5p, let-7f-5p, and miR-423-5p (66; **Figure 1**). Accumulation of the aforementioned miR-150-5p or miR-21-5p, which are dysregulated in various AChR+ MG subtypes, does not differ between MuSK+ MG patients and healthy controls.

As the majority of blood samples are stored as serum, most studies have analyzed circulating miRNAs in serum. Serum and plasma contain miRNAs; however, their concentrations cannot be automatically presumed to be interchangeable (67). Recently, the miRNA profile was also analyzed in the plasma of patients with MuSK+ MG (68). Out of 179 different miRNAs, only two were distinctly different in MuSK+ MG patients; miR-210-3p and miR-324-3p were downregulated in MuSK+ MG plasma compared to healthy controls (68). None of these miRNAs have previously been reported dysregulated in immune diseases; however, miR-210-3p has been found dysregulated in several cancers (69) and miR-324-3p has been mentioned as a potential biomarker in the diagnosis of osteoporosis (70).

## Link Between Elevated Circulating miRNAs in MG and Disease Pathophysiology

Both miR-150-5p and miR-21-5p are so-called immuno-miRs and considered important T cell regulators (71). In AChR+ EOMG, the effector organ is the thymus, which is often characterized by hyperplasia as well as ectopic germinal centers consisting of infiltrating B cells (9, 72). miR-150 regulates proliferation, apoptosis, and differentiation of natural killer (NK), T, and B cells (71, 73, 74) and is a marker of lymphocyte activation (75). In addition to being elevated in the serum of EOMG patients, higher miR-150 levels are also found in other autoimmune conditions, including multiple sclerosis (MS) (76), HIV-1 infection (77), and certain cancers (78).

A recent study indicated that miR-150 expression was much higher in the thymus of AChR+ EOMG patients compared to healthy controls, in particular in the mantle zone of germinal centers containing B cells, although not directly related to the degree of thymus hyperplasia (79). In peripheral blood mononuclear cells (PBMCs), miR-150 was also downregulated in the $CD4^+$ T cells of EOMG patients compared to healthy controls. The results from this study suggest that increased serum levels of miR-150-5p, which were also detected in this study, result from the released miR-150 from activated peripheral $CD4^+$ T cells (79). Furthermore, miR-150 treatment of PBMCs affects the main proto-oncogene MYB, and thus, miR-150 could play a role in EOMG both at the thymic level and in the periphery by modulating the expression of target genes and peripheral cell survival (79). One hypothesis is that miR-150 could be regulated by its release into the extracellular space (80). Similar to the observations in MG, other studies demonstrate reduced miR-150-5p in PBMCs from patients with Sjogren's syndrome, while levels are still increased in the serum and salivary glands (81, 82), in analogy with miR-150-5p in PBMCs *versus* the thymus in MG.

Another immuno-miR, miR-21-5p is expressed at higher levels in regulatory T cells (Tregs) (71) and associated with other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (24, 83). Similar to miR-150, miR-21 also plays an important role for T cells (84, 85), with higher miR-21 levels expressed in Tregs.

The third miRNA in AChR+ MG, miR-30e-5p, was somewhat contradictorily downregulated in EOMG (48) and upregulated in LOMG (56). miR-30e-5p has also been associated with SLE (86). Intriguingly, the low-density lipoprotein receptor-related protein 6 (LRP6), one of the critical co-receptors for Wnts (a family of genes that encode secretory glycoproteins), is a direct target of miR-30e (87), and thus there is a potential role for miR-30e in regulating muscle homeostasis.

The let-7 miRNA family members have been extensively studied because of their broad functional role in various cellular processes, including neuronal development and embryogenesis (88, 89). Similar to MuSK+ MG patients, the accumulation of circulating serum let-7a is observed in patients with secondary progressive MS (76). Intriguingly, let-7 miRNAs stimulate the Toll-like receptor 7 (TLR7) and thereby activate T cells (90). In addition, engagement of TLR7 in $CD4^+$ T cells induces T cell unresponsiveness (91). Interestingly, at least two of the identified miRNAs, let-7a-5p and let-7f-5p, are also upregulated in PBMCs isolated from TAMG patients (92), whereas let-7f-5p is instead downregulated in the thymus of AChR+ EOMG patients (93).

As of yet, studies on circulating miRNAs in Lrp4 and agrin antibody-seropositive MG are lacking. In these MG subtypes, the antibodies as such have been suggested as biomarkers for disease (94). Further, considering the important role of agrin/Lrp4/MuSK signaling in the maintenance of the neuromuscular junction, deeper understanding of these serological subtypes is needed (94).

# INTRACELLULAR MIRNAS IN MG

Studies of intracellular miRNAs in MG patients have focused mainly on PBMCs and the thymus. The first study of PBMCs revealed that 44 miRNAs are dysregulated in MG patients' PBMCs and that let-7c expression is specifically downregulated (95). Further, miR-320a, a miRNA that modulates the expression of inflammatory cytokines, was shown to be downregulated in PBMCs from an undefined cohort of MG patients (96). Another group reported the upregulation of miR-146a in the PBMCs of AChR+ EOMG patients (97), suggesting that miR-146a might have an effect on the activation of AChR-specific B cells through the regulation of TLR4 and NF-κB. Furthermore, miR-15a expression is reduced in the PBMCs of MG patients and levels are much lower in patients with OMG compared to GMG (98). Yet another miRNA, miR-181a, is downregulated in the PBMCs of MG patients, with a negative correlation between the miR-181a level and QMG score as well as AChR antibody levels (99). Reduced miR-181c expression in the PBMCs of AChR+ MG patients also seems to correlate with elevated serum levels of the interleukins IL-7 and IL-17 (100). A recent study observed that AChR+ MG patients non-responsive to immunosuppressive treatment had lower levels of miR-323b-3p, miR-409-3p, and

miR-485-3p and higher levels of miR-181d-5p and miR-340-3p in PBMCs compared to those MG patients responding to immunosuppression (101).

The first study that analyzed miRNAs in thymus cells studied TAMG and found that miR-125a-5p, which has an important role in cancer and immune processes, was significantly upregulated (92). In female EOMG patients, thymic miRNA expression analysis revealed that the most downregulated miRNAs were miR-20b-3p, miR-892-3p, and miR-7-5p (93). The most upregulated miRNAs were miR-486-5p and miR-125-5p, whereas miR-7-5p was more downregulated in the thymuses of MG patients who had high-degree thymic hyperplasia. In the MGTX Study (50), thymuses from non-thymomatous MG patients were used for miRNA and mRNA expression analysis (102). When comparing germinal center (GC)-positive samples to GC-negative ones, 38 miRNAs involved in immune response showed differences in expression (102). Regulator of G-protein signaling 13 (RGS13) is expressed in GC B cells and thymic epithelial cells. Therefore, predicted regulators of RGS13, miR-139-5p and miR-452-5p, were further analyzed and found to be downregulated (102).

## CONCLUDING REMARKS

Myasthenia gravis is a heterogeneous autoimmune disorder with several subgroups, greatly in need of easily accessible biomarkers that can aid in monitoring the disease course. The studies discussed in this review focus mainly on some of these specific subgroups of MG, including subgroups defined by antibodies (AChR+ *versus* MuSK+ MG), clinical features (GMG *versus* OMG), and age of onset (EOMG *versus* LOMG). Since there are obvious differences in the miRNA profiles between these different MG entities, further development of subgroup-specific circulating miRNA detection tests would allow for personalized medical treatment for MG patients. In the case of EOMG *versus* LOMG, they share certain upregulated miRNAs (miR-150-5p and miR-21-5p), whereas the levels of miR-30e-5p are lower in EOMG and higher in LOMG. Due to the large variations in treatment response and also in disease course over time, a crucial future need is to personalize treatment by identifying biomarkers that will predict treatment response. In this sense, circulating miRNAs could serve as indicators of disease progression for individual patients. It will be of great importance in future studies to also examine changes in miRNAs over shorter time periods to study the intra-individual and inter-individual fluctuations more closely. Differences in the response to various therapeutic agents are also subject to further studies before this will be entirely unraveled. Furthermore, all studies referred to regarding extracellular miRNAs were conducted in European populations, except for the international study on thymectomy (MGTX), and there may be important changes in the miRNA levels related to ethnicity as well as sex, which remain to be explored.

## AUTHOR CONTRIBUTIONS

All authors contributed to manuscript drafting and revision and read and approved the submitted version.

## FUNDING

The study was supported by the Swedish Research Council (grant no. 2016-02184 to AP and TP, grant nos. 2014-02048 and 2014-07603 to AP) and Göran Gustafsson Foundation for Medical Research (to AP).

## ACKNOWLEDGMENTS

We are grateful to Dr. Laura O'Connor for proofreading and to all our international collaborators, in particular Drs. Le Panse and Berrih-Aknin, Dr. Kaminski and the MGTX Study group, Dr. Evoli, Drs. Maddison and Wong. We would also like to thank all patients who contributed with their blood samples for miRNA analysis.

## REFERENCES

1. Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurol*. (2015) 14:1023–36. doi: 10.1016/S1474-4422(15)00145-3
2. Sabre L, Westerberg E, Liik M, Punga AR. Diversity in mental fatigue and social profile of patients with myasthenia gravis in two different Northern European countries. *Brain Behav*. (2017) 7:e00653. doi: 10.1002/brb3.653
3. Pevzner A, Schoser B, Peters K, Cosma NC, Karakatsani A, Schalke B, et al. Anti-LRP4 autoantibodies in AChR- and MuSK-antibody-negative myasthenia gravis. *J Neuro*. (2012) 259:427–35. doi: 10.1007/s00415-011-6194-7
4. Zhang B, Tzartos JS, Belimezi M, Ragheb S, Bealmear B, Lewis RA, et al. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. *Arch Neurol*. (2012) 69:445–51. doi: 10.1001/archneurol.2011.2393
5. Zisimopoulou P, Evangelakou P, Tzartos J, Lazaridis K, Zouvelou V, Mantegazza R. A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis. *J Autoimmun*. (2014) 52:139–45. doi: 10.1016/j.jaut.2013.12.004
6. Gasperi C, Melms A, Schoser B, Zhang Y, Meltoranta J, Risson V, et al. Anti-agrin autoantibodies in myasthenia gravis. *Neurology*. (2014) 82:1976–83. doi: 10.1212/WNL.0000000000000478
7. Zhang B, Shen C, Bealmear B, Ragheb S, Xiong WC, Lewis RA, et al. Autoantibodies to agrin in myasthenia gravis patients. *PLoS One*. (2014) 9:e91816. doi: 10.1371/journal.pone.0091816
8. Berrih-Aknin S, Panse R. Le. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J Autoimmun*. (2014) 52:90–100. doi: 10.1016/j.jaut.2013.12.011
9. Cron MA, Maillard S, Villegas J, Truffault F, Sudres M, Dragin N, et al. Thymus involvement in early-onset myasthenia gravis. *Ann N Y Acad Sci*. (2018) 1412:137–45. doi: 10.1111/nyas.13519
10. Kerty E, Elsais A, Argov Z, Evoli A, Gilhus NE. EFNS/ENS guidelines for the treatment of ocular myasthenia. *Eur J Neurol*. (2014) 21:687–93. doi: 10.1111/ene.12359

11. Meriggioli MN, Sanders DB. Muscle autoantibodies in myasthenia gravis: beyond diagnosis? *Exp Rev Clin Immunol*. (2012) 8:427–38. doi: 10.1586/eci.12.34
12. Benatar M, Sanders DB, Burns TM, Cutter GR, Guptill JT, Baggi F, et al. Recommendations for myasthenia gravis clinical trials. *Muscle Nerve*. (2012) 45:909–17. doi: 10.1002/mus.23330
13. Kaminski HJ, Kusner LL, Wolfe GI, Aban I, Minisman G, Conwit R, et al. Biomarker development for myasthenia gravis. *Ann N Y Acad Sci*. (2012) 1275:101–6. doi: 10.1111/j.1749-6632.2012.06787.x
14. FDA-NIH Biomarker Working Group. *BEST (Biomarkers, EndpointS, and other Tools) Resource*. Bethesda, MD: Food and Drug Administration (2016).
15. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. (1993) 75:843–54. doi: 10.1016/0092-8674(93)90529-y
16. Punga T, Kamel W, Akusjärvi G. Old and new functions for the adenovirus virus-associated RNAs. *Future Virol*. (2013) 8:343–56. doi: 10.2217/fvl.13.19
17. Motameny S, Wolters S, Nurnberg P, Schumacher B. Next generation sequencing of miRNAs - strategies, resources and methods. *Genes (Basel)*. (2010) 1:70–84. doi: 10.3390/genes1010070
18. Alles J, Fehlmann T, Fischer U, Backes C, Galata V, Minet M, et al. An estimate of the total number of true human miRNAs. *Nucleic Acids Res*. (2019) 47:3353–64. doi: 10.1093/nar/gkz097
19. Michlewski G, Caceres JF. Post-transcriptional control of miRNA biogenesis. *RNA*. (2019) 25:1–16. doi: 10.1261/rna.068692.118
20. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. *Cell*. (2003) 115:209–16. doi: 10.1016/s0092-8674(03)00801-8
21. Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. *PLoS Biol*. (2005) 3:e85. doi: 10.1371/journal.pbio.0030085
22. Garofalo M, Condorelli GL, Croce CM, Condorelli G. MicroRNAs as regulators of death receptors signaling. *Cell Death Differ*. (2010) 17:200–8. doi: 10.1038/cdd.2009.105
23. Li N, Long B, Han W, Yuan S, Wang K. microRNAs: important regulators of stem cells. *Stem Cell Res Ther*. (2017) 8:110. doi: 10.1186/s13287-017-0551-0
24. Chen JQ, Papp G, Szodoray P, Zeher M. The role of microRNAs in the pathogenesis of autoimmune diseases. *Autoimmun Rev*. (2016) 15:1171–80. doi: 10.1016/j.autrev.2016.09.003
25. Hata A, Lieberman J. Dysregulation of microRNA biogenesis and gene silencing in cancer. *Sci Signal*. (2015) 8:re3. doi: 10.1126/scisignal.2005825
26. Maegdefessel L. The emerging role of microRNAs in cardiovascular disease. *J Intern Med*. (2014) 276:633–44. doi: 10.1111/joim.12298
27. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. (2008) 18:997–1006. doi: 10.1038/cr.2008.282
28. Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, et al. Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem*. (2008) 54:482–90. doi: 10.1373/clinchem.2007.097972
29. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol*. (2008) 141:672–5. doi: 10.1111/j.1365-2141.2008.07077.x
30. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. (2008) 105:10513–8. doi: 10.1073/pnas.0804549105
31. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. (2007) 9:654–9. doi: 10.1038/ncb1596
32. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. (2010) 56:1733–41. doi: 10.1373/clinchem.2010.147405
33. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res*. (2011) 39:7223–33. doi: 10.1093/nar/gkr254
34. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*. (2008) 10:1470–6. doi: 10.1038/ncb1800
35. Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles*. (2014) 3:1–14. doi: 10.3402/jev.v3.24641
36. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. (2015) 527:329–35. doi: 10.1038/nature15756
37. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun*. (2011) 2:282. doi: 10.1038/ncomms1285
38. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, et al. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat Commun*. (2013) 4:2980. doi: 10.1038/ncomms3980
39. Mori MA, Ludwig RG, Garcia-Martin R, Brandao BB, Kahn CR. Extracellular miRNAs: from biomarkers to mediators of physiology and disease. *Cell Metab*. (2019) 30:656–73. doi: 10.1016/j.cmet.2019.07.011
40. Bar C, Thum T, de Gonzalo-Calvo D. Circulating miRNAs as mediators in cell-to-cell communication. *Epigenomics*. (2019) 11:111–3. doi: 10.2217/epi-2018-0183
41. Ortega FJ, Mercader JM, Catalan V, Moreno-Navarrete JM, Pueyo N, Sabater M, et al. Targeting the circulating microRNA signature of obesity. *Clin Chem*. (2013) 59:781–92. doi: 10.1373/clinchem.2012.195776
42. Wang R, Hong J, Cao Y, Shi J, Gu W, Ning G, et al. Elevated circulating microRNA-122 is associated with obesity and insulin resistance in young adults. *Eur J Endocrinol*. (2015) 172:291–300. doi: 10.1530/EJE-14-0867
43. Domanska-Senderowska D, Laguette MN, Jegier A, Cieszczyk P, September AV, Brzezianska-Lasota E. MicroRNA profile and adaptive response to exercise training: a review. *Int J Sports Med*. (2019) 40:227–35. doi: 10.1055/a-0824-4813
44. Rome S. Use of miRNAs in biofluids as biomarkers in dietary and lifestyle intervention studies. *Genes Nutr*. (2015) 10:33. doi: 10.1007/s12263-015-0483-1
45. Tiberio P, Callari M, Angeloni V, Daidone MG, Appierto V. Challenges in using circulating miRNAs as cancer biomarkers. *Biomed Res Int*. (2015) 2015:731479. doi: 10.1155/2015/731479
46. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. *J Cell Mol Med*. (2014) 18:371–90. doi: 10.1111/jcmm.12236
47. Witwer KW. Circulating microRNA biomarker studies: pitfalls and potential solutions. *Clin Chem*. (2015) 61:56–63. doi: 10.1373/clinchem.2014.221341
48. Punga T, Le Panse R, Andersson M, Truffault F, Berrih-Aknin S, Punga AR. Circulating miRNAs in myasthenia gravis: miR-150-5p as a new potential biomarker. *Ann Clin Transl Neurol*. (2014) 1:49–58. doi: 10.1002/acn3.24
49. Punga AR, Andersson M, Alimohammadi M, Punga T. Disease specific signature of circulating miR-150-5p and miR-21-5p in myasthenia gravis patients. *J Neurol Sci*. (2015) 356:90–6. doi: 10.1016/j.jns.2015.06.019
50. Wolfe GI, Kaminski HJ I, Aban B, Minisman G, Kuo HC, Marx A, et al. Randomized trial of thymectomy in myasthenia gravis. *N Engl J Med*. (2016) 375:511–22. doi: 10.1056/NEJMoa1602489
51. Molin CJ, Sabre L, Weis CA, Punga T, Punga AR. Thymectomy lowers the myasthenia gravis biomarker miR-150-5p. *Neurol Neuroimmunol Neuroinflamm*. (2018) 5:e450. doi: 10.1212/NXI.0000000000000450
52. Palagani A, Op de Beeck K, Naulaerts S, Diddens J, Sekhar Chirumamilla C, Van Camp G, et al. Ectopic microRNA-150-5p transcription sensitizes glucocorticoid therapy response in MM1S multiple myeloma cells but fails to overcome hormone therapy resistance in MM1R cells. *PLoS One*. (2014) 9:e113842. doi: 10.1371/journal.pone.0113842
53. Westerberg E, Molin CJ, Lindblad I, Emtner M, Punga AR. Physical exercise in myasthenia gravis is safe and improves neuromuscular parameters and physical performance-based measures: a pilot study. *Muscle Nerve*. (2017) 56:207–14. doi: 10.1002/mus.25493
54. Nogales-Gadea G, Ramos-Fransi A, Suarez-Calvet X, Navas M, Rojas-Garcia R, Mosquera JL, et al. Analysis of serum miRNA profiles of myasthenia gravis patients. *PLoS One*. (2014) 9:e91927. doi: 10.1371/journal.pone.0091927
55. Weis CA, Schalke B, Strobel P, Marx A. Challenging the current model of early-onset myasthenia gravis pathogenesis in the light of the MGTX trial and histological heterogeneity of thymectomy specimens. *Ann N Y Acad Sci*. (2018) 1413:82–91. doi: 10.1111/nyas.13563

56. Sabre L, Maddison P, Sadalage G, Ambrose PA, Punga AR. Circulating microRNA miR-21-5p, miR-150-5p and miR-30e-5p correlate with clinical status in late onset myasthenia gravis. *J Neuroimmunol*. (2018) 321:164–70. doi: 10.1016/j.jneuroim.2018.05.003
57. Punga AR, Punga T. Circulating microRNAs as potential biomarkers in myasthenia gravis patients. *Ann N Y Acad Sci*. (2018) 1412:33–40. doi: 10.1111/nyas.13510
58. Grob D, Brunner N, Namba T, Pagala M. Lifetime course of myasthenia gravis. *Muscle Nerve*. (2008) 37:141–9. doi: 10.1002/mus.20950
59. Kusner LL, Puwanant A, Kaminski HJ. Ocular myasthenia: diagnosis, treatment, and pathogenesis. *Neurologist*. (2006) 12:231–9. doi: 10.1097/01.nrl.0000240856.03505.b5
60. Wong SH, Petrie A, Plant GT. Ocular myasthenia gravis: toward a risk of generalization score and sample size calculation for a randomized controlled trial of disease modification. *J Neuroophthalmol*. (2016) 36:252–8. doi: 10.1097/WNO.0000000000000350
61. Sabre L, Maddison P, Wong SH, Sadalage G, Ambrose PA, Plant GT, et al. miR−30e−5p as predictor of generalization in ocular myasthenia gravis. *Ann Clin Transl Neurol*. (2019) 6:243–51. doi: 10.1002/acn3.692
62. Kupersmith MJ. Does early immunotherapy reduce the conversion of ocular myasthenia gravis to generalized myasthenia gravis? *J Neuroophthalmol*. (2003) 23:249–50. doi: 10.1097/00041327-200312000-00001
63. Kupersmith MJ. Ocular myasthenia gravis: treatment successes and failures in patients with long-term follow-up. *J Neurol*. (2009) 256:1314–20. doi: 10.1007/s00415-009-5120-8
64. Chunjie N, Huijuan N, Zhao Y, Jianzhao W, Xiaojian Z. Disease-specific signature of serum miR-20b and its targets IL-8 and IL-25, in myasthenia gravis patients. *Eur Cytokine Netw*. (2015) 26:61–6. doi: 10.1684/ecn.2015.0367
65. Evoli A, Alboini PE, Damato V, Iorio R, Provenzano C, Bartoccioni E, et al. Myasthenia gravis with antibodies to MuSK: an update. *Ann N Y Acad Sci*. (2018) 1412:82–9. doi: 10.1111/nyas.13518
66. Punga T, Bartoccioni E, Lewandowska M, Damato V, Evoli A, Punga AR. Disease specific enrichment of circulating let-7 family microRNA in MuSK+ myasthenia gravis. *J Neuroimmunol*. (2016) 292:21–6. doi: 10.1016/j.jneuroim.2016.01.003
67. Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. *PLoS One*. (2012) 7:e41561. doi: 10.1371/journal.pone.0041561
68. Sabre L, Guptill JT, Russo M, Juel VC, Massey JM, Howard JF Jr., et al. Circulating microRNA plasma profile in MuSK+ myasthenia gravis. *J Neuroimmunol*. (2018) 325:87–91. doi: 10.1016/j.jneuroim.2018.10.003
69. Bavelloni A, Ramazzotti G, Poli A, Piazzi M, Focaccia E, Blalock W, et al. MiRNA-210: a current overview. *Anticancer Res*. (2017) 37:6511–21. doi: 10.21873/anticanres.12107
70. Feichtinger X, Muschitz C, Heimel P, Baierl A, Fahrleitner-Pammer A, Redl H, et al. Bone-related circulating MicroRNAs miR-29b-3p, miR-550a-3p, and miR-324-3p and their association to bone microstructure and histomorphometry. *Sci Rep*. (2018) 8:4867. doi: 10.1038/s41598-018-22844-2
71. Kroesen BJ, Teteloshvili N, Smigielska-Czepiel K, Brouwer E, Boots AM, van den Berg A, et al. Immuno-miRs: critical regulators of T-cell development, function and ageing. *Immunology*. (2015) 144:1–10. doi: 10.1111/imm.12367
72. Corsiero E, Nerviani A, Bombardieri M, Pitzalis C. Ectopic lymphoid structures: powerhouse of autoimmunity. *Front Immunol*. (2016) 7:430. doi: 10.3389/fimmu.2016.00430
73. Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci USA*. (2007) 104:7080–5. doi: 10.1073/pnas.0702409104
74. Zhou L, Park JJ, Zheng Q, Dong Z, Mi Q. MicroRNAs are key regulators controlling iNKT and regulatory T-cell development and function. *Cell Mol Immunol*. (2011) 8:380–7. doi: 10.1038/cmi.2011.27
75. de Candia P, Torri A, Gorletta T, Fedeli M, Bulgheroni E, Cheroni C, et al. Intracellular modulation, extracellular disposal and serum increase of MiR-150 mark lymphocyte activation. *PLoS One*. (2013) 8:e75348. doi: 10.1371/journal.pone.0075348
76. Gandhi R, Healy B, Gholipour T, Egorova S, Musallam A, Hussain MS, et al. Circulating microRNAs as biomarkers for disease staging in multiple sclerosis. *Ann Neurol*. (2013) 73:729–40. doi: 10.1002/ana.23880
77. Munshi SU, Panda H, Holla P, Rewari BB, Jameel S. MicroRNA-150 is a potential biomarker of HIV/AIDS disease progression and therapy. *PLoS One*. (2014) 9:e95920. doi: 10.1371/journal.pone.0095920
78. Ma Y, Zhang P, Wang F, Zhang H, Yang J, Peng J, et al. miR-150 as a potential biomarker associated with prognosis and therapeutic outcome in colorectal cancer. *Gut*. (2012) 61:1447–53. doi: 10.1136/gutjnl-2011-301122
79. Cron MA, Maillard S, Truffault F, Gualeni AV, Gloghini A, Fadel E, et al. Causes and consequences of miR-150-5p dysregulation in myasthenia gravis. *Front Immunol*. (2019) 10:539. doi: 10.3389/fimmu.2019.00539
80. Stamatopoulos B, Van Damme M, Crompot E, Dessars B, Housni HE, Mineur P, et al. Opposite prognostic significance of cellular and serum circulating microRNA-150 in patients with chronic lymphocytic leukemia. *Mol Med*. (2015) 21:123–33. doi: 10.2119/molmed.2014.00214
81. Alevizos I, Alexander S, Turner RJ, Illei GG. MicroRNA expression profiles as biomarkers of minor salivary gland inflammation and dysfunction in Sjogren's syndrome. *Arthritis Rheum*. (2011) 63:535–44. doi: 10.1002/art.30131
82. Lopes AP, Hillen MR, Chouri E, Blokland SLM, Bekker CPJ, Kruize AA, et al. Circulating small non-coding RNAs reflect IFN status and B cell hyperactivity in patients with primary Sjogren's syndrome. *PLoS One*. (2018) 13:e0193157. doi: 10.1371/journal.pone.0193157
83. Hu R, O'Connell RM. MicroRNA control in the development of systemic autoimmunity. *Arthritis Res Ther*. (2013) 15:202. doi: 10.1186/ar4131
84. Smigielska-Czepiel K, van den Berg A, Jellema P, Slezak-Prochazka I, Maat H, van den Bos H, et al. Dual role of miR-21 in CD4+ T-cells: activation-induced miR-21 supports survival of memory T-cells and regulates CCR7 expression in naive T-cells. *PLoS One*. (2013) 8:e76217. doi: 10.1371/journal.pone.0076217
85. Stagakis E, Bertsias G, Verginis P, Nakou M, Hatziapostolou M, Kritikos H, et al. Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression. *Ann Rheum Dis*. (2011) 70:1496–506. doi: 10.1136/ard.2010.139857
86. Kim BS, Jung JY, Jeon JY, Kim HA, Suh CH. Circulating hsa-miR-30e-5p, hsa-miR-92a-3p, and hsa-miR-223-3p may be novel biomarkers in systemic lupus erythematosus. *HLA*. (2016) 88:187–93. doi: 10.1111/tan.12874
87. Wang J, Guan X, Guo F, Zhou J, Chang A, Sun B, et al. miR-30e reciprocally regulates the differentiation of adipocytes and osteoblasts by directly targeting low-density lipoprotein receptor-related protein 6. *Cell Death Dis*. (2013) 4:e845. doi: 10.1038/cddis.2013.356
88. Gurtan AM, Ravi A, Rahl PB, Bosson AD, JnBaptiste CK, Bhutkar A, et al. Let-7 represses Nr6a1 and a mid-gestation developmental program in adult fibroblasts. *Genes Dev*. (2013) 27:941–54. doi: 10.1101/gad.215376.113
89. Patterson M, Gaeta X, Loo K, Edwards M, Smale S, Cinkornpumin J, et al. let-7 miRNAs can act through notch to regulate human gliogenesis. *Stem Cell Reports*. (2014) 3:758–73. doi: 10.1016/j.stemcr.2014.08.015
90. Wang S, Tang Y, Cui H, Zhao X, Luo X, Pan W, et al. Let-7/miR-98 regulate Fas and Fas-mediated apoptosis. *Genes Immun*. (2011) 12:149–54. doi: 10.1038/gene.2010.53
91. Dominguez-Villar M, Gautron AS, de Marcken M, Keller MJ, Hafler DA. TLR7 induces anergy in human CD4(+) T cells. *Nat Immunol*. (2015) 16:118–28. doi: 10.1038/ni.3036
92. Li J, Qiu D, Chen Z, Du W, Liu J, Mo X. Altered expression of miR-125a-5p in thymoma-associated myasthenia gravis and its down-regulation of foxp3 expression in Jurkat cells. *Immunol Lett*. (2016) 172:47–55. doi: 10.1016/j.imlet.2016.02.005
93. Cron MA, Maillard S, Delisle F, Samson N, Truffault F, Foti M, et al. Analysis of microRNA expression in the thymus of Myasthenia Gravis patients opens new research avenues. *Autoimmun Rev*. (2018) 17:588–600. doi: 10.1016/j.autrev.2018.01.008
94. Yan M, Xing GL, Xiong WC, Mei L. Agrin and LRP4 antibodies as new biomarkers of myasthenia gravis. *Ann N Y Acad Sci*. (2018) 1413:126–35. doi: 10.1111/nyas.13573

95. Jiang L, Cheng Z, Qiu S, Que Z, Bao W, Jiang C, et al. Altered let-7 expression in Myasthenia gravis and let-7c mediated regulation of IL-10 by directly targeting IL-10 in Jurkat cells. *Int Immunopharmacol*. (2012) 14:217–23. doi: 10.1016/j.intimp.2012.07.003
96. Cheng Z, Qiu S, Jiang L, Zhang A, Bao W, Liu P, et al. MiR-320a is downregulated in patients with myasthenia gravis and modulates inflammatory cytokines production by targeting mitogen-activated protein kinase 1. *J Clin Immunol*. (2013) 33:567–76. doi: 10.1007/s10875-012-9834-5
97. Lu J, Yan M, Wang Y, Zhang J, Yang H, Tian FF, et al. Altered expression of miR-146a in myasthenia gravis. *Neurosci Lett*. (2013) 555:85–90. doi: 10.1016/j.neulet.2013.09.014
98. Liu XF, Wang RQ, Hu B, Luo MC, Zeng QM, Zhou H, et al. MiR-15a contributes abnormal immune response in myasthenia gravis by targeting CXCL10. *Clin Immunol*. (2016) 164:106–13. doi: 10.1016/j.clim.2015.12.009
99. Liu X, Luo M, Meng H, Zeng Q, Xu L, Hu B, et al. MiR-181a regulates CD4(+) T cell activation and differentiation by targeting IL-2 in the pathogenesis of myasthenia gravis. *Eur J Immunol*. (2019). doi: 10.1002/eji.201848007 [Online ahead of print].
100. Zhang Y, Guo M, Xin N, Shao Z, Zhang X, Zhang Y, et al. Decreased microRNA miR-181c expression in peripheral blood mononuclear cells correlates with elevated serum levels of IL-7 and IL-17 in patients with myasthenia gravis. *Clin Exp Med*. (2016) 16:413–21. doi: 10.1007/s10238-015-0358-1
101. Cavalcante P, Mizrachi T, Barzago C, Scandiffio L, Bortone F, Bonanno S, et al. MicroRNA signature associated with treatment response in myasthenia gravis: a further step towards precision medicine. *Pharmacol Res*. (2019) 148:104388. doi: 10.1016/j.phrs.2019.104388
102. Sengupta M, Wang BD, Lee NH, Marx A, Kusner LL, Kaminski HJ. MicroRNA and mRNA expression associated with ectopic germinal centers in thymus of myasthenia gravis. *PLoS One*. (2018) 13:e0205464. doi: 10.1371/journal.pone.0205464

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 March 2020
doi: 10.3389/fimmu.2020.00142

# miR-146a in Myasthenia Gravis Thymus Bridges Innate Immunity With Autoimmunity and Is Linked to Therapeutic Effects of Corticosteroids

*Federica Bortone[1], Letizia Scandiffio[1], Stefania Marcuzzo[1], Silvia Bonanno[1], Rita Frangiamore[1], Teresio Motta[2], Carlo Antozzi[1], Renato Mantegazza[1], Paola Cavalcante[1†] and Pia Bernasconi[1*†]*

[1] Neurology IV—Neuroimmunology and Neuromuscular Diseases Unit, Fondazione I.R.C.C.S. Istituto Neurologico Carlo Besta, Milan, Italy, [2] Department of Pathological Anatomy, ASST—Bergamo Est Ospedale Bolognini Seriate, Bergamo, Italy

OPEN ACCESS

**Edited by:**
Sonia Berrih-Aknin,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

**Reviewed by:**
Mélanie A. Cron,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Helen Thomas,
St Vincents Institute of Medical Research, Australia

***Correspondence:**
Pia Bernasconi
pia.bernasconi@istituto-besta.it

[†] These authors share senior authorship

**Specialty section:**
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

**Received:** 06 November 2019
**Accepted:** 20 January 2020
**Published:** 10 March 2020

**Citation:**
Bortone F, Scandiffio L, Marcuzzo S, Bonanno S, Frangiamore R, Motta T, Antozzi C, Mantegazza R, Cavalcante P and Bernasconi P (2020) miR-146a in Myasthenia Gravis Thymus Bridges Innate Immunity With Autoimmunity and Is Linked to Therapeutic Effects of Corticosteroids. Front. Immunol. 11:142. doi: 10.3389/fimmu.2020.00142

Toll-like receptor (TLR)-mediated innate immune responses are critically involved in the pathogenesis of myasthenia gravis (MG), an autoimmune disorder affecting neuromuscular junction mainly mediated by antiacetylcholine receptor antibodies. Considerable evidence indicate that uncontrolled TLR activation and chronic inflammation significantly contribute to hyperplastic changes and germinal center (GC) formation in the MG thymus, ultimately leading to autoantibody production and autoimmunity. miR-146a is a key modulator of innate immunity, whose dysregulation has been associated with autoimmune diseases. It acts as inhibitor of TLR pathways, mainly by targeting the nuclear factor kappa B (NF-κB) signaling transducers, interleukin 1 receptor associated kinase 1 (IRAK1) and tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6); miR-146a is also able to target c-REL, inducible T-cell costimulator (ICOS), and Fas cell surface death receptor (FAS), known to regulate B-cell function and GC response. Herein, we investigated the miR-146a contribution to the intrathymic MG pathogenesis. By real-time PCR, we found that miR-146a expression was significantly downregulated in hyperplastic MG compared to control thymuses; contrariwise, IRAK1, TRAF6, c-REL, and ICOS messenger RNA (mRNA) levels were upregulated and negatively correlated with miR-146a levels. Microdissection experiments revealed that miR-146a deficiency in hyperplastic MG thymuses was not due to GCs, but restricted to the GC-surrounding medulla, characterized by IRAK1 overexpression. We also showed higher c-REL and ICOS mRNA levels, and lower FAS mRNA levels, in GCs than in the remaining medulla, according to the contribution of these molecules in GC formation. By double immunofluorescence, an increased proportion of IRAK1-expressing dendritic cells and macrophages was found in hyperplastic MG compared to control thymuses, along with GC immunoreactivity for c-REL. Interestingly, in corticosteroid-treated MG patients intrathymic miR-146a and mRNA target levels were comparable to those of controls, suggesting that immunosuppressive therapy may restore the microRNA (miRNA) levels. Indeed, an effect of prednisone on miR-146a expression was demonstrated *in vitro* on peripheral

blood cells. Serum miR-146a levels were lower in MG patients compared to controls, indicating dysregulation of the circulating miRNA. Our overall findings strongly suggest that defective miR-146a expression could contribute to persistent TLR activation, lack of inflammation resolution, and hyperplastic changes in MG thymuses, thus linking TLR-mediated innate immunity to B-cell-mediated autoimmunity. Furthermore, they unraveled a new mechanism of action of corticosteroids in inducing control of autoimmunity in MG via miR-146a.

**Keywords: myasthenia gravis, thymus, autoimmunity, innate immunity, miR-146a**

## INTRODUCTION

Myasthenia gravis (MG) is a prototypical B-cell-mediated autoimmune disorder affecting neuromuscular junction, mainly caused by autoantibodies against the postsynaptic acetylcholine receptor (AChR), which lead to invalidating weakness and fatigability of skeletal muscles (1). The bulk of MG therapy consists of symptomatic treatment by acetylcholinesterase inhibitors, non-specific immunosuppression with corticosteroids, and thymectomy as a natural course disease-modifying intervention in selected patients (1–3). However, complete stable remission is only rarely achieved, and ∼10% of patients are treatment refractory (2), highlighting the need to better understand the specific disease-associated pathogenic events, to develop more effective therapeutic strategies.

The involvement of thymus in AChR-MG pathogenesis is now widely accepted. This organ is the prime site of autosensitization and autoimmunity to AChR (4, 5), and thymectomy has beneficial effect in a high proportion of patients (6, 7). Approximately 80% of all AChR-MG patients presents thymic morphological and functional changes, including hyperplasia and thymoma (5). Hyperplasia is the most common alteration in early-onset MG; it is characterized by the expansion of perivascular spaces fused with the thymic medulla, which abnormally contains abundant B-lymphocyte infiltrates organized in ectopic germinal centers (GCs) forming follicles (8). In terms of immunoglobulin gene diversification, mutation, and selection, GCs in MG thymus do not differ from those observed in the lymphoid follicles of peripheral lymphatic organs, but they are uniquely surrounded by plasma cells and muscle-like myoid cells expressing AChR and other muscle proteins, which can set up an antigen-driven reaction (8). Chronic inflammation and persistent activation of Toll-like receptor (TLR)-mediated innate immune pathways have been critically implicated in the intrathymic MG pathogenesis, supporting the existence of a dangerous cross-talk between innate immunity and autoimmunity (9–13). Indeed, a hallmark of hyperplastic MG thymus is the significant overexpression of pro-inflammatory cytokines and chemokines [e.g. interleukin-6 (IL-6), type I interferons (IFNs), CXCL13, CCL21], along with upregulation of TLRs, particularly TLR3, TLR4, TLR7, and TLR9 (8, 10–13). The contribution of TLRs in autoimmunity can be explained by the ability of these receptors to stimulate maturation of antigen-presenting cells and production of type I IFNs and other inflammatory cytokines, which in turn cause priming of adaptive immune cells, such as autoreactive T cells (8). In addition, TLR7 and 9 stimuli may function as costimulatory signals for proliferation, maturation, and survival of B cells, thus compromising B-cell tolerance and promoting autoimmune response perpetuation (11, 12). Recently, Robinet and colleagues demonstrated that the combined use of TLR agonists induces thymic hyperplastic changes and triggers MG symptoms in mice, suggesting that tertiary lymphoid genesis, and consequently autoreactivity, in MG thymus could result from dysregulated TLR signaling (14). What exactly causes uncontrolled TLR activation, and loss of the fine regulation of TLR pathways, in hyperplastic MG thymus is not totally understood.

MicroRNAs (miRNAs) are ∼22 nucleotide long small non-coding RNA molecules recognized to play a critical role in fine-tune regulation of gene expression (15). They modulate many biological processes, including cell-cycle progression, apoptosis, inflammation, and both innate and adaptive immune response (15). Thus, their involvement in several pathophysiological conditions, including cancer and autoimmunity (16–19), is not surprising. One of the most important miRNAs known to orchestrate immune and inflammatory signaling, and to play a central role in innate immunity, is miR-146a-5p (hereinafter called miR-146a) (20). Its dysregulated expression has been reported in different inflammatory and autoimmune pathologies, including systemic lupus erythematosus (SLE) (21), rheumatoid arthritis (RA) (22, 23), multiple sclerosis (MS) (24), and sepsis (25, 26). miR-146a gene is located within the MIR3142HG host gene on chromosome 5 (5q33.3), and its promoter locus presents binding sites for several transcription factors, including nuclear factor kappa B (NF-κB), IRF3/7, and c-myc (27, 28). Interestingly, in a kind of feedback mechanism, miR-146a targets two NF-κB signaling transducers, the tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) and the interleukin 1 receptor associated kinase 1 (IRAK1), which are key components of the MyD88-dependent TLR pathways (28). By targeting TRAF6 and IRAK1, miR-146a acts as potent inhibitor of TLR-mediated innate immune responses, preventing an overstimulation of the inflammatory response and ensuring immune tolerance (20, 28). In addition, miR-146a is able to regulate B-cell function and GC response. Indeed, its deficiency has been shown to promote the activation of c-REL, a NF-κB subunit implicated in B-cell proliferation, differentiation, and GC development (29, 30), which was reported to be a direct target of the miRNA in B cells (31). Moreover, as demonstrated in mice by Pratama et al. (32), miR-146a limits the

**TABLE 1 |** Summary of the main features of acetylcholine receptor myasthenia gravis (AChR-MG) patients and controls included in the study.

| | Thymus | | PBMCs and Serum | |
|---|---|---|---|---|
| | Normal (n = 10) | Hyperplastic MG (n = 27) | Healthy controls (n = 11) | MG patients (n = 31)[a] |
| Sex (F:M) | 4:6 | 23:4 | 7:4 | 21:10 |
| Age at onset (years, mean ± SD) | – | 26.5 ± 8.9 | – | 36.8 ± 15.4[b] |
| Age at thymectomy (years, mean ± SD) | 26.0 ± 16.1 | 29.2 ± 8.1 | – | – |
| Age at blood collection (years, mean ± SD) | – | – | 33.4 ± 8.7 | 41.5 ± 14.5 |
| Number of corticosteroid-treated patients | – | 15 | – | 13 |

[a] *Serum was available for 21 of the 31 MG patients at the same time of PBMC collection (age at onset 38.0 ± 13.9; age at blood collection 43.3 ± 15.3).*
[b] *Information on age at onset was not available in 6 of the 31 patients.*

accumulation of follicular T helper (Tfh) cells and GC B cells by targeting the inducible T-cell costimulator (ICOS) and its ligand (ICOSL), which are also critically involved in GC formation (33). Furthermore, enhanced miR-146a expression was associated with downregulation of Fas cell surface death receptor (FAS) in naïve B cells, unbalancing lymphocyte homeostasis and leading to hyper lymphoproliferation, and GC formation (34).

The ability of miR-146a to control TLR signaling and GC development makes it a good candidate to play a role in the intrathymic MG pathogenesis, since altered miR-146a expression could well-contribute to uncontrolled TLR activation and dysregulated B-cell function, which characterize hyperplastic MG thymuses.

Previous studies showed a significant upregulation of miR-146a in peripheral blood mononuclear cells (PBMCs) of MG patients compared to healthy controls (35), and clinical amelioration was observed in experimental autoimmune myasthenia gravis (EAMG) animals treated with antagomiR-146a (36). However, the possible contribution of miR-146a to autoimmunity development in MG thymus, and its perpetuation in peripheral blood, has never been thoroughly investigated.

In the present study, we performed a comprehensive analysis of miR-146a expression, along with that of its target genes, in hyperplastic thymuses and peripheral blood of MG patients. Our data revealed a possible role of miR-146a as key molecular link between intrathymic innate immunity and B-cell-mediated autoimmunity in MG.

# METHODS

## Patients and Biological Samples

The study included 27 follicular hyperplastic thymuses from early-onset (<50 years) AChR-positive MG patients who underwent thymectomy as a therapeutic treatment and 10 non-pathological thymuses from patients without autoimmune diseases who underwent cardiovascular surgery (**Table 1**). Of the MG patients, 15 were treated with corticosteroids, and 12 were untreated or treated only with cholinesterase inhibitors at the time of thymectomy. MG thymuses were classified as follicular hyperplastic at the Department of Pathological Anatomy, Azienda Ospedaliera Bolognini (Seriate, Bergamo) according to the presence of at least one GC for thymic section: 5–15 GCs for section were present in thymuses from corticosteroid-naïve patients; 1–3 GCs for section were observed in corticosteroid-treated patients. For each thymus, some fragments were fixed in 10% formalin for histopathological classification; other fragments were snap frozen in optimal cutting temperature (OCT) and stored at −80 °C pending molecular and immunofluorescence analyses.

Since serum and PBMCs from MG patients and controls included in the thymus analysis were not available, peripheral blood was collected from an independent group of 31 not thymectomized AChR-positive MG patients, of whom 13 were under treatment with corticosteroids, and from 11 healthy controls (**Table 1**), to collect PBMCs and serum. PBMCs were isolated by Lymphoprep (Axis-Shield, Dundee, Scotland) according to the manufacturer's instructions, frozen in fetal bovine serum (FBS) plus 10% dimethyl sulfoxide (DMSO) (Euroclone, Milan, Italy), and stored in liquid nitrogen until use.

None of the MG patients displayed any infectious diseases. The study was approved by the Ethics Committee of the Fondazione I.R.C.C.S. Istituto Neurologico Carlo Besta in Milan (Approval No. 586/2014), and each patient and control provided written informed consent for thymectomy and use of thymus specimens or blood for research purposes.

## Laser-Capture Microdissection

Seven of the 27 (**Table 1**) snap-frozen hyperplastic MG thymuses were subjected to laser-capture microdissection (LCM) of GCs using a Nikon Eclipse TE2000-S microscope (Nikon GMBH, Germany), equipped with a laser microdissector CellCut (MMI). For each thymus, six to ten 15-μm thick serial sections were mounted on membrane slides for LCM, stained by 50% hematoxylin and fixed in RNase-free 75–100% ethanol. Sections before and after the microdissected ones were stained for CD20, a B-cell marker, to identify GCs. From each MG thymic sample, at least 15 GCs (from consecutive serial sections) were microdissected and pooled in a single cap; sections devoid of the microdissected GCs were collected in separate caps. Whole

sections from 4 of the 10 control thymuses (**Table 1**) were also collected.

The isolated tissue fragments of each series were incubated in lysis buffer (RNeasy Micro Kit, Qiagen, Valencia, CA) at 37 °C for 1 h and centrifuged at 800 × *g* for 5 min; lysates were then stored at −80 °C until use.

## *In vitro* Treatment of PBMCs With Prednisone

PBMCs isolated from four MG patients corticosteroid-naïve at time of bleeding and from three healthy controls were plated in a 48-well plate ($0.5 \times 10^6$ cells/well) in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 0.1% β-mercaptoethanol, and 0.1% concanavalin A, and kept at 37 °C in a humidified 5% $CO_2$ atmosphere for 24 h. Cells were treated with a non-toxic dose of 0.1 μM prednisone (Sigma-Aldrich, Darmstadt, Germany) (37), for 3 days, collected in TRIzol after 6, 24, 48, and 72 h of treatment and stored at −80 °C for RNA extraction. Concanavalin A was added to the medium as lymphocyte mitogen, for allowing PBMCs to proliferate and avoiding spontaneous cell death without stimuli up to 72 h of prednisone treatment. This was important to test the impact of the drug without bias due to spontaneous cell death. Concanavalin A, or other mitogens, are widely used in *in vitro* studies performed on PBMCs, such as studies exploring PBMC response to prednisone or other corticosteroid drugs (37–39). Cell viability was evaluated by Countess II Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA).

## RNA Isolation

Total RNA was extracted from frozen thymic fragments and treated and untreated PBMCs using the TRIzol method (Thermo Fisher Scientific), previously reported to efficiently recover miRNAs and showing comparable results, in different tissue samples, to those obtained using specific kits for miRNA enrichment (e.g. MirVana kit) (40, 41). To note, the use of this kind of kits is considered optimal for miRNA isolation when unbiased high-throughput approaches are used downstream, since they allow detection of different miRNAs with the same efficiency, avoiding the risk to lose some molecules (41). Here, we aimed at assessing the expression of one candidate miRNA, miR-146a, along with its messenger RNA (mRNA) target genes, and obtained evidence of efficient miR-146a amplification in TRIzol-extracted RNA samples using the TaqMan real-time PCR protocol described below.

For LCM samples and sera, we used the RNeasy Micro Kit and the miRNeasy Serum/Plasma Kit (Qiagen), respectively. All extractions were performed according to manufacturer's instructions. Quality and concentration of RNA were evaluated by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

## Reverse Transcription and Real-Time PCR

Total RNA samples were reverse transcribed using a TaqMan MicroRNA Reverse Transcription Kit with primers specific for miR-146a and for human small nuclear RNA (snRNA) U6, included as endogenous control. Complementary DNAs (cDNAs) (corresponding to 15 ng total RNA) were amplified in duplicates by quantitative real-time PCR, using predesigned TaqMan MicroRNA assays, on ViiA7 Real-Time PCR system (Thermo Fisher Scientific). For gene expression analyses, cDNA samples were prepared from total RNA using Superscript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) and subjected to real-time PCR reactions in duplicates with predesigned functionally tested TaqMan gene expression assays specific for IRAK1, TRAF6, c-REL, ICOS, FAS, and interferon regulatory factor 8 (IRF8) (Thermo Fisher Scientific). Human 18S was used as endogenous control for the normalization of gene expression data (Thermo Fisher Scientific). U6 and 18S were stably expressed across control and the MG samples (normally distributed Ct values with SD ≤ 0.5). Transcriptional levels of miR-146a and target genes were expressed as relative values normalized with U6 or 18S, respectively, using the formula $2^{-\Delta Ct} \times 100$.

## Double Immunofluorescence

Double immunofluorescence stainings were performed on 6-μm thick serial sections of snap-frozen follicular hyperplastic thymuses from five corticosteroid-naïve and four corticosteroid-treated MG patients and six control thymuses. Sections were fixed in 4% paraformaldehyde (PFA) for 10 min and incubated in cold methanol for 10 min and in 5% bovine serum albumin (BSA) for 1 h; then, they were immunostained overnight at 4 °C with a combination of primary antibodies against IRAK1 (1:50, rabbit polyclonal, Thermo Fisher Scientific), c-REL (1:50, rabbit polyclonal, Thermo Fisher Scientific), CD20 (1:300, clone L26, Agilent Dako, Santa Clara, CA), CD11c (1:20, clone B-ly6, BD Pharmigen, San Jose, CA), cytokeratin (CK) (1:100, clone MNF116, Agilent Dako), and CD68 (1:100, clone KP1, Agilent Dako). Sections were then incubated for 1 h with a mixture of Cy2-conjugated goat antimouse immunoglobulin G (IgG) and Cy3-conjugated goat antirabbit IgG (Jackson ImmunoResearch Laboratories, West Baltimore Pike, West Grove, PA); nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). As negative control, primary antibodies were omitted or replaced with isotype-specific IgGs (Agilent Dako). Fluorescence images were captured by the C1 laser scanning confocal microscope system (Nikon) and analyzed using Image J software (version 1.48). CD20/IRAK1, CD11c/IRAK1, and CD68/IRAK1 double-positive cells were counted by two experienced operators blinded to the diagnosis and treatment on at least four randomly selected adjacent fields per section at × 60 magnification.

## Statistical Analysis

The non-parametric distributed data, tested via Shapiro–Wilk test, were analyzed by Kruskal–Wallis test with Dunn's *post-hoc* test for multiple comparisons or by Mann–Whitney test for comparison of two groups, as indicated in figure legends. Differences were considered statistically significant when the *p* values were $<0.05$. Nonparametric Spearman correlation test was applied to evaluate the correlation between miR-146a and its target expression levels in thymic tissues. Receiver operating

characteristic (ROC) curves were used to assess the sensitivity and specificity of miR-146a in serum as biomarker for MG. GraphPad Prism v5.0 (La Jolla, CA) was used for data elaboration and statistical analyses.

# RESULTS

## miR-146a Deficiency in Follicular Hyperplastic MG Thymus

The expression of miR-146a was assessed in follicular hyperplastic thymuses from early-onset AChR-positive MG patients and normal thymuses from patients without autoimmune diseases. Patients were stratified in corticosteroid-naïve and corticosteroid-treated patients according to the therapeutic treatment before thymectomy. The first group was characterized by a higher number of GCs per thymic section compared to the second one (5–15 vs. 1–3 GCs), in line with the corticosteroids' ability to reduce thymic GCs in MG patients (42). Interestingly, a significant lower expression of miR-146a was observed in hyperplastic thymuses from corticosteroid-naïve MG patients compared to normal thymuses (**Figure 1A**). This decrease was not found in thymuses from corticosteroid-treated patients, suggesting that corticosteroids may affect miR-146a expression (**Figure 1A**). No significant difference in the intrathymic miRNA levels was found between male and female patients and controls (data not shown). We then analyzed the transcriptional levels of IRAK1, TRAF6, c-REL, and ICOS, well-known miR-146a targets critically involved in innate immune response activation and GC formation (20, 28, 30–32), which are key events in the intrathymic MG pathogenesis (4, 5). IRAK1, c-REL, and ICOS mRNA levels showed an opposite trend compared to that of miR-146a: they were increased in thymuses of corticosteroid-naïve patients compared to both immunosuppressed MG patients' and normal thymuses (**Figure 1B**). These results supported an effect of corticosteroid therapy on the miR-146a/mRNA target axis. Regarding the expression of TRAF6, although difference among the sample groups did not reach statistical significance, corticosteroid-naïve hyperplastic MG thymuses showed increased mRNA levels of this gene, as observed for the other miR-146a targets (**Figure 1B**).

A negative correlation was found between miR-146a levels and the mRNA levels of IRAK1, c-REL, and ICOS in MG and control tissues (**Figure 1C**), in accordance with the existence of a functional relationship between the miRNA and its three target genes (20, 28, 30–32).

## miR-146a Expression in Germinal Centers of Hyperplastic MG Thymuses

Based on the hypothesis that miR-146a/mRNA target changes observed in MG thymuses could be related to GC presence and to structural changes in GC organization during corticosteroid treatment, we next compared miR-146a, along with IRAK-1, c-REL, and ICOS expression, in microdissected GCs, and the corresponding GC-surrounding tissue (WS-GCs), from follicular hyperplastic MG thymuses (**Figure 2A**) and whole sections from normal thymuses. To control GC microdissection quality, the IRF8 transcript was analyzed in all the samples as GC marker (43, 44). As expected, IRF8 mRNA levels were higher in microdissected GCs, both from corticosteroid-naïve and corticosteroid-treated patients, compared to WS-GC sections of follicular hyperplastic thymuses and whole thymic sections from controls (**Figure 2B**). In pathological thymuses from untreated MG patients, miR-146a was expressed at lower levels in WS-GC tissues, but it was expressed in GCs, indicating that the miR-146a deficiency observed in hyperplastic MG thymuses was not directly related to GC presence. Indeed, miR-146a levels did not correlate with the GC number in the hyperplastic thymic sections (data not shown). In corticosteroid-treated patients, the miRNA levels were higher in both GC and WS-GC tissue, compared to the levels observed in corticosteroid-naïve patients, in line with the hypothesis that corticosteroids influence miR-146a expression (**Figure 2B**). IRAK1 showed the opposite expression trend of miR-146a: its transcriptional levels were higher in WS-GCs, and lower in GCs, in untreated patients' thymuses compared to normal thymuses; moreover, they were reduced in the WS-GCs of immunosuppressed patients (**Figure 2C**). This suggested that defective miR-146a expression in the hyperplastic thymic medulla, out of GCs, might lead to IRAK1 overexpression; in addition, miR-146a induction by the immunosuppressive treatment seems to normalize/reduce IRAK1 expression. As regard c-REL and ICOS, the increased expression levels previously found in hyperplastic MG thymuses (**Figure 1B**) were due to GC presence. Indeed, microdissected GCs from untreated and treated patients showed the highest c-REL and ICOS levels (**Figure 2C**). In corticosteroid-treated patients, lower expression of c-REL was observed in WS-GCs compared to the same tissues from untreated patients and normal thymuses, in line with the increased expression of miR-146a in WS-GCs.

Based on the literature data showing a contribution of miR-146a to GC formation via FAS (34), we also analyzed FAS expression in the microdissected samples. In untreated MG patients, FAS mRNA levels were lower in GCs than in the corresponding WS-GCs (**Figure 2C**), sustaining the hypothesis of a miR-146a contribution to GC formation via Fas downregulation in B cells. In corticosteroid-treated patients, WS-GCs presented higher levels of FAS mRNA compared to the thymic samples of untreated patients and controls, indicative of an impact of the corticosteroid treatment in inducing programmed cell death in thymic cell populations (**Figure 2C**).

In line with previous data showing that FAS is a direct target of miR-146a in B cells (34), we found a negative correlation between miR-146a and FAS mRNA levels in GCs and WS-GCs from MG thymuses (**Figure 2D**).

## Increased Expression of IRAK1 in Myeloid Dendritic Cells and Macrophages of Hyperplastic MG Thymuses

As described above, in follicular hyperplastic MG thymuses, IRAK1 expression was increased in medullary area out of GCs, indicating that other thymic cell populations than GC cells overexpressed this molecule in the pathological tissues. Since IRAK1 is a critical component of TLR signaling pathways,

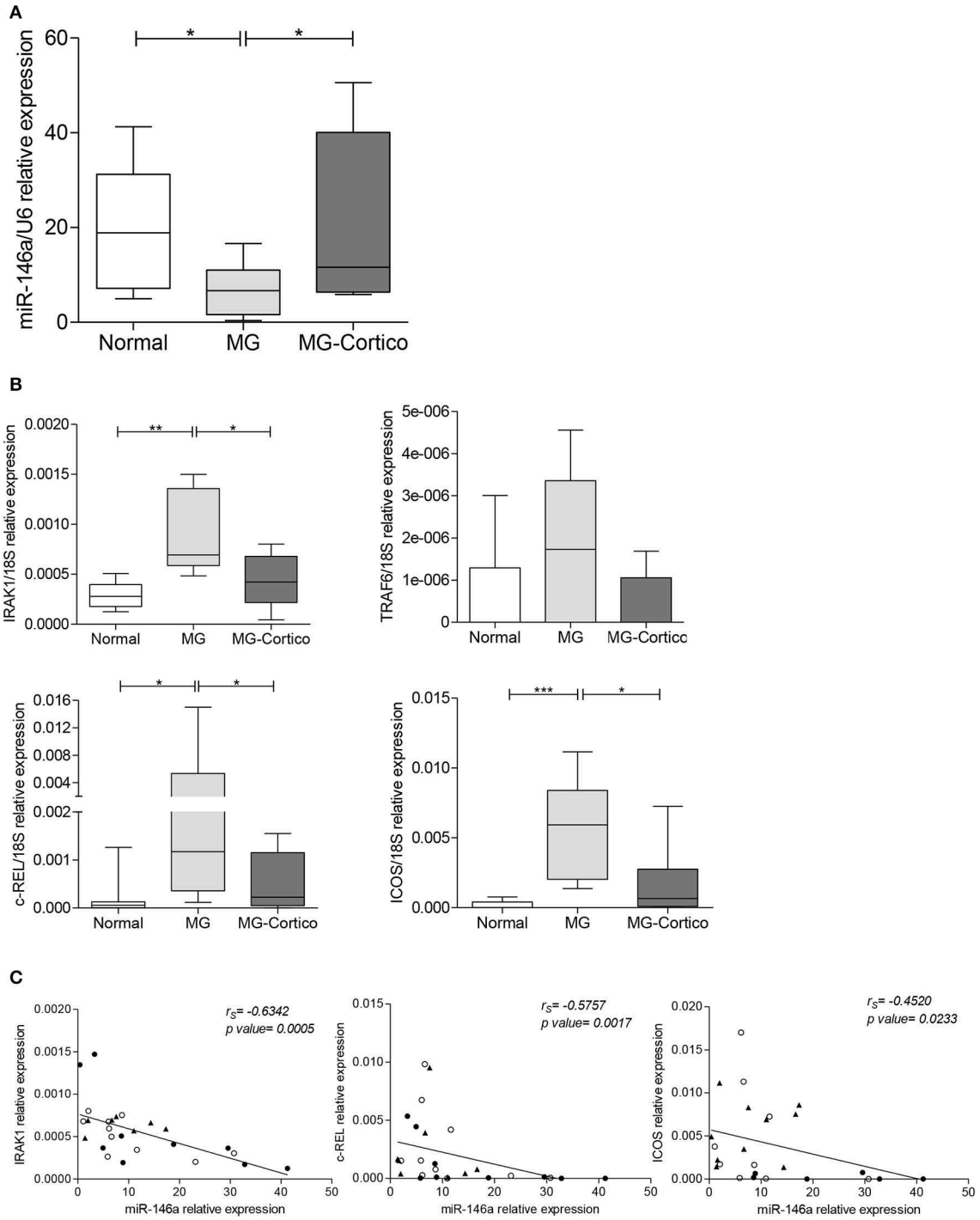



**FIGURE 1 |** Decreased expression of miR-146a in follicular hyperplastic myasthenia gravis (MG) thymuses and increased transcriptional levels of its target genes, IRAK1, TRAF6, c-REL, and ICOS. **(A)** Real-time PCR analysis of miR-146a levels in normal thymuses ($n = 10$) and follicular hyperplastic thymuses from MG patients classified based on treatment before thymectomy in untreated or treated only with cholinesterase inhibitors ($n = 12$, MG) and treated with corticosteroids ($n = 15$, MG-Cortico). **(B)** Real-time PCR analysis to assess the expression of miR-146a gene targets IRAK1, TRAF6, c-REL, and ICOS in the same normal and MG thymuses. In **(A,B)** boxplots, miR-146a and target expression levels were expressed as relative values ($2^{-\Delta Ct} \times 100$) normalized toward the endogenous small nuclear RNA (snRNA) U6 (miR-146a) or 18S (target genes); dark horizontal lines represent means, with the box representing the 25 and 75th percentiles and the whiskers the 5 and 95th percentiles. *p* values were assessed by the Kruskal–Wallis test followed by the Dunn's *post hoc* test, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. **(C)** Negative correlation estimated by Spearman's correlation analysis between miR-146a levels and messenger RNA (mRNA) levels of IRAK1, c-REL, and ICOS in the thymus of 10 controls (•), 12 corticosteroid-naïve (○), and 15 corticosteroid-treated (▲) MG patients.

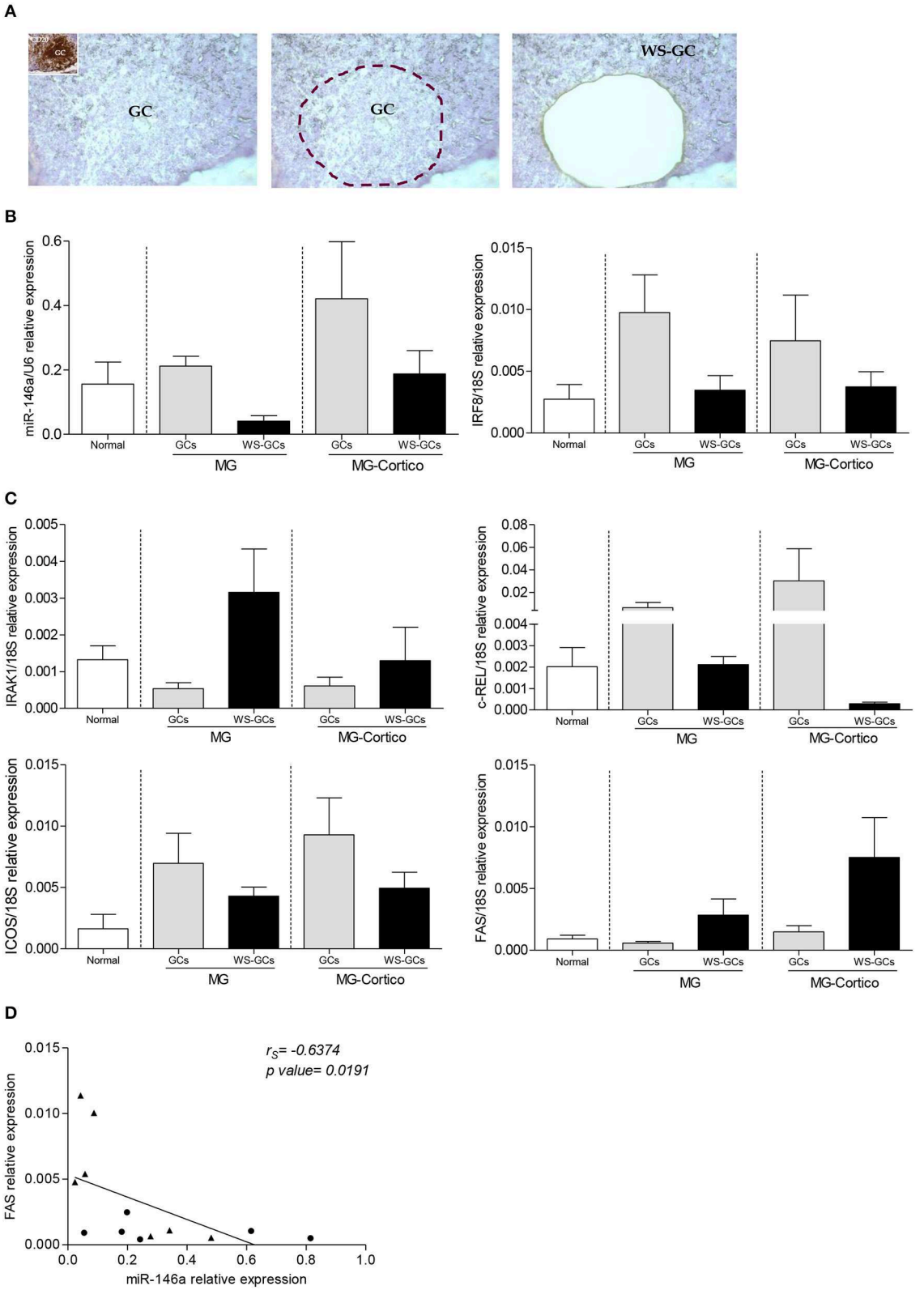



**FIGURE 2 |** Analysis of miR-146a and its target gene messenger RNA (mRNA) levels in microdissected germinal centers (GCs) of hyperplastic myasthenia gravis (MG) thymuses. **(A)** Representative image showing a follicular hyperplastic MG thymic section containing a GC subjected to laser capture microdissection. The inset in the

*(Continued)*

**FIGURE 2 |** first panel corresponds to an adjacent section of the same thymus stained for the B-cell marker CD20. Expression of miR-146a and IRF8 **(B)** and miR-146a targets IRAK1, c-REL, ICOS, and FAS **(C)** in normal thymus sections ($n = 4$), whole thymic sections devoid of GCs (WS-GCs), and the corresponding microdissected GCs, from corticosteroid-naïve ($n = 4$, MG) and corticosteroid-treated ($n = 3$, MG-Cortico) MG patients. In the graphs, miR-146a, IRF8, and miRNA target gene levels were expressed as relative values ($2^{-\Delta Ct} \times 100$) normalized toward the endogenous small nuclear RNA (snRNA) U6 (miR-146a) or 18S (IRF8 and target genes). **(D)** Negative correlation estimated by Spearman's correlation analysis between miR-146a levels and mRNA levels of FAS in GCs (•) and WS-GCs (▲) from hyperplastic MG thymuses.

we investigated its expression in thymic cells, known to overexpress TLRs (i.e. TLR4, TLR7, and TLR9) (10, 11), including CK-positive thymic epithelial cells (TECs), CD11c-positive myeloid dendritic cells (mDCs), and CD68-positive macrophages. CD20-positive B cells were included in the analysis since diffuse B-cell lymphoid infiltrates consistently characterize medulla of hyperplastic MG thymuses (9, 11). By double immunofluorescence, we observed IRAK1 expression in a proportion of infiltrating B cells of hyperplastic MG thymuses from untreated patients, but scarcely in GCs and B-cell infiltrates of pathological tissues from corticosteroid-treated patients and B cells of normal thymuses (**Figure 3A**). Indeed, although differences did not reach statistical significance, the percentage of diffuse CD20-positive B cells expressing IRAK1 was higher in thymuses from untreated MG patients compared to control and corticosteroid-treated MG patients' thymuses (**Figure 3B**). MG and control thymuses did not show marked difference in IRAK1 expression in TECs (**Figure 3A**). On the contrary, the protein expression in mDCs and macrophages was increased in thymuses from untreated MG patients compared to controls and tissues from immunosuppressed patients, in terms of both intensity of the immunostaining and number of positive cells (**Figures 3A,B**).

By considering IRAK1-positive cells, irrespective of the phenotype, the immunofluorescence analysis showed an increased percentage of these cells in the thymus of corticosteroid-naïve MG patients compared to control thymuses (**Figure 3B**). According to molecular data, this percentage was reduced in thymuses from patients treated with corticosteroids before thymectomy, supporting a corticosteroid effect in IRAK1 normalization within the MG thymus, which could occur via miR-146a.

## Marked c-REL Expression in Germinal Center and Infiltrating B Cells of MG Thymuses

By immunofluorescence, we found a strong immunoreactivity for c-REL of follicular hyperplastic thymuses from corticosteroid-naïve MG patients compared to normal thymuses. This immunoreactivity was marked in GCs, highlighting the role of c-REL in GC formation (**Figure 4**). In addition, enhanced c-REL expression was observed in infiltrating B cells of the thymic medulla in untreated MG patients, indicating that increased levels of c-REL in hyperplastic MG thymuses were mainly due to GC and infiltrating B cells. In contrast, reduced c-REL expression was observed in thymuses of patients treated with corticosteroids before thymectomy (**Figure 4**), suggesting a possible impact of immunosuppressive drugs on GCs and infiltrating B cells via the miR-146a/c-REL axis.

## Dysregulated Expression of miR-146a in Serum and PBMCs of MG Patients

To check for possible miR-146a dysregulation also in peripheral blood of MG patients, we analyzed the miRNA expression in serum and PBMCs of a cohort of MG patients and healthy controls (**Table 1**) by real-time PCR. We found a significant downregulation of miR-146a in the serum of corticosteroid-naïve MG patients compared to healthy controls (**Figure 5A**), as observed in the thymus, whereas in corticosteroid-treated patients, serum miR-146a levels were comparable to those of controls. In line with previous literature data (35), we found that miR-146a expression was significantly increased in PBMCs of corticosteroid-naïve patients compared to controls, suggesting a possible defective miR-146a release in serum by PBMCs. miR-146a increase in PBMCs was not observed in immunosuppressed patients (**Figure 5A**), again indicating differences between untreated patients and patients under immunosuppressive therapy. Thus, to understand the effect of corticosteroids on miR-146a expression, we performed *in vitro* treatment of PBMCs from corticosteroid-naïve MG patients and healthy controls with prednisone for 6, 24, 48, and 72 h. We did not observe differences in miR-146a levels at 0, 6, 24, 48, and 72 h between MG and control PBMCs. In both MG and control cells, we observed that the drug was able to increase miR-146a levels with a peak at 24 h of treatment (**Figure 5B**). These data were in line with the increased expression levels of miR-146a observed in thymuses from corticosteroid-treated compared to untreated patients. Lower expression of miR-146a in PBMCs of corticosteroid-treated compared to corticosteroid-naïve patients could be explained by considering that corticosteroids may induce miR-146a expression, but this induction might be accompanied by an increased release of the miRNA in serum. Indeed, serum levels of miR-146a were higher in patients under immunosuppressive therapy compared to untreated patients (**Figure 5B**).

Potential value of serum miR-146a as biomarker for MG was evaluated by ROC curve analysis. Of interest, in our cohort of AChR-positive patients and controls, we obtained sensitivity and specificity performance results that suggested a possible role of miR-146a as disease biomarker for AChR-MG (**Figure 5C**): area under curve (AUC) was 0.782 (95% CI, 0.5999–0.9648) ($p = 0.02$).

# DISCUSSION

MiRNA dysregulation is critically involved in the development of several autoimmune diseases (18, 19, 45). A number of studies showed alterations of miRNAs in PBMCs and serum of

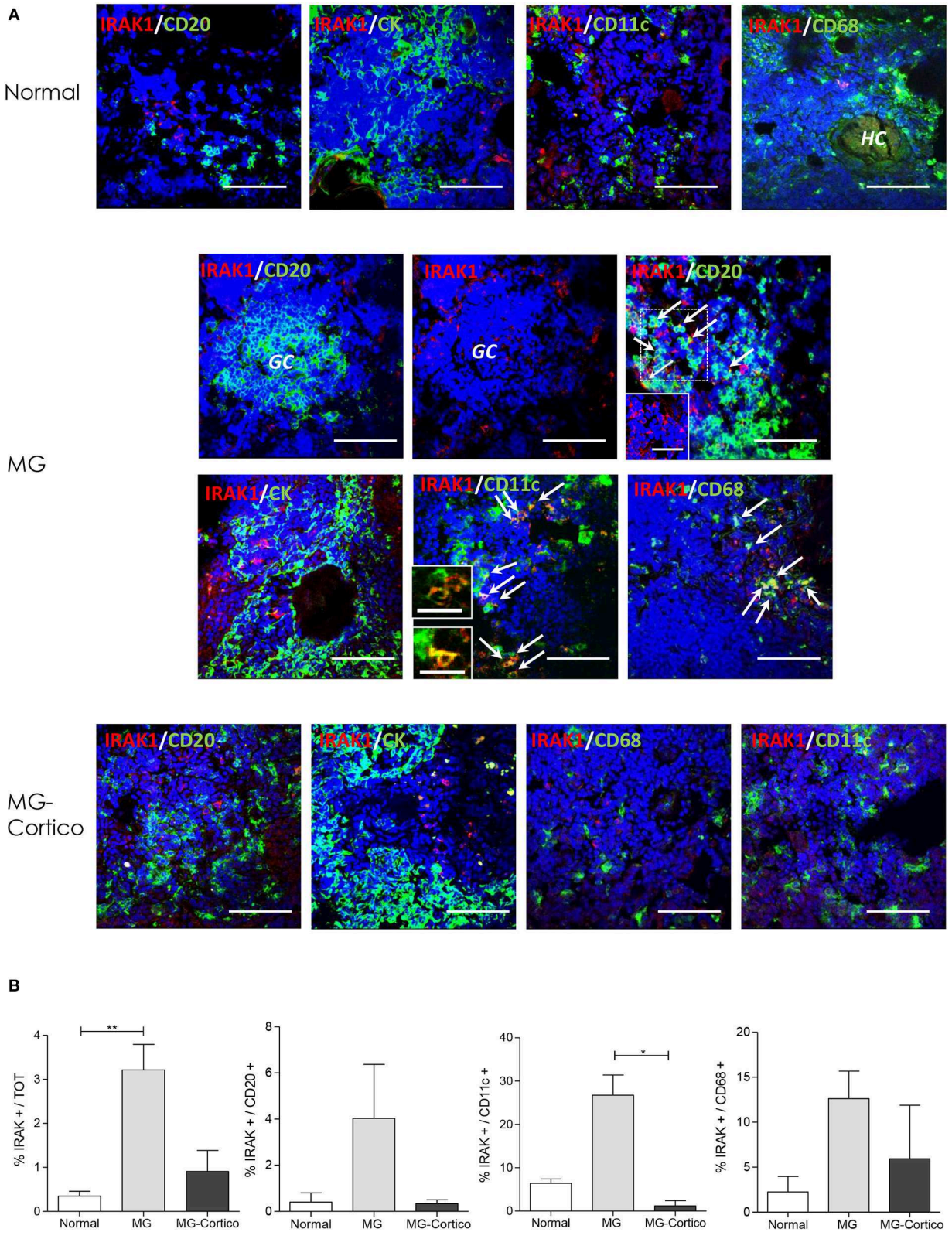



**FIGURE 3 |** Increased expression of interleukin 1 receptor associated kinase 1 (IRAK1) in infiltrating B cells, myeloid dendritic cells (mDCs), and macrophages of hyperplastic myasthenia gravis (MG) thymuses. **(A)** Double immunofluorescence stainings of normal thymus (upper panels) and hyperplastic MG thymuses from

*(Continued)*

**FIGURE 3 |** corticosteroid-naïve (MG) and corticosteroid-treated (MG-Cortico) MG patients (middle and lower panels) for the expression of IRAK1 (red) in combination with: CD20 (green), marker for B cells; cytokeratin (CK, green), marker of epithelial cells; CD11c (green), marker for mDCs; and CD68 (green), marker for macrophages. The arrows in the middle panels indicate double-positive cells. Blue staining shows 4′,6-diamidino-2-phenylindole (DAPI)-positive nuclei. The inset in MG IRAK-1/CD20 panel shows cells stained only for IRAK1 present in the main panel (dashed box). The insets in MG IRAK1/CD11c panel show enlargement of mDCs cells expressing IRAK1, present in the main panel. HC, Hassall's corpuscle. Magnification bars: 50 μm in the main panels and in MG and MG-Cortico IRAK1/CD20 insets; 20 μm in the MG IRAK1/CD11c insets. **(B)** Mean percentage (±SEM) of IRAK1-positive cells estimated on total DAPI-positive cells, total CD20-positive B cells, total CD11c-positive mDCs, and total CD68-positive macrophages in six control thymuses, five corticosteroid-naïve (MG), and four corticosteroid-treated (MG-Cortico) MG patients (four adjacent fields for sample group). *p* values were assessed by the Kruskal–Wallis test for multiple comparisons followed by the Dunn's *post-hoc* test, $^{*}p < 0.05$; $^{**}p < 0.01$.

myasthenic patients, suggesting a key role of these molecules also in the immune pathophysiology of MG (46–50). The contribution of miRNAs to the intrathymic MG pathogenesis has been poorly explored. A recent miRNome profiling performed in hyperplastic MG thymuses highlighted the role of specific miRNAs (e.g. miR-7 and miR-125a) in thymic changes, as well as inflammatory pathways and immune dysregulation, associated with MG (51). miR-146a is one of the first miRNAs identified to be involved in the modulation of innate and adaptive immune system, whose role in autoimmunity has been reported in different studies (20–24). Physiologically, it is induced by TLR ligands in an NF-κB-dependent manner and acts as inhibitor of innate immune responses by targeting IRAK1 and TRAF6, two key effectors of TLR signaling (20). Its ability to control TLR pathways allows inflammation resolution, avoiding persisting inflammatory reactions that could be dangerous and favor chronic inflammatory and autoimmune conditions (20, 28). At the same time, miR-146a has been reported to participate to GC development, by targeting c-REL, ICOS, and FAS (30–32, 34).

Chronic inflammation, abnormal TLR activation, and GC formation characterize hyperplastic thymus of patients affected by MG (5, 8), but the contribution of miR-146a to the intrathymic MG pathogenesis has not been deeply investigated. In the present study, we analyzed the expression of miR-146a, and its above-mentioned targets, in follicular hyperplastic MG thymuses and normal thymuses from patients without autoimmune diseases. Interestingly, we provided evidence of dysregulated miR-146a/mRNA target pattern in MG thymuses. Indeed, we showed that miR-146a expression was defective in hyperplastic thymus of patients untreated, or treated only with cholinesterase inhibitors before thymectomy, compared to controls. This deficiency was associated with a significant increased expression of IRAK1, c-REL, and ICOS, whose expression levels negatively correlated with those of miR-146a, supporting a functional miR-146a/mRNA target relationship. Considering the critical role of IRAK1 in TLR signaling (20, 28), and of c-REL and ICOS in GC formation (29, 33), these results strongly suggested that miR-146a downregulation could significantly contribute to persistent innate immune activation, sustained inflammation, and follicular hyperplastic changes in the thymus of MG patients. In line with this hypothesis, gene knockout studies, aimed at investigating the miR-146a function, showed that deficiency of this miRNA may lead to an excessive IL-6 and TNF-α production, hyperresponsiveness to bacterial lipopolysaccharide (LPS), chronic inflammation, and spontaneous autoimmunity (52, 53). Not surprisingly, miR-146a-deficient expression has been described in other autoimmune conditions, than MG. In particular, miR-146a levels were found to be lower in PBMCs and serum of patients with SLE compared to controls (21, 54). Underexpression of miR-146a in these patients was shown to contribute to activation of type I IFN pathways, and its overexpression reduced the induction of these pathways in PBMCs (55), also suggesting that the miR-146a decrement that we observed in MG thymuses could favor type I IFN overproduction, which is an intrathymic hallmark of MG patients (56). Interestingly, in a study by Rosato et al. (57), low miR-146a expression, correlating with elevated levels of IRAK1 and type I IFNs, was associated with Epstein–Barr virus (EBV) infection in cells expressing the EBV-encoded EBNA2, thus suggesting a possible relationship between the observed miR-146a reduction and the EBV presence that we previously found in hyperplastic MG thymuses (9). Moreover, decreased expression of miR-146a has been reported to contribute to abnormal regulatory T cell (Treg) phenotype in RA patients with active disease, and correlated with joint inflammation (58). Likewise, defective Tregs in hyperplastic MG thymuses (59) could be related to miR-146a decrease, a hypothesis to be deeply explored. Indeed, miR-146a is one of the miRNAs prevalently expressed in Tregs and is critical for their suppressor function by targeting the signal transducer and activator transcription 1 (STAT1), so that its deletion may result in tolerance breakdown (60).

Of note, the analysis of miR-146a/mRNA target expression in hyperplastic thymuses from MG patients treated with corticosteroids before thymectomy revealed that the immunosuppressive therapy was able to normalize the intrathymic miRNA levels, along with the transcriptional levels of IRAK1, c-REL, and ICOS. Indeed, miR-146a expression was increased in immunosuppressed compared to untreated patients. This finding was in line with previous observations demonstrating that miR-146a is a glucocorticoid-inducible miRNA, along with mir-26b, mir-125a-5p, mir-150-5p, and mir-184 (61). Accordingly, normalization of IRAK1, c-REL, and ICOS mRNA levels in corticosteroid-treated patients was likely due to miR-146a restoration, as an effect of the therapy. However, corticosteroids are potent modulators of immune system with a strong impact on immune system cells and ability to modify miRNA pattern at different levels; thus, their effects on miR-146a expression could be both direct or indirect. Since immunosuppressive treatment is known to affect the number of GCs in the MG thymus (42), we also hypothesized that differences in thymic miR-146a/mRNA target pattern between corticosteroid-naïve and corticosteroid-treated patients could be

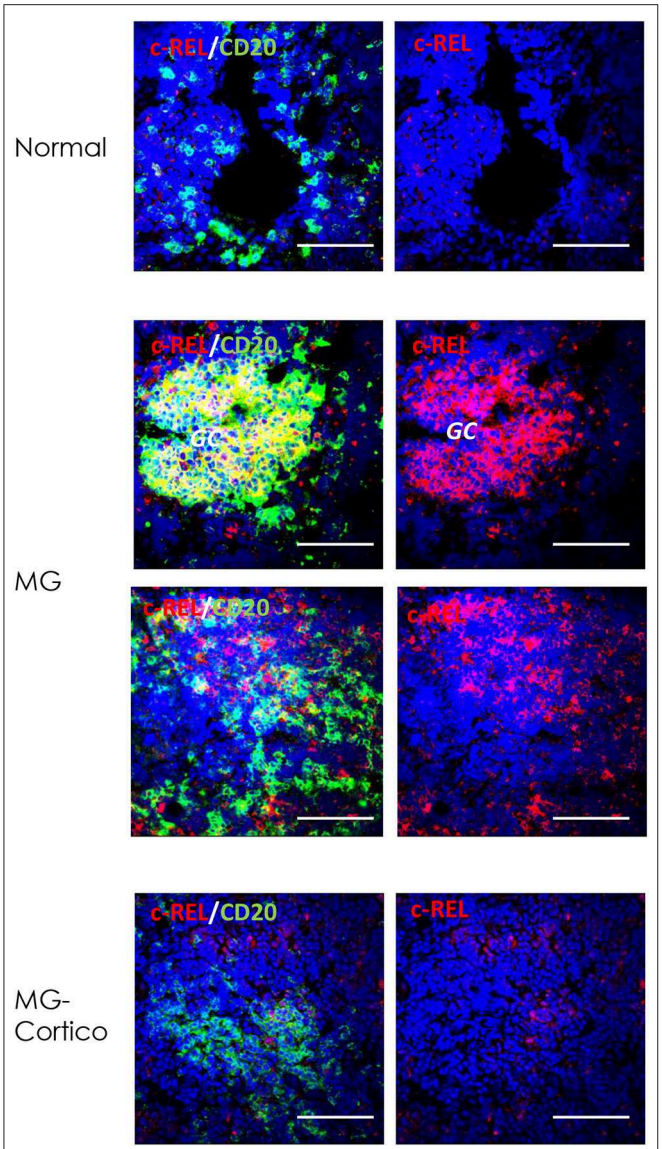



**FIGURE 4 |** Immunoreactivity for c-REL of germinal center (GC) and infiltrating B cells of hyperplastic myasthenia gravis (MG) thymuses. Double immunofluorescence stainings of normal thymus (upper panels) and hyperplastic MG thymic sections from untreated (MG) and corticosteroid-treated (MG-Cortico) patients (middle and lower panels) for c-REL (red), alone (right panels) or in combination with CD20 (green) (left panels), marker of B cells. Blue staining shows DAPI-positive nuclei. Magnification bars: 50 μm in all panels.

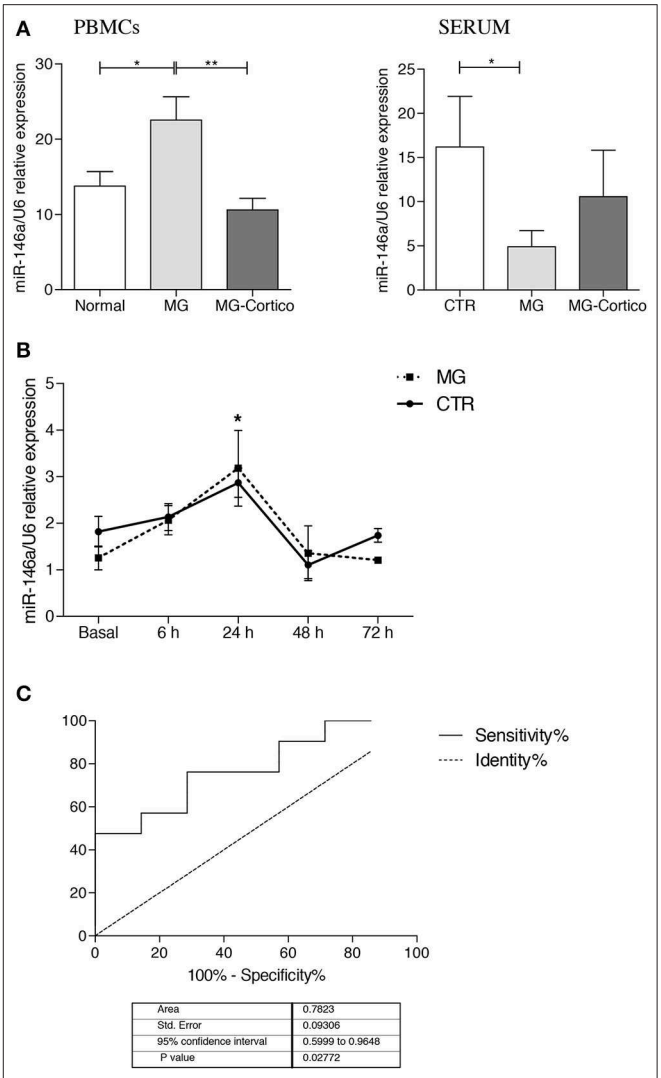



**FIGURE 5 |** Peripheral expression of miR-146a in myasthenia gravis (MG). **(A)** Real-time PCR analysis of miR-146a expression in peripheral blood mononuclear cells (PBMCs) (left graph) and serum (right graph) from MG patients ($n = 31$, 13 of whom were corticosteroid-treated, MG-Cortico) and healthy controls ($n = 11$). miR-146a levels were expressed as relative values ($2^{-\Delta Ct} \times 100$) normalized toward the endogenous small nuclear RNA (snRNA) U6. $p$ values were assessed by Kruskal–Wallis test followed by the Dunn's *post hoc* test. $^{*}p < 0.05$; $^{**}p < 0.01$. **(B)** *In vitro* assay of miR-146a expression in PBMCs from four MG patients and three healthy controls, treated with prednisone at 6, 24, 48, and 72 h. miR-146a levels were analyzed by real-time PCR and expressed as relative values ($2^{-\Delta Ct} \times 100$) normalized toward U6 snRNA. In the graph, the mean relative expression levels ± SEM of miR-146a estimated in cells from the four MG patients and from the three controls at basal condition and after 6, 24, 48, and 72 h of treatment with prednisone are shown. $p$ values were assessed by Kruskal–Wallis test followed by the Dunn's *post hoc* test. $^{*}p < 0.05$ (24 h of treatment vs. basal condition). **(C)** Receiver operating characteristic (ROC) curves used to assess the sensitivity and specificity of miR-146a in serum as biomarker for MG.

related to GC changes upon treatment. However, by performing LCM experiments, we demonstrated that miR-146a deficiency in hyperplastic MG thymuses was not related to GCs. Indeed, in untreated patients, miR-146a was downregulated in the GC-surrounding medulla, where IRAK1 was upregulated, but it was expressed in GCs. Moreover, its levels did not correlate with the GC number in the MG thymuses. In treated patients, miR-146a expression was higher in both GCs and WS-GCs compared to untreated patients, again supporting an effect of corticosteroids in inducing the miRNA expression. Our data also highlighted the role of c-REL and ICOS in GC formation and maintenance in MG thymuses. Indeed, c-REL and ICOS were expressed at higher levels in GCs of both untreated and

corticosteroid-treated patients than in control thymuses. Of interest, in MG GCs and WS-GCs, a negative relationship was found between miR-146a expression and mRNA levels of FAS, another recognized direct target of miR-146a (34), indicating a key contribution of the miRNA to GC formation via FAS downregulation. The role of FAS in GCs is strongly supported by studies performed in B-cell-specific FAS-deficient mice, found to develop fatal lymphoproliferation due to B-cell activation, and by the observation that ablation of FAS specifically in GC B cells reproduced lymphoproliferation (62). Downregulation of FAS in GCs by miR-146a was also reported to cause autoimmune lymphoproliferative syndrome in mice (34), which is indicative of a critical role of miR-146a in B-cell homeostasis and GC response through FAS. Consistent with these observations, Cho et al. demonstrated that not only elevated levels of miR-146a in B cells are important in controlling humoral autoimmunity by targeting CD40 signaling pathways but also that specific deletion of miR-146a in T cells increases Tfh cell number enhancing GC reactions (31), thus sustaining that miR-146a expression is required for maintenance of GC reactions.

In line with LCM data, by double immunofluorescence, we found that IRAK1 was not expressed in GCs, but mainly in MG mDCs and macrophages, thymic cell populations characterized, in MG patients, by overexpression of TLRs, particularly TLR4, as reported in our previous studies (10, 11). Literature data showed that miR-146a efficiently targets TRAF6 and IRAK1 in DCs and modulates the production of pro-inflammatory cytokines by these cells (63). Thus, miR-146a deficiency observed in the medulla of hyperplastic MG thymuses could well-contribute to IRAK1 upregulation and TLR-mediated inflammatory activation in mDCs and macrophages. B cells of GCs, along with B-cell lymphoid infiltrates, were markedly labeled for c-REL, strengthening the role of this molecular factor in GC development and B-cell dysregulation in MG thymuses. Heise et al. (29) reported that GC B-cell-specific deletion of c-REL led to the collapse of established GCs and was associated with the failure to activate a metabolic program that promotes cell growth, unequivocally demonstrating the role of c-REL in GCs. Along with c-REL, ICOS, a recognized target of miR-146a that we found to be overexpressed in MG thymuses, actively participate in GC formation (32). Its blockage has been found to prevent Tfh and GC B-cell accumulation (32), suggesting that miR-146a break may significantly promote or enhance GC response also via ICOS in MG thymus.

Unfortunately, prethymectomy serum and PBMCs from patients included in the thymus analysis were not available. However, to verify whether miR-146a dysregulation also characterized MG peripheral blood, we investigated the miRNA expression in serum and PBMCs of a group of not thymectomized MG patients and controls. In untreated patients, miR-146a was downregulated in serum, as observed in SLE patients (54), but it was upregulated in PBMCs compared to controls, as previously reported in the literature for MG patients (35). Increased miR-146a levels, positively correlating with levels of proinflammatory cytokines, were reported in PBMCs, and particularly CD4-positive T cells, from patients with RA (22, 64). In an independent study, higher miR-146a levels were found in IL-17-producing T cells from RA patients with high disease activity, suggesting a role of miR-146a in the differentiation of Th17 cells (65). Similarly, by considering the key function of miR-146a in controlling Treg activity (60), the dysregulated miR-146a expression that we found in the MG peripheral blood could well-affect the T-cell inflammatory phenotype, contributing to the Th17/Treg unbalance characteristic of MG patients (66). miR-146a overexpression in PBMCs was also demonstrated in MS patients, and again, it was associated with increased Th1/Th17 cytokine overexpression (e.g. IL-17, IFN-γ, TNF-α) (67). Variation in serum and cellular miR-146a levels in an opposite manner led us to hypothesize a possible defective release of the miRNA from PBMCs to serum in MG patients. However, the exact mechanisms underlying miR-146a dysregulation in MG blood, as well as the miRNA impact on circulating immune system cells, needs to be further explored.

In corticosteroid-treated patients, PBMC levels of miR-146a were comparable to those of controls, again revealing an effect of immunosuppressive treatment on miR-146a expression. By *in vitro* experiments, we observed that treatment of PBMCs with prednisone was able to increase the expression of miR-146a in both MG and control cells, according to the already reported role of corticosteroids to induce the expression of this miRNA (61). Hence, this increase may explain normalization of miR-146a levels in hyperplastic MG thymuses of patients treated with corticosteroids before thymectomy. Since serum, but not PBMC, levels of miR-146a were higher in treated compared to untreated MG patients, we hypothesized that immunosuppressive drugs might induce miR-146a expression in PBMCs accompanied by high release in the serum. In addition, corticosteroids could affect viability of specific cell populations expressing the miRNA in PBMCs or thymus of treated patients; thus, their exact impact on miR-146a expression needs to be deeply studied.

Finally, ROC curve analyses provided sensitivity and specificity results indicative of a possible role of miR-146a in serum as biomarker for MG, whose usefulness for monitoring the disease progression or prognosis deserves future investigation. However, since the miRNA was widely implicated in other inflammatory and autoimmune pathologies (21–26), it could represent not a biomarker specific for MG, but a more general marker of an inflammatory autoimmune condition, with potential utility in more than one autoimmune disease.

Our overall findings thus revealed that miR-146a expression is defective in follicular hyperplastic MG thymuses and that loss of fine regulation of innate and adaptive immune response by the miRNA may significantly contribute to the intrathymic MG pathogenesis. The ability of miR-146a to control TLR signaling pathways, and consequently inflammation, along with GC formation, makes the miR-146a/mRNA target axis a promising candidate target of innovative treatments for counteracting B-cell-mediated autoimmunity in MG.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The study was approved by the Ethics Committee of the Fondazione I.R.C.C.S. Istituto Neurologico Carlo Besta (Milan, Italy; Approval No. 586/2014). All patients and controls signed an informed consent form for the use of biological samples for research purpose.

## AUTHOR CONTRIBUTIONS

FB participated in sample collection, performed immunofluorescence analyses and cell counting, elaborated the experimental data, performed statistical analysis, and drafted the manuscript. LS was involved in sample collection and molecular analyses and performed the *in vitro* experiments. SM contributed to the study design and participated in data discussion and elaboration. SB and RF performed clinical data collection and were involved in data discussion. TM contributed to collection of thymic samples and performed histological classification of MG thymuses. CA and RM provided clinical data, interpretation of results, participated in data discussion and critically revised the manuscript. PC obtained funding, contributed to the study conception and design, provided interpretation of results, and was involved in manuscript writing and editing. PB contributed to the study setup and experimental design, monitored the experimental activity, participated in data discussion, and critically revised the manuscript.

## FUNDING

This work was supported by the Italian Ministry of Health (Grants Nos. GR-2013-02358564 and RF-2016-02364384, and Annual Research funding).

## ACKNOWLEDGMENTS

We wish to thank Chiara Vantaggiato, Claudia Malacarne, Eleonoria Giagnorio, Cristina Cappelletti, Francesca Andreetta, and Ornella Simoncini (Neurology IV – Neuroimmunology and Neuromuscular Diseases Unit, Fondazione I.R.C.C.S. Istituto Neurologico Carlo Besta, Milan, Italy) for participating in biological sample collection. We also thank the Italian Association of Mysthenia Gravis (A.I.M., Associazione Italiana Miastenia e Malattie Immunodegenerative – Amici del Besta Onlus) for the kind support in the research activity.

## REFERENCES

1. Mantegazza R, Cavalcante P. Diagnosis and treatment of myasthenia gravis. *Curr Opin Rheumatol*. (2019) 31:623–33. doi: 10.1097/BOR.0000000000000647
2. Mantegazza R, Antozzi C. When myasthenia gravis is deemed refractory: clinical signposts and treatment strategies. *Ther Adv Neurol Disord*. (2018) 11:1756285617749134. doi: 10.1177/1756285617749134
3. Berrih-Aknin S, Le Panse R. Thymectomy in myasthenia gravis: when, why, and how? *Lancet Neurol*. (2019) 18:225–26. doi: 10.1016/S1474-4422(18)30467-8
4. Berrih-Aknin S. Myasthenia Gravis: paradox versus paradigm in autoimmunity. *J Autoimmun*. (2014) 52:1–28. doi: 10.1016/j.jaut.2014.05.001
5. Cavalcante P, Cufi P, Mantegazza R, Berrih-Aknin S, Bernasconi P, Le Panse R. Etiology of myasthenia gravis: innate immunity signature in pathological thymus. *Autoimmun Rev*. (2013) 12:863–74. doi: 10.1016/j.autrev.2013.03.010
6. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo HC, Marx A, et al. MGTX study group. Randomized trial of thymectomy in myasthenia gravis. *N Engl J Med*. (2016) 375:511–22. doi: 10.1056/NEJMoa1602489
7. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo HC, Marx A, et al. MGTX Study Group. Long-term effect of thymectomy plus prednisone versus prednisone alone in patients with non-thymomatous myasthenia gravis: 2-year extension of the MGTX randomised trial. *Lancet Neurol*. (2019) 18:259–68. doi: 10.1016/S1474-4422(18)30392-2
8. Cron MA, Maillard S, Villegas J, Truffault F, Sudres M, Dragin N, et al. Thymus involvement in early-onset myasthenia gravis. *Ann N Y Acad Sci*. (2018) 1412:137–45. doi: 10.1111/nyas.13519
9. Cavalcante P, Serafini B, Rosicarelli B, Maggi L, Barberis M, Antozzi C, et al. Epstein-Barr virus persistence and reactivation in myasthenia gravis thymus. *Ann Neurol*. (2010) 67:726–38. doi: 10.1002/ana.21902
10. Cordiglieri C, Marolda R, Franzi S, Cappelletti C, Giardina C, Motta T, et al. Innate immunity in myasthenia gravis thymus: pathogenic effects of Toll-like receptor 4 signaling on autoimmunity. *J Autoimmun*. (2014) 52:74–89. doi: 10.1016/j.jaut.2013.12.013
11. Cavalcante P, Galbardi B, Franzi S, Marcuzzo S, Barzago C, Bonanno S, et al. Increased expression of Toll-like receptors 7 and 9 in myasthenia gravis thymus characterized by active Epstein-Barr virus infection. *Immunobiology*. (2016) 221:516–27. doi: 10.1016/j.imbio.2015.12.007
12. Cavalcante P, Barzago C, Baggi F, Antozzi C, Maggi L, Mantegazza R, et al. Toll-like receptors 7 and 9 in myasthenia gravis thymus: amplifiers of autoimmunity? *Ann N Y Acad Sci*. (2018) 1413:11–24. doi: 10.1111/nyas.13534
13. Cufi P, Dragin N, Weiss JM, Martinez-Martinez P, De Baets MH, Roussin R, et al. Implication of double-stranded RNA signaling in the etiology of autoimmune myasthenia gravis. *Ann Neurol*. (2013) 73:281–93. doi: 10.1002/ana.23791
14. Robinet M, Maillard S, Cron MA, Berrih-Aknin S, Le Panse R. Review on Toll-like receptor activation in myasthenia gravis: application to the development of new experimental models. *Clin Rev Allergy Immunol*. (2017) 52:133–47. doi: 10.1007/s12016-016-8549-4
15. Liu H, Lei C, He Q, Pan Z, Xiao D, Tao Y. Nuclear functions of mammalian microRNAs in gene regulation, immunity and cancer. *Mol Cancer*. (2018) 17:64. doi: 10.1186/s12943-018-0765-5
16. Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet*. (2011) 12:861–74. doi: 10.1038/nrg3074
17. Dai X, Kaushik AC, Zhang J. The emerging role of major regulatory RNAs in cancer control. *Front Oncol*. (2019) 9:920. doi: 10.3389/fonc.2019.00920
18. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun*. (2009) 32:189–94. doi: 10.1016/j.jaut.2009.02.012
19. Singh RP, Massachi I, Manickavel S, Singh S, Rao NP, Hasan S, et al. The role of miRNA in inflammation and autoimmunity. *Autoimmun Rev*. (2013) 12:1160–5. doi: 10.1016/j.autrev.2013.07.003
20. Saba R, Sorensen DL, Booth SA. MicroRNA-146a: a dominant, negative regulator of the innate immune response. *Front Immunol*. (2014) 5:578. doi: 10.3389/fimmu.2014.00578
21. Löfgren SE, Frostegård J, Truedsson L, Pons-Estel BA, D'Alfonso S, Witte T, et al. Genetic association of miRNA-146a with systemic lupus erythematosus in Europeans through decreased expression of the gene. *Genes Immun*. (2012) 13:268–74. doi: 10.1038/gene.2011.84
22. Pauley KM, Satoh M, Chan AL, Bubb MR, Reeves WH, Chan EK. Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Res Ther*. (2008) 10:R101. doi: 10.1186/ar2493

23. Churov AV, Oleinik EK, Knip M. MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. *Autoimm Rev*. (2015) 14:1029–37. doi: 10.1016/j.autrev.2015.07.005
24. Ma X, Zhou J, Zhong Y, Jiang L, Mu P, Li Y, et al. Expression, regulation and function of microRNAs in multiple sclerosis. *Int J Med Sci*. (2014) 11:810–8. doi: 10.7150/ijms.8647
25. Han Y, Li Y, Jiang Y. The prognostic value of plasma microRNA-155 and microRNA-146a level in severe sepsis and sepsis-induced acute lung injury patients. *Clin Lab*. (2016) 62:2355–60. doi: 10.7754/Clin.Lab.2016.160511
26. Zhou J, Chaudhry H, Zhong Y, Ali MM, Perkins LA, Owens WB, et al. Dysregulation in microRNA expression in peripheral blood mononuclear cells of sepsis patients is associated with immunopathology. *Cytokine*. (2015) 71:89–100. doi: 10.1016/j.cyto.2014.09.003
27. Paterson MR, Kriegel AJ. miR-146a/b: a family with shared seeds and different roots. *Physiol Genomics*. (2017) 49:243–52. doi: 10.1152/physiolgenomics.00133.2016
28. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NFkappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA*. (2006) 103:12481–6. doi: 10.1073/pnas.0605298103
29. Heise N, De Silva NS, Silva K, Carette A, Simonetti G, Pasparakis M, et al. Germinal center B cell maintenance and differentiation are controlled by distinct NF-κB transcription factor subunits. *J Exp Med*. (2014) 211:2103–18. doi: 10.1084/jem.20132613
30. Li B, Wang X, Choi IY, Wang YC, Liu S, Pham AT, et al. miR-146a modulates autoreactive Th17 cell differentiation and regulates organ-specific autoimmunity. *J Clin Invest*. (2017) 127:3702–16. doi: 10.1172/JCI94012
31. Cho S, Lee HM, Yu IS, Choi YS, Huang HY, Hashemifar SS, et al. Differential cell-intrinsic regulations of germinal center B and T cells by miR-146a and miR-146b. *Nat Commun*. (2018) 9:2757. doi: 10.1038/s41467-018-05196-3
32. Pratama A, Srivastava M, Williams NJ, Papa I, Lee SK, Dinh XT, et al. MicroRNA-146a regulates ICOS-ICOSL signalling to limit accumulation of T follicular helper cells and germinal centres. *Nat Commun*. (2015) 6:6436. doi: 10.1038/ncomms7436
33. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity*. (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
34. Guo Q, Zhang J, Li J, Zou L, Zhang J, Xie Z, et al. Forced miR-146a expression causes autoimmune lymphoproliferative syndrome in mice via downregulation of Fas in germinal center B cells. *Blood*. (2013) 121:4875–83. doi: 10.1182/blood-2012-08-452425
35. Lu J, Yan M, Wang Y, Zhang J, Yang H, Tian FF, et al. Altered expression of miR-146a in myasthenia gravis. *Neurosci Lett*. (2013) 555:85–90. doi: 10.1016/j.neulet.2013.09.014
36. Zhang J, Jia G, Liu Q, Hu J, Yan M, Yang B, et al. Silencing miR-146a influences B cells and ameliorates experimental autoimmune myasthenia gravis. *Immunology*. (2015) 144:56–67. doi: 10.1111/imm.12347
37. Goleva E, Jackson LP, Gleason M, Leung DY. Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients. *J Allergy Clin Immunol*. (2012) 129:687–93. doi: 10.1016/j.jaci.2011.12.001
38. Sugiyama K, Kawada T, Sato H, Hirano T. Comparison of suppressive potency between prednisolone and prednisolone sodium succinate against mitogen-induced blastogenesis of human peripheral blood mononuclear cells *in-vitro*. *J Pharm Pharmacol*. (2001) 53:727–33. doi: 10.1211/0022357011775857
39. Hirano T, Akashi T, Kido T, Oka K, Shiratori T, Miyaoka M. Immunosuppressant pharmacodynamics on peripheral-blood mononuclear cells from patients with ulcerative colitis. *Int Immunopharmacol*. (2002) 2:1055–63. doi: 10.1016/S1567-5769(02)00077-2
40. Masotti A, Caputo V, Da Sacco L, Pizzuti A, Dallapiccola B, Bottazzo GF. Quantification of small non-coding RNAs allows an accurate comparison of miRNA expression profiles. *J Biomed Biotechnol*. (2009) 2009:659028. doi: 10.1155/2009/659028
41. El-Khoury V, Pierson S, Kaoma T, Bernardin F, Berchem G. Assessing cellular and circulating miRNA recovery: the impact of the RNA isolation method and the quantity of input material. *Sci Rep*. (2016) 6:19529. doi: 10.1038/srep19529
42. Truffault F, de Montpreville V, Eymard B, Sharshar T, Le Panse R, Berrih-Aknin S. Thymic germinal centers and corticosteroids in myasthenia gravis: an immunopathological study in 1035 cases and a critical review. *Clin Rev Allergy Immunol*. (2017) 52:108–24. doi: 10.1007/s12016-016-8558-3
43. Lee CH, Melchers M, Wang H, Torrey TA, Slota R, Qi CF, et al. Regulation of the germinal center gene program by interferon (IFN) regulatory factor 8/IFN consensus sequence-binding protein. *J Exp Med*. (2006) 203:63–72. doi: 10.1084/jem.20051450
44. Martinez A, Pittaluga S, Rudelius M, Davies-Hill T, Sebasigari D, Fountaine TJ, et al. Expression of the interferon regulatory factor 8/ICSBP-1 in human reactive lymphoid tissues and B-cell lymphomas: a novel germinal center marker. *Am J Surg Pathol*. (2008) 32:1190–200. doi: 10.1097/PAS.0b013e318166f46a
45. Chen JQ, Papp G, Szodoray P, Zeher M. The role of microRNAs in the pathogenesis of autoimmune diseases. *Autoimmun Rev*. (2016) 15:1171–80. doi: 10.1016/j.autrev.2016.09.003
46. Punga AR, Andersson M, Alimohammadi M, Punga T. Disease specific signature of circulating miR-150-5p and miR-21-5p in myasthenia gravis patients. *J Neurol Sci*. (2015) 356:90–6. doi: 10.1016/j.jns.2015.06.019
47. Punga T, Le Panse R, Andersson M, Truffault F, Berrih-Aknin S, Punga AR. Circulating miRNAs in myasthenia gravis: miR-150-5p as a new potential biomarker. *Ann Clin Transl Neurol*. (2014) 1:49–58. doi: 10.1002/acn3.24
48. Wang J, Zheng S, Xin N, Dou C, Fu L, Zhang X, et al. Identification of novel microRNA signatures linked to experimental autoimmune myasthenia gravis pathogenesis: down-regulated miR-145 promotes pathogenetic Th17 cell response. *J Neuroimmune Pharmacol*. (2013) 8:1287–302. doi: 10.1007/s11481-013-9498-9
49. Cron MA, Maillard S, Truffault F, Gualeni AV, Gloghini A, Fadel E, et al. Causes and consequences of miR-150-5p dysregulation in myasthenia gravis. *Front Immunol*. (2019) 10:539. doi: 10.3389/fimmu.2019.00539
50. Barzago C, Lum J, Cavalcante P, Srinivasan KG, Faggiani E, Camera G, et al. A novel infection- and inflammation-associated molecular signature in peripheral blood of myasthenia gravis patients. *Immunobiology*. (2016) 221:1227–36. doi: 10.1016/j.imbio.2016.06.012
51. Cron MA, Maillard S, Delisle F, Samson N, Truffault F, Foti M, et al. Analysis of microRNA expression in the thymus of myasthenia gravis patients opens new research avenues. *Autoimmun Rev*. (2018) 17:588–600. doi: 10.1016/j.autrev.2018.01.008
52. Testa U, Pelosi E, Castelli G, Labbaye C. miR-146 and miR-155: two key modulators of immune response and tumor development. *Noncoding RNA*. (2017) 3:E22. doi: 10.3390/ncrna3030022
53. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-155 is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med*. (2011) 208:1189–201. doi: 10.1084/jem.20101823
54. Wang G, Tam LS, Li EK, Kwan BC, Chow KM, Luk CC, et al. Serum and urinary cell-free miR-146a and miR-155 in patients with systemic lupus erythematosus. *J Rheumatol*. (2010) 37:2516–22. doi: 10.3899/jrheum.100308
55. Tang Y, Luo X, Cui H, Ni X, Yuan M, Guo Y, et al. MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. *Arthritis Rheum*. (2009) 60:1065–75. doi: 10.1002/art.24436
56. Cufi P, Dragin N, Ruhlmann N, Weiss JM, Fadel E, Serraf A, et al. Central role of interferon-beta in thymic events leading to myasthenia gravis. *J Autoimmun*. (2014) 52:44–52. doi: 10.1016/j.jaut.2013.12.016
57. Rosato P, Anastasiadou E, Garg N, Lenze D, Boccellato F, Vincenti S, et al. Differential regulation of miR-21 and miR-146a by Epstein-Barr virus-encoded EBNA2. *Leukemia*. (2012) 26:2343–52. doi: 10.1038/leu.2012.108
58. Zhou Q, Haupt S, Kreuzer JT, Hammitzsch A, Proft F, Neumann C, et al. Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis. *Ann Rheum Dis*. (2015) 74:1265–74. doi: 10.1136/annrheumdis-2013-204377
59. Gradolatto A, Nazzal D, Truffault F, Bismuth J, Fadel E, Foti M, et al. Both Treg cells and Tconv cells are defective in the myasthenia gravis thymus: roles of IL-17 and TNF-α. *J Autoimmun*. (2014) 52:53–63. doi: 10.1016/j.jaut.2013.12.015
60. Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, et al. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell*. (2010) 142:914–29. doi: 10.1016/j.cell.2010.08.012
61. Palagani A, Op de Beeck K, Naulaerts S, Diddens J, Sekhar Chirumamilla C, Van Camp G, et al. Ectopic microRNA-150-5p transcription sensitizes glucocorticoid therapy response in MM1S multiple myeloma cells but fails to overcome hormone therapy resistance in MM1R cells. *PLoS ONE*. (2014) 9:e113842. doi: 10.1371/journal.pone.0113842

62. Hao Z, Duncan GS, Seagal J, Su YW, Hong C, Haight J, et al. Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity*. (2008) 29:615–27. doi: 10.1016/j.immuni.2008.07.016
63. Park H, Huang X, Lu C, Cairo MS, Zhou X. MicroRNA-146a and microRNA-146b regulate human dendritic cell apoptosis and cytokine production by targeting TRAF6 and IRAK1 proteins. *J Biol Chem*. (2015) 290:2831–41. doi: 10.1074/jbc.M114.591420
64. Abou-Zeid A, Saad M, Soliman E. microRNA 146a expression in rheumatoid arthritis: association with tumor necrosis factor-alpha and disease activity. *Genet Test Mol Biomark*. (2011) 15:807–12. doi: 10.1089/gtmb.2011.0026
65. Niimoto T, Nakasa T, Ishikawa M, Okuhara A, Izumi B, Deie M, et al. microRNA-146a expresses in interleukin-17 producing T cells in rheumatoid arthritis patients. *BMC Musculoskelet Disord*. (2010) 11:209. doi: 10.1186/1471-2474-11-209
66. Villegas JA, Van Wassenhove J, Le Panse R, Berrih-Aknin S, Dragin N. An imbalance between regulatory T cells and T helper 17 cells in acetylcholine receptor-positive myasthenia gravis patients. *Ann N Y Acad Sci*. (2018) 1413:154–62. doi: 10.1111/nyas.13591
67. Fenoglio C, Cantoni C, De Riz M, Ridolfi E, Cortini F, Serpente M, et al. Expression and genetic analysis of miRNAs involved in CD4+ cell activation in patients with multiple sclerosis. *Neurosci Lett*. (2011) 504:9–12. doi: 10.1016/j.neulet.2011.08.021

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 17 March 2020
doi: 10.3389/fimmu.2020.00403

# MuSK EAMG: Immunological Characterization and Suppression by Induction of Oral Tolerance

*Debby Reuveni*[1], *Revital Aricha*[1], *Miriam C. Souroujon*[1,2†] *and Sara Fuchs*[1*]

[1] *Department of Immunology, Weizmann Institute of Science, Rehovot, Israel,* [2] *Department of Natural Sciences, The Open University of Israel, Ra'anana, Israel*

Myasthenia gravis (MG) with antibodies to the muscle-specific receptor tyrosine kinase (MuSK) is a distinct sub-group of MG, affecting 5–8% of all MG patients. MuSK, a receptor tyrosine kinase, is expressed at the neuromuscular junctions (NMJs) from the earliest stages of synaptogenesis and plays a crucial role in the development and maintenance of the NMJ. MuSK-MG patients are more severely affected and more refractory to treatments currently used for MG. Most patients require long-term immunosuppression, stressing the need for improved treatments. Ideally, preferred treatments should specifically delete the antigen-specific autoimmune response, without affecting the entire immune system. Mucosal tolerance, induced by oral or nasal administration of an auto-antigen through the mucosal system, resulting in an antigen-specific immunological systemic hyporesponsiveness, might be considered as a treatment of choice for MuSK-MG. In the present study we have characterized several immunological parameters of murine MuSK-EAMG and have employed induction of oral tolerance in mouse MuSK-EAMG, by feeding with a recombinant MuSK protein one week before disease induction. Such a treatment has been shown to attenuate MuSK-EAMG. Both induction and progression of disease were ameliorated following oral treatment with the recombinant MuSK fragment, as indicated by lower clinical scores and lower anti-MuSK antibody titers.

**Keywords: myasthenia gravis, muscle-specific receptor tyrosine kinase (MuSK), neuromuscular junction (NMJ), oral tolerance, T regulatory cells**

OPEN ACCESS

***Edited by:***
*Anna Rostedt Punga, Uppsala University, Sweden*

***Reviewed by:***
*William Donald Phillips, University of Sydney, Australia*
*Maartje G. Huijbers, Leiden University Medical Center, Netherlands*

***Correspondence:**
*Sara Fuchs*
*sara.fuchs@weizmann.ac.il*

†*Deceased*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *05 November 2019*
***Accepted:*** *20 February 2020*
***Published:*** *17 March 2020*

***Citation:***
*Reuveni D, Aricha R, Souroujon MC and Fuchs S (2020) MuSK EAMG: Immunological Characterization and Suppression by Induction of Oral Tolerance. Front. Immunol. 11:403. doi: 10.3389/fimmu.2020.00403*

## INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease characterized by skeletal muscle weakness as a result of an immunological attack at the neuromuscular junction (NMJ). In 5–8% of MG patients the autoantibodies present in the sera are against the muscle-specific receptor tyrosine kinase (MuSK) classifying these patients as a distinct MG sub-group called MuSK-MG (1). MuSK is a tyrosine kinase receptor expressed from early stages of synaptogenesis at the NMJ and has been shown to play a critical role in NMJ development and maintenance (2). Low-density lipoprotein receptor-related protein 4 (LRP4) and MuSK act together as a receptor for Agrin, a motor-neuron-derived matrix proteoglycan. Agrin binding results in dimerization of MuSK and LRP4 followed by activation of MuSK (3). However, it is still unknown how these molecules regulate NMJ formation (4).

The use of currently immunosuppressive therapies for MuSK-MG patients is challenging as patients are prone to develop severe facial weakness and bulbar symptoms, including dysphagia, dysarthria and respiratory crisis with some atrophy of facial muscles making the treatment less effective (5, 6). Thymus alterations are common in Acetylcholine receptor (AChR)-MG patients whereas in MuSK-MG patients, thymus histology is mostly normal-for-age, with scattered lymphoid infiltrates (7). Among the treatments available, the use of acetyl-cholinesterase inhibitors is unsatisfactory and thymectomy does not improve the course of disease (1). Rituximab, a B cell depleting agent, was recently shown to benefit patients in uncontrolled studies; yet, data from controlled prospective studies on the use of rituximab in MuSK-MG patients are not available, thus, leaving immunosuppression as the mainstay of treatment (8). The severe form of MuSK-MG requires emergent and aggressive treatment to manage respiratory distress. A marked improvement in disease symptoms is achieved by corticosteroids but disease flares are frequent during dosage tapering, as a result the patients depend on treatment thus, it is crucial to develop improved and better treatment modalities.

To date, general immunosupression is the mainstay treatment for autoimmune diseases. The main challenge for immunologists is to develop novel treatments that will manipulate specifically or correct the abnormal immune response leaving the overall immune response intact.

Specific systemic tolerance to an antigen can be achieved by exposing mucosal surfaces to a particular antigen (9), and it is now accepted that it plays a crucial role in preventing disorders such autoimmunity and food allergies. While the process is not fully understood, recent years have seen a number of important advances due to expansion of knowledge in cellular immunology. One of the most important developments in the field has been the realization that the microbiota has dramatic effects on immune function throughout the body. It encouraged scientists to modulate many experimental autoimmune diseases by induction of mucosal tolerance to a specific autoantigen (10).

We have shown in our previous studies that mucosal (oral or nasal) administration of torpedo AChR prior to disease induction resulted in EAMG suppression and was accompanied by inhibition of the humoral response as well as cellular responses to AChR (11, 12). Moreover, EAMG clinical manifestation was suppressed when the antigen was administered during the acute phase of the disease (13).

Shigemoto et al. have developed a MuSK-EAMG model (14) in which FVB/N complement-deficient mice are immunized with a recombinant MuSK protein and a month after 100% of these mice synchronously develop MuSK-EAMG. The use of this model is particularly useful for the development and testing of novel therapeutic strategies as disease progression is predictable and the model resembles well the human disease.

**Abbreviations:** MG, Myasthenia gravis; MuSK, Muscle specific tyrosine kinase; NMJ, Neural muscular junction; AChR, Acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; CFA, complete Freund's adjuvant; IFA, Incomplete Freund's adjuvant; TGFβ, transforming growth factor beta; Foxp3, Forkhead box P3; IL-18, Interleukin 18; IL-15, Interleukin 15; OVA, Ovalbumin protein and Tregs, T regulatory cells.

In this study we have induced the MUSK-EAMG experimental model disease in mice and characterized some of its immunological properties in order to apply it as a model for therapeutic experiments. Specifically, we report on our successful efforts to induce mucosal tolerance to the MuSK antigen by oral application of the recombinant extracellular domain of the MuSK protein.

# MATERIALS AND METHODS

## Animals

Female FVB/N mice aged 6-8 weeks were obtained from the Animal Breeding Center of The Weizmann Institute of Science, Rehovot, Israel and were maintained at the Institute's animal facilities. All the experiments in this study were performed according to the institutional guidelines for animal care.

## Production of Recombinant Rat MuSK

pCEP-PU vector containing the His-tagged extracellular domain of recombinant rat MuSK [aa 21-491; (15)] was kindly provided by A.R. Punga (Upsala, Sweden). The plasmid was transfected, using Lipofectamine 2000 from Invitrogen (Carlsbad, CA) into HEK 293 EBNA cells. Large-scale production of the recombinant protein was performed at the Proteomics Unit of the Weizmann Institute. The recombinant MuSK protein was produced by the mammalian cells and was secreted to the medium under serum free conditions. Cell supernatant was subjected to a Ni-NTA super-flow column (Qiagen, Hilden, Germany) for protein purification. The purity of the protein obtained following Ni affinity chromatography and gel filtration was ∼95%. Concentration was determined at OD 280 nm.

## Induction and Clinical Evaluation of MuSK-EAMG

On day 0, adult female mice were anesthetized (Ketamine: 111 mg/kg and Xylazine: 22 mg/kg) and immunized subcutaneously, each with a total volume of 200 μl of recombinant MuSK (20 or 40 μg/mouse), emulsified in complete Freund's adjuvant (CFA) from Sigma-Aldrich (St. Louis, MO), as follows: 20 μl in each hind foot pads, 40 μl at the base of the tail and 20 μl in each of 6 well-separated sites on the back. On day 14 post-injection, all mice were boosted by 6 well-separated sites on the back with 20–40 μg MuSK emulsified with incomplete Freund's adjuvant (IFA) from Sigma-Aldrich (St. Louis, MO). Control mice were immunized by CFA and IFA only.

Mice were observed, weighed and scored blindly on alternate days, for the clinical severity of disease as follows: 0 - Healthy mouse; 1 - Body weight loss; 2 - Body weight loss, weakness, prominent cervicothoracic hump; 3 - Body weight loss, weakness, prominent cervicothoracic hump, tremor and ungroomed fur; 4 - Dead.

The method of blinding employed in our experiments was as follows: FVB/N mice were randomly divided into experimental groups: MuSK and CFA. One operator was assigned to the experimental treatments (MuSK immunization) while a second person who assessed the mice (weight, scoring) remained blinded to the experimental groups until the end of the experiment.

All experimental groups consisted of 10 mice each, unless otherwise specified and all experiments were repeated 2–3 times.

## Anti-MuSK Antibodies

Sera of treated mice were collected by retro-orbital bleeding 6 weeks following disease induction. The levels of anti-MuSK antibodies were determined by standard ELISA as follows: Microtiter plates were coated with recombinant MuSK protein (10 mg/100 ml in Tris-Cl, pH 8.0), and reacted with 100 µl of the tested mouse serum, at a dilution of 1:1000 for total IgG. Rabbit anti-Mouse alkaline phosphatase antibody (1:10000 Jackson, immunoresearch laboratories; West Grove, PA) was added followed by alkaline phosphatase-conjugated streptavidin. Antibody levels were evaluated by measuring the optical density at 405 nm.

## Immunofluorescence Flow Cytometry

Flow cytometry analysis was performed on splenocytes of MuSK-immunized mouse and of control mouse. Spleen cells were suspended in FACS wash buffer (PBS, 5% BSA) and incubated for 60 min at 4°C in the dark with antibodies to the tested cell surface molecules. Cells were washed and analyzed on a FACScan flow cytometer. The following antibodies were used for flow cytometry: FITC-conjugated anti-mouse CD4 (L3T4) and APC-conjugated anti-mouse CD25 (PC61.5). For intracellular staining, cells were fixed and permeabilized using the fixation/permeabilization kit from e-Bioscience (San Diego, CA) followed by staining with PE-conjugated anti-FoxP3 antibody (e-Bioscience).

## RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from mice splenocytes and muscles at the end of the experiment (8 weeks following immunization with MuSK). Extraction was performed using the high pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The concentration of total RNA was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was prepared and quantitative real-time reverse transcription (PCR) was performed using the LightCycler system (Roche) according to the manufacturer's instructions. Primer sequences (forward and reverse, respectively) are given in **Table 1**.

## MuSK-Specific Oral Tolerance Induction

Musk-specific oral tolerance was attempted by feeding of FVB/N mice with recombinant MuSK, essentially as described by us for the induction of oral tolerance of AChR-EAMG by a recombinant AChR fragment (11). FVB/N mice were fed, by means of a feeding tube, each with either recombinant MuSK (120 µg/100 µl/mouse) or with a control irrelevant protein (Ovalbumin, Sigma), both diluted in PBS. Treatment was initiated one week before disease induction and continued 3 times a week, until the end of the experiment.

Disease evaluation was performed as mentioned above: FVB/N mice were randomly divided into experimental groups: MuSK and OVA oral administration. One operator was assigned to the experimental treatments (feeding by gavage and MuSK immunization) while a second person who assessed the mice (weight, scoring) remained blinded to the experimental groups until the end of the experiment.

**TABLE 1** | Primer sequences.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| MuSK | 5′-GGCCGTGTAAGACCAG-3′ | 5′-GGAACGTAACCGGGAT-3′ |
| TGFβ | 5′-CAAGGGCTACCAT GCCAACT-3′ | 5′-CCGGGTTGTGTTGGTTGT AGA-3′ |
| Foxp3 | 5′-TGCTCCATACCTTGAACA C-3′ | 5′-CACTATATAGTCACCCCA AC-3′ |
| Cathepsin-l | 5′-GTTCTGGTGGTTGGCT-3′ | 5′-GTAGTGTCCGTAAGTCCT-3′ |
| IL-18 | 5′-TCCCAGACCAGACTGATA A-3′ | 5′-CTGGCACACGTTTCTGA-3′ |
| IL-15 | 5′-CTGGCACACGTTTCTGA-3′ | 5′-CAGCAGGTGGAGGTACCT TAA-3′ |
| β-actin | 5′-TACTGCCCTGGCTCCTAG CA-3′ | 5′-TGGACAGTGAGGCCAGGA TAG-3′. |

*β-actin was used as the house-keeping gene.*

## Statistics

The results are presented as mean ± SEM. Two-way ANOVA test was used to compare disease assessment vs. control and treatment vs. control groups all along the experiment. Differences in mean values were compared between treatment and control groups by the Student's *t*-test. $p$ values $< 0.05$ were considered as significant.

# RESULTS

## Induction of MuSK-EAMG

MuSK-EAMG, an experimental model of MuSK-MG, has been established in our lab in FVB/N female mice, according to Mori et al. (14), which observed that these mice are highly susceptible to MuSK-EAMG induction. 8 weeks old female FVB/N mice were immunized with recombinant MuSK protein (20 or 40 µg/mouse, as indicated) in CFA on day 0 and boosted 14 days later, with a similar dose of antigen, in incomplete Freund's adjuvant (IFA). All immunized mice manifested disease symptoms including severe muscle weakness and tremors within 2 weeks from the second injection. At the end of the experiment (35 days after disease induction) the CFA control group had a clinical score of 0, the MuSK 20 µg had a clinical score of 3 ± 0.6 (SD), and the MuSK 40 µg had a clinical score of 2.5 ± 1 (SD) (**Figure 1A**). These symptoms were observed synchronously in all animals, along with the appearance of a prominent cervicothoracic hump, indicating weak cervical extensor muscles and ungroomed fur. In addition, it should be noted that the MuSK-injected mice exhibited weight loss, corresponding to the progression of disease (**Figure 1B**). In contrast, control mice injected with PBS in CFA did not exhibit weight loss or any symptoms of disease (**Figures 1A,B**). Similar disease severity and antibody levels were observed following immunization with either 20 or 40 µg/mouse (**Figure 1A**) and for further experiments we have used 20 µg of MuSK for disease induction.

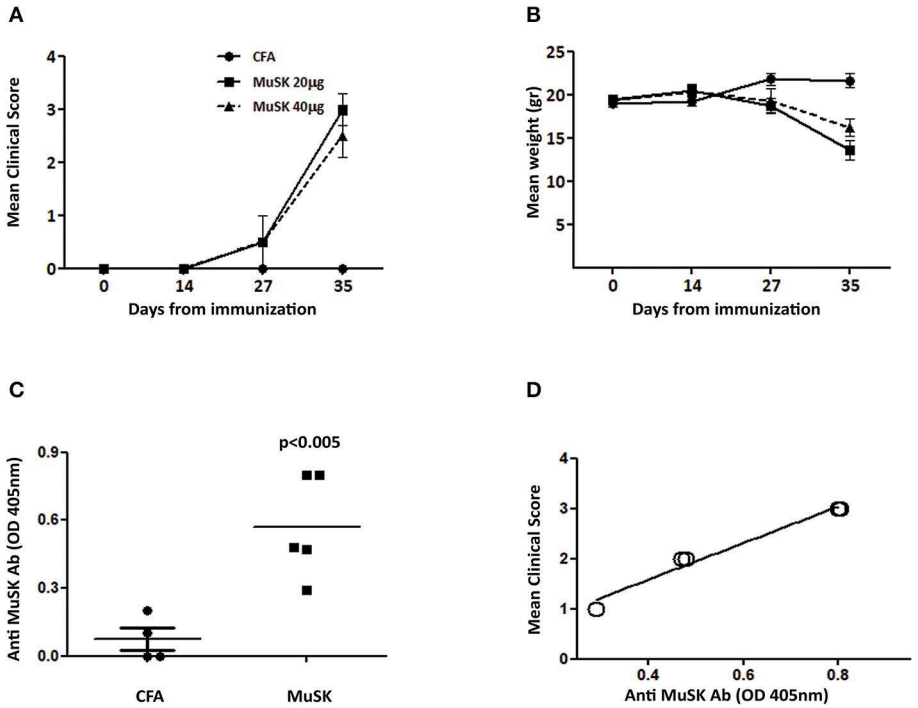



**FIGURE 1 |** Clinical characterization and antibody titers in MuSK-EAMG, induced in FVB/N mice. FVB/N female mice were immunized twice with 20–40 μg (as indicated) of recombinant MuSK in CFA, or with CFA alone, as a control ($n = 6$). Mice were followed up for clinical score **(A)** and weight loss changes **(B)**. Anti-MuSK antibody titer was tested 4 weeks following immunization, by ELISA **(C)**, and correlation with disease severity was tested **(D)**. $P < 0.001$ in **(A,B)**. Analyzed by the two-way ANOVA test.

## Anti-MuSK Antibody Titers Correlate With Disease Severity

Anti-MuSK IgG antibodies were analyzed by ELISA and were detected in all MuSK-immunized mice, whereas control CFA-immunized mice had no detectable antibodies to MuSK (**Figure 1C**). Interestingly, in contrast to AChR-EAMG, in which disease severity has no correlation to the levels of anti-AChR autoantibody titers, in MuSK EAMG - there seems to be a good correlation between anti-MuSK antibody and disease severity (**Figure 1D**). Such a correlation has been also observed and reported in MuSK-MG patients (5).

## MuSK- Immunized Mice Show Specific Muscle Damage

In order to test whether the induction of MuSK-EAMG results in muscle damage, the mRNA expression of several genes was examined in samples derived from masseter muscles from sick (MuSK-immunized) and control mice.

The initiation of protein degradation involved among others the lysosomal endopeptidase enzyme Cathepsin l. We have observed that the level of cathepsin 1 mRNA expression is significantly increased in MuSK-immunized mice, as depicted in **Figure 2A**, indicating muscle damage in sick mice. Likewise, there is also a significant increase in the expression of MuSK in MuSK-immunized mice, probably as a compensatory mechanism. In addition, IL-15, which is highly expressed in skeletal muscle and is believed to be a myokine, improve muscle glucose homeostasis and oxidative metabolism, was decreased in MuSK immunized mice (**Figures 2B,C**, respectively).

## Treg Cell Frequency Is Decreased in MuSK-EAMG Mice

We have analyzed the T cell subpopulations of MuSK-immunized mice, and of control, adjuvant-immunized mice. FACS analyses were performed on spleen cells, 4 weeks after disease induction. To test whether there are changes in the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells in MuSK-EAMG mice, their splenocytes were stained for CD4, CD25 and Foxp3, by specific antibodies. As shown in **Figure 3**, the percentage of CD4+CD25+$Foxp3^+$ cells among $CD4^+CD25^+$ cells, in the spleens of MuSK-EAMG mice (**Figures 3B,C**) is lower when compared to the percentage of such cells from healthy adjuvant-immunized controls (**Figures 3A,C**) These findings suggest that alterations in the Treg cell population may be involved in the immunopathology of MuSK-MG. Furthermore, we have observed a significant decrease in FoxP3 mRNA expression (**Figure 3D**) in MuSK immunized mice, as compared to control CFA-immunized mice. The decreased expression of FoxP3 in MuSK- immunized mice is in agreement with the flow cytometry results (**Figures 3A–C**), supporting a reduced frequency of Treg cells, as a result of the induced disease. In addition, we have also observed elevated levels of IL-18 mRNA (**Figure 3E**) whereas the expression levels of TGFβ were not changed in MuSK immunized mice when compared to CFA immunized mice.

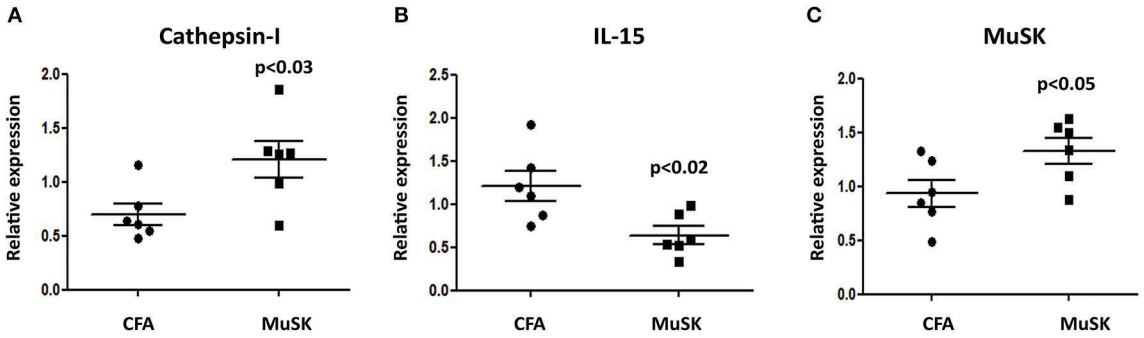



**FIGURE 2 |** MuSK immunized mice exhibit specific muscle damage. FVB/N mice were sacrificed 5 weeks after disease induction and RNA was isolated from masseter muscles. The expression levels of cathepsin-l **(A)**, IL-15 **(B)** and MuSK **(C)** were analyzed by quantitative real time RT-PCR and compared to the levels obtained in CFA-immunized control mice. β-actin was used as an internal control for normalization. All data are presented as mean ± SEM. Unpaired Student *t* test was employed. Representative out of two experiments ($n = 6$).

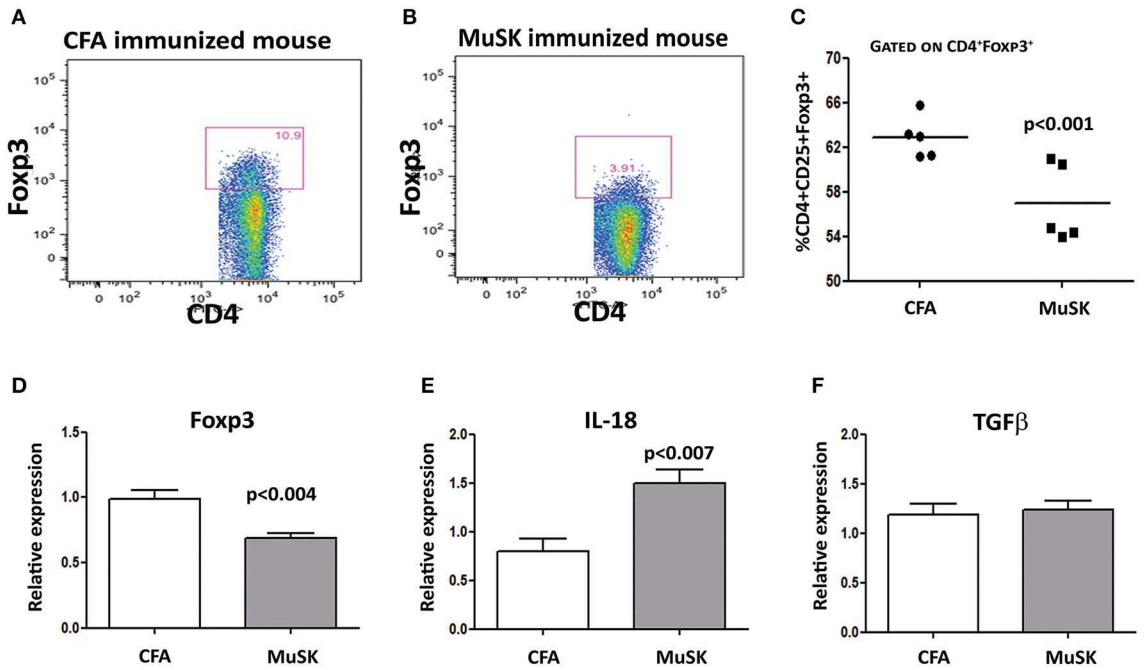



**FIGURE 3 |** Decreased Treg cell frequencies in spleens of MuSK-EAMG mice immunized mice. Spleens from control and MuSK immunized mice were harvested at the end of the experiments and analyzed by Flow cytometry and rt-PCR analyses. Representative Flow cytometry analysis of control immunized mouse **(A)** and MuSK immunized mouse **(B)**. Graphical summary of the frequency of CD4+CD25+FoxP3+ cells **(C)**. Expression levels of FoxP3, IL-18 and TGF-β (**D–F**, respectively) were evaluated by RT-PCR. β-actin was used as an inner control for normalization. All data are presented as mean ± SEM. Unpaired Student *t* test has been employed. Representative out of two experiments ($n = 12$).

## Suppression of MuSK-EAMG Following Induction of Oral Tolerance to MuSK Protein

Toward developing an antigen-specific treatment for MuSK-MG, we have attempted to develop an oral tolerance approach by feeding with recombinant MuSK-protein, by a similar protocol employed by us previously (11, 12) oral tolerance induction in AChR-EAMG. For these experiments we have first prepared large amounts of recombinant rat-MuSK protein, as described above in the Materials and Methods section. Preliminary experiments indicated that a dose of 120 μg recombinant MuSK/dose/mouse was optimal (Data not shown).

The oral tolerance experiment was initiated one week before disease induction and continued for 3 times a week, until the end of the experiment. Clinical scores and weights were evaluated, blinded, 3 times a week in 3 different experiments; each experimental group consisted of 10 mice.

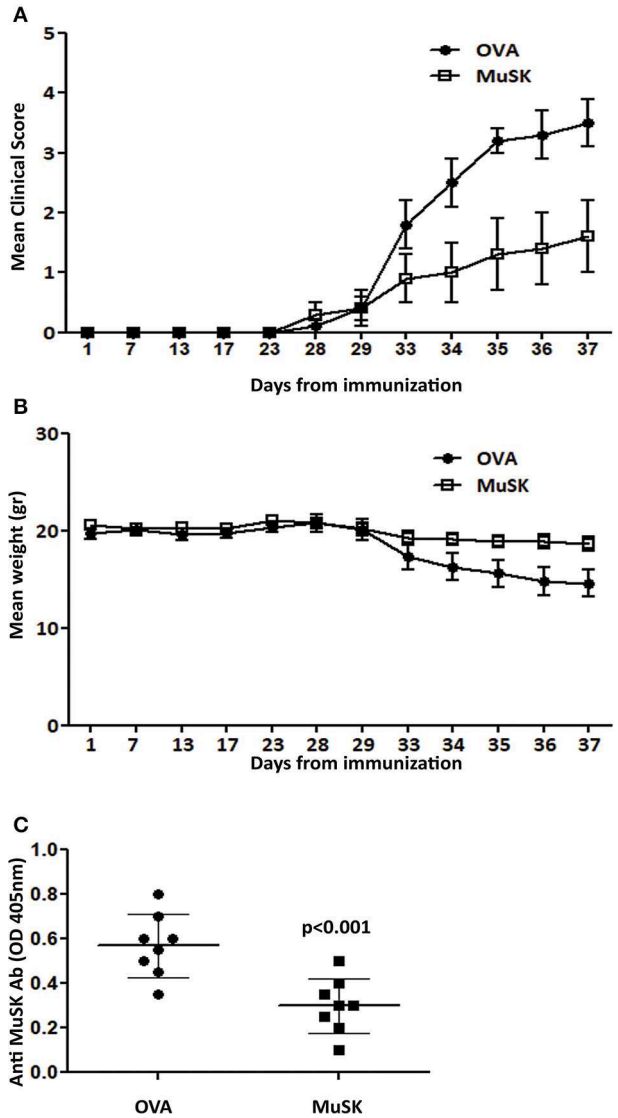



**FIGURE 4 |** Oral treatment suppresses MuSK-MG. Mice were fed with recombinant MuSK protein or OVA protein as a control, 3 times a week, starting one week before MuSK immunization and until the end of the experiment. Mean clinical score **(A)** and weight changes **(B)** in mice fed with 120μg MuSK or OVA/mouse/dose. Total MuSK specific IgG, tested by ELISA **(C)**. $n \geq 8$ mice for each group, in 3 different experiments. $P < 0.001$ in **(A,B)**. Analyzed by the two-way ANOVA test. Representative out of three experiments.

Administration of recombinant rat MuSK protein resulted in a significant therapeutic effect in MuSK- treated mice, accompanied also by monitoring the levels of weight changes, as depicted in **Figures 4A,B**, respectively. Thus, the severity of disease was significantly lower in the group of mice fed with recombinant MuSK than in the control group of mice (**Figure 4A**), whereas, the weight loss was less pronounced with disease progression, in the MuSK-fed group (**Figure 4B**).

Concomitantly with the effect of oral tolerance induction on MuSK-EAMG progression, a suppressive effect on MuSK-specific IgG antibody has also been observed. Thus, the titers of total MuSK-specific IgG in the sera of mice that were fed with MuSK, were lower than the titers in mice in the control OVA-fed mice (**Figure 4C**).

## The Effect of Oral Tolerance Induction on Cytokine Profile

In light of our previous results showing the alterations in the Treg sub-population in MuSK-immunized mice, we decided to evaluate the effect of oral tolerance treatment on Treg associated genes by RT-PCR from spleens of MuSK and OVA fed mice. As shown in **Figure 5**, mice that were orally treated by recombinant MuSK had significantly increased expression levels of Foxp3 and TGF-β, which are essential for Treg induction, activation and maintenance when compared to OVA-fed mice. A marked decrease in IL-18 expression was observed in MuSK-fed mice, as compared to OVA-fed mice. These results imply that oral treatment may have a protective effect on MuSK immunized mice perhaps by induction of tolerance rather than by anergy induction of T-cells.

# DISCUSSION

The present study was aimed to characterize immunological parameters of MuSK EAMG and to assess selective therapeutic strategy for MuSK myasthenia gravis.

We utilized a recombinant MuSK protein for immunization and showed that 100% of mice developed MuSK myasthenia gravis, which was synchronously developed within a month after immunization. MuSK immunized mice manifested human disease symptoms, including severe muscle weakness. This could relate to cathepsin-l up-regulation in the muscles of MuSK immunized mice, an endopeptidase that participates in pathological responses leading to muscle loss. Cathepsin-l was also shown to be up-regulated in the muscles of rats immunized with torpedo AChR (16). Additionally, MuSK immunized mice had specific anti-MuSK antibodies, which correlated with disease severity. This was in agreement with other reports that showed that serum anti-MuSK antibodies from patients correlated with clinical symptoms and response to immunotherapy. In a clinical study, the clinical score and disease classification correlated with anti-MuSK antibody distribution in 83 samples from 40 patients, and treatment with immunosuppressive agents resulted in a significant decrease in the MuSK IgG levels in individual patients (17).

The pro-inflammatory cytokine IL-18 was shown to be involved in the production of IFN-γ as well as the production of IL-12 that shifts the immune response toward a Th-1 phenotype. It was shown that IL-18 plays a role in the pathogenesis of many diseases. IL-18 knockout mice were resistant to EAMG induction (18). In our model, MuSK immunized mice manifested elevated expression levels of IL-18, indicating an inflammatory response upon MuSK immunization that may result in a dominant Th1 response.

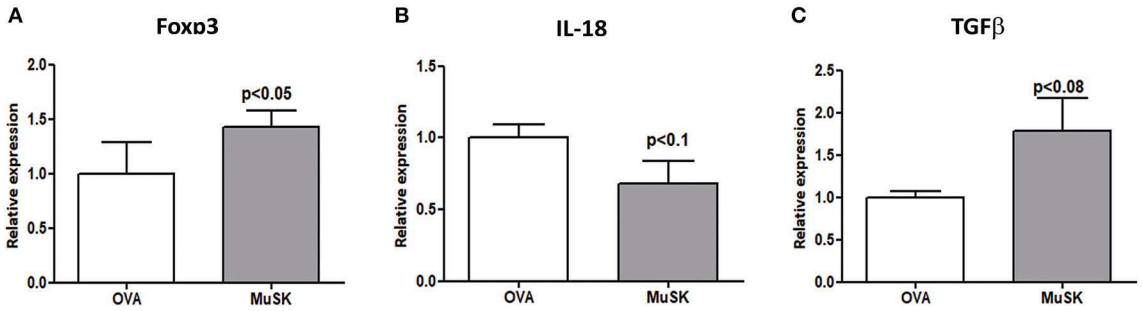



**FIGURE 5 |** MuSK-fed mice display increased levels of Tregs associated genes. Spleens were harvested at the end of the experiment and the RNA was isolated. Expression levels of Foxp3, IL-18, and TGF-β (**A–C**, respectively) were evaluated by RT-PCR. β-actin was used as an inner control for normalization. All data are presented as mean ± SEM ($n \geq 5$). Unpaired Student *t* test has been employed.

Homeostasis and self-tolerance is achieved mainly by immune regulatory T (Treg) cells. Dysfunction and/or altered Treg cell numbers can result in the development of autoimmune diseases (19). Animal models revealed that development of autoimmunity was due to defects in the CD4+CD25+FoxP3+ Treg cell population (20). Treg cells impairment is evident in several autoimmune diseases such as type 1 diabetes, multiple sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease (IBD), autoimmune hepatitis and psoriasis (21, 22). Although there is no consensus on decreased percentage of Treg cells in MG patients, including MuSK-MG, many studies report that these cells have reduced suppressive activity. (23, 24) but there is no evidence to their role in MuSK myasthenia gravis. Our results demonstrated dysregulation of $CD4^+CD25^+Foxp3^+$ Treg cells in MuSK-EAMG mice; the percentage of CD4+CD25+$Foxp3^+$ out of $CD4^+$ cells in the spleen of MuSK-EAMG mice is reduced compared to healthy adjuvant-immunized controls, as was also for Foxp3 mRNA expression.

Induction of oral tolerance involves many mechanisms that modulate the immune response against auto antigens. Oral tolerance has an impact on the numbers and function of Treg cells, on the secretion of pro and anti-inflammatory cytokines and on Th-1/Th-2 effector cells (25). This observation led to attempts to regulate many autoimmune diseases by induction of mucosal tolerance to auto antigens (10, 26–28). We have previously shown that EAMG was prevented in rats by oral or nasal administration of AChR-derived recombinant fragments when treatment started prior immunization, moreover, ongoing disease was suppressed when mucosal tolerance treatment started at the acute or chronic phase of the disease (11, 29). Mucosal tolerance induction resulted in a marked decrease in AChR specific T cell proliferative response and IL-2 production in addition to reduced levels of AChR auto-antibodies titers. The immune response shifted toward Th-2/Th-3 in addition to down-regulation of co-stimulatory factors. The underlying mechanism for the mucosal tolerance induced by the AChR fragments was shown to be active suppression and not clonal anergy.

Here we demonstrate for the first time an attempt to modulate MuSK myasthenia gravis by feeding mice with low doses of recombinant MuSK. The immune response acts differently to the dose of the antigen administered. High doses of antigens leads to anergy or apoptosis of antigen specific immune cells while administration of low doses of antigen leads to induction of antigen specific Tregs (30–32).

Administration of recombinant MuSK protein resulted in a significant therapeutic effect in treated mice, accompanied by a corresponding effect in weight loss. Additionally, the titer of total MuSK specific IgG in the serum at the end of the experiment was lower in MuSK fed mice, indicating amelioration of the disease. Moreover, expression level of Foxp3 and TGFβ were elevated in MuSK fed-mice suggesting that oral administration of MuSK modulated Tregs.

Taken together, our results demonstrated oral tolerance efficacy in MuSK-EAMG model. This therapeutic potential should be further explored and considered as a novel approach for MuSK-MG treatment and hopefully be effective and safe for the benefit of MuSK-MG patients.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Weizmann Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

DR, SF, and MS designed and guided the research. DR and RA performed the animal experiments and analyzed most of the experiments. DR and SF wrote the manuscript. All authors reviewed and approved the manuscript.

## FUNDING

This work was supported by grants from The Association Francaise Contre les Myopathies (AFM), The European Commission (FIGHT-MG, contract # FP7 HEALTH-2009-242-210) and The Chief Scientist Office, Israel Ministry of Health to MS and SF.

## ACKNOWLEDGMENTS

We wish to thank Drs, Anna Punga and Phillipo Oliveri for the generous gift of the pCEP-PU vector containing the His-tagged extracellular domain of recombinant rat MuSK (aa 21-491). This research that was inspired by our colleague and friend, Miriam C. Souroujon, is dedicated to her memory with love.

## REFERENCES

1. Evoli A, Lindstrom J. Myasthenia gravis with antibodies to MuSK: another step toward solving mystery? *Neurology*. (2011) 77:1783–4. doi: 10.1212/WNL.0b013e3182377fa6
2. Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, et al. Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron*. (1995) 15:573–84. doi: 10.1016/0896-6273(95)90146-9
3. Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, et al. Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell*. (2008) 135:334–42. doi: 10.1016/j.cell.2008.10.002
4. Wu H, Xiong WC, Mei L. To build a synapse: signaling pathways in neuromuscular junction assembly. *Development*. (2010) 137:1017–33. doi: 10.1242/dev.038711
5. Evoli A, Tonali PA, Padua L, Monaco ML, Scuderi F, Batocchi AP, et al. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. *Brain*. (2003) 126:2304–11. doi: 10.1093/brain/awg223
6. Sanders DB, B Tucker-Lipscomb, Massey JM. A simple manual muscle test for myasthenia gravis: validation and comparison with the QMG score. *Ann N Y Acad Sci*. (2003) 998:440–4. doi: 10.1196/annals.1254.057
7. Leite MI, Strobel P, Jones M, Micklem K, Moritz R, Gold R, et al. Fewer thymic changes in MuSK antibody-positive than in MuSK antibody-negative MG. *Ann Neurol*. (2005) 57:444–8. doi: 10.1002/ana.20386
8. Diaz-Manera J, Martinez-Hernandez E, Querol L, Klooster R, Rojas-Garcia R, Suarez- Calvet X, et al. Long-lasting treatment effect of rituximab in MuSK myasthenia. *Neurology*. (2012) 78:189–93. doi: 10.1212/WNL.0b013e3182407982
9. Weiner HL, van Rees EP. Mucosal tolerance. *Immunol Lett*. (1999) 69:3–4. doi: 10.1016/S0165-2478(99)00092-9
10. Mowat AM. To respond or not to respond - a personal perspective of intestinal tolerance. *Nat Rev Immunol*. (2018) 18:405–15. doi: 10.1038/s41577-018-0002-x
11. Im SH, Barchan D, Fuchs S, Souroujon MC. Suppression of ongoing experimental myasthenia by oral treatment with an acetylcholine receptor recombinant fragment. *J Clin Invest*. (1999) 104:1723–30. doi: 10.1172/JCI8121
12. Im SH, Barchan D, Fuchs S, Souroujon MC. Mechanism of nasal tolerance induced by a recombinant fragment of acetylcholine receptor for treatment of experimental myasthenia gravis. *J Neuroimmunol*. (2000) 111:161–8. doi: 10.1023/A:1003810030414
13. Drachman DB, Okumura S, Adams RN, McIntosh KR. Oral tolerance in myasthenia gravis. *Ann N Y Acad Sci*. (1996) 778:258–72. doi: 10.1111/j.1749-6632.1996.tb21134.x
14. Mori S, Kubo S, Akiyoshi T, Yamada S, Miyazaki T, Hotta H, et al. Antibodies against muscle-specific kinase impair both presynaptic and postsynaptic functions in a murine model of myasthenia gravis. *Am J Pathol*. (2012) 180:798–810. doi: 10.1016/j.ajpath.2011.10.031
15. Jones G, Moore C, Hashemolhosseini S, Brenner HR. Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. *J Neurosci*. (1999) 19:3376–83. doi: 10.1523/JNEUROSCI.19-09-03376.1999
16. Aricha R, Feferman T, Souroujon MC, Fuchs S. Overexpression of phosphodiesterases in experimental autoimmune myasthenia gravis: suppression of disease by a phosphodiesterase inhibitor. *FASEB J*. (2006) 20:374–6. doi: 10.1096/fj.05-4909fje
17. Meriggioli MN, Sanders DB. Muscle autoantibodies in myasthenia gravis: beyond diagnosis? *Expert Rev Clin Immunol*. (2012) 8:427–38. doi: 10.1586/eci.12.34
18. Shi FD, Wang HB, Li H, Hong S, Taniguchi M, Link H, et al. Natural killer cells determine the outcome of B cell-mediated autoimmunity. *Nat Immunol*. (2000) 1:245–51. doi: 10.1038/79792
19. Richards CD. Innate immune cytokines, fibroblast phenotypes, and regulation of extracellular matrix in lung. *J Interferon Cytokine Res*. (2017) 37:52–61. doi: 10.1089/jir.2016.0112
20. Sakaguchi S, Ono R, Setoguchi M, Yagi H, Hori S, Fehervari Z, et al. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. (2006) 212:8–27. doi: 10.1111/j.0105-2896.2006.00427.x
21. Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. *Nat Rev Immunol*. (2010) 10:849–59. doi: 10.1038/nri2889
22. Longhi MS, Ma Y, Mitry RR, Bogdanos DP, Heneghan M, Cheeseman P, et al. Effect of CD4+ CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. *J Autoimmun*. (2005) 25:63–71. doi: 10.1016/j.jaut.2005.05.001
23. Adolfo J, Van Wassenhove VJ, Le Panse R, Berrih-Aknin S, Dragin N. An imbalance between regulatory T cells and T helper 17 cells in acetylcholine receptor-positive myasthenia gravis patients. *Ann N Y Acad Sci*. (2018) 1413:154–62. doi: 10.1111/nyas.13591
24. Yi JS, Guidon A, Sparks S, Osborne R, Juel VC, Massey JM, et al. Characterization of CD4 and CD8 T cell responses in MuSK myasthenia gravis. *J Autoimmun*. (2014) 52:130–8. doi: 10.1016/j.jaut.2013.12.005
25. Pabst O, Mowat AM. Oral tolerance to food protein. *Mucosal Immunol*. (2012) 5:232–9. doi: 10.1038/mi.2012.4
26. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science*. (1994) 265:1237–40. doi: 10.1126/science.7520605
27. Kuhn C, Rezende RM, da Cunha AP, Valette F, Quintana FJ, Chatenoud L, et al. Mucosal administration of CD3-specific monoclonal antibody inhibits diabetes in NOD mice and in a preclinical mouse model transgenic for the CD3 epsilon chain. *J Autoimmun*. (2017) 76:115–22. doi: 10.1016/j.jaut.2016.10.001
28. Buerth C, Mausberg AK, Heininger MK, Hartung HP, Kieseier BC, Ernst JF. Oral tolerance induction in experimental autoimmune encephalomyelitis with candida utilis expressing the immunogenic MOG35-55 peptide. *PLoS ONE*. (2016) 11:e0155082. doi: 10.1371/journal.pone.0155082
29. Barchan D, Souroujon MC, Im SH, Antozzi C, Fuchs S. Antigen-specific modulation of experimental myasthenia gravis: nasal tolerization with recombinant fragments of the human acetylcholine receptor alpha-subunit. *Proc Natl Acad Sci USA*. (1999) 96:8086–91. doi: 10.1073/pnas.96.14.8086

30. Miller A, Lider O, Roberts AB, Sporn MB, Weiner HL. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci USA*. (1992) 89:421–5. doi: 10.1073/pnas.89.1.421
31. Melamed D, Friedman A. Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin. *Eur J Immunol.* (1993) 23:935–42. doi: 10.1002/eji.1830230426
32. Whitacre CC, Gienapp IE, Orosz CG, Bitar DM. Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. *J Immunol.* (1991) 147:2155–63.

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 16 April 2020
doi: 10.3389/fimmu.2020.00664

# Thymoma Associated Myasthenia Gravis (TAMG): Differential Expression of Functional Pathways in Relation to MG Status in Different Thymoma Histotypes

*Yosuke Yamada[1], Cleo-Aron Weis[1], Julian Thelen[1], Carsten Sticht[2], Berthold Schalke[3], Philipp Ströbel[4] and Alexander Marx[1]**

[1] Institute of Pathology, University Medical Centre Mannheim, Heidelberg University, Mannheim, Germany, [2] Medical Faculty Mannheim, Medical Research Center, Heidelberg University, Mannheim, Germany, [3] Department of Neurology, University of Regensburg, Regensburg, Germany, [4] Institute of Pathology, University Medical Center Göttingen, University of Göttingen, Göttingen, Germany

OPEN ACCESS

**Edited by:**
Sonia Berrih-Aknin,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

**Reviewed by:**
Yuriko Nagane,
Hanamaki General Hospital, Japan
Roberta Ricciardi,
University of Pisa, Italy

***Correspondence:**
Alexander Marx
alexander.marx@umm.de

**Specialty section:**
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

**Received:** 09 December 2019
**Accepted:** 23 March 2020
**Published:** 16 April 2020

**Citation:**
Yamada Y, Weis C-A, Thelen J, Sticht C, Schalke B, Ströbel P and Marx A (2020) Thymoma Associated Myasthenia Gravis (TAMG): Differential Expression of Functional Pathways in Relation to MG Status in Different Thymoma Histotypes. Front. Immunol. 11:664. doi: 10.3389/fimmu.2020.00664

A unique feature of thymomas is their unrivaled frequency of associated myasthenia gravis (MG). Previous studies reported that MG+ thymomas contain a larger number of mature "pre-emigrant" CD4+ T cells than MG- thymomas and that most thymomas do not contain AIRE expressing cells irrespective of MG status. These findings suggest that CD4+ T cells that mature inside the abnormal microenvironment of thymomas and egress to the blood are critical to the development of thymoma-associated MG (TAMG) irrespective of thymoma histotype. However, underlying mechanisms have remained enigmatic. To get hints to mechanisms underlying TAMG, we pursue three hypotheses: (i) Functional pathways with metabolic and immunological relevance might be differentially expressed in TAMG(+) compared to TAMG(-) thymomas; (ii) differentially enriched pathways might be more evident in immature lymphocyte-poor (i.e., tumor cell/stroma-rich) thymoma subgroups; and (iii) mechanisms leading to TAMG might be different among thymoma histological subtypes. To test these hypotheses, we compared the expression of functional pathways with potential immunological relevance ($N$ = 380) in relation to MG status separately in type AB and B2 thymomas and immature lymphocyte-rich and lymphocyte-poor subgroups of these thymoma types using the TCGA data set. We found that <10% of the investigated pathways were differentially upregulated or downregulated in MG+ compared to MG- thymomas with significant differences between AB and B2 thymomas. The differences were particularly evident, when epithelial cell/stroma-rich subsets of type AB and B2 thymomas were analyzed. Unexpectedly, some MG-associated pathways that were significantly upregulated in AB thymomas were significantly downregulated in B2 thymomas, as exemplified by the oxidative phosphorylation pathway. Conversely, the MG-associated pathway related to macrophage polarization was downregulated in MG+ AB thymoma and upregulated in MG+ B2 thymoma. We conclude that functional pathways are significantly associated with TAMG, and that some mechanisms leading to TAMG might be different

among thymoma histological subtypes. Functions related to metabolisms, vascular and macrophage biology are promising new candidate mechanisms potentially involved in the pathogenesis of TAMG. More generally, the results imply that future studies addressing pathomechanisms of TAMG should take the histotype and abundance of tumor cells and non-neoplastic stromal components of thymomas into account.

**Keywords: thymoma, myasthenia gravis, autoimmunity, the cancer genome atlas (TCGA), functional pathways, metabolism, macrophage polarization**

## INTRODUCTION

Thymomas are tumors that appear to be derived from or show differentiation toward thymic epithelial cells, with a resemblance to the normal thymic histological architecture, such as discrete lobulation, perivascular spaces, and admixed immature T cells. Thymomas are classified into several subtypes according to the morphology of the tumor cells and the proportion of associated immature T cells (i.e., type A, AB, B1, B2, B3, and other rare subtypes) (1).

One of the unique features of thymomas is their frequent association with autoimmune diseases, especially myasthenia gravis (MG). As comprehensively described in other articles in this issue, MG is characterized by autoantibodies against components of the neuromuscular junction and divided into several subgroups based on clinical features and the causative antibody. The subgroup that is associated with thymoma and almost consistently with anti-acetylcholine receptor (AChR) antibodies, is termed thymoma-associated MG (TAMG) (2).

Taking into account that most thymoma subtypes contain immature T cells and are likely involved in the "education" of such T cells like the normal thymus, it has been hypothesized that the "non-tolerogenic" microenvironment in thymomas plays a key role in the pathogenesis of TAMG. Indeed, MG+ thymomas of all major histotypes (except for type A thymomas) contain significantly more mature "pre-emigrant" CD4+ T cells than MG- thymomas (3). Besides, the polymorphism of the non-MHC gene, *CTLA4* that affects T cell receptor signaling appears to correlate with TAMG (4). On the other hand and again across all major histotypes, almost all thymomas show a reduced intratumoral generation of regulatory T cells (Tregs) (5), attenuated MHC class II expression (6), and deficient expression of the autoimmune regulator, AIRE irrespective of MG status (7). Together, these findings suggest that CD4+ effector T cells that mature inside the abnormal microenvironment of thymomas and egress from them to the blood are critical to the development TAMG in thymopoietically active thymomas. Also, a recent comprehensive analysis of thymic epithelial tumors conducted as TCGA (The Cancer Genome Atlas) project has reported meaningful findings associated with TAMG, such as the higher prevalence of aneuploidy and overexpression of genes with sequence similarity with *CHRNA1*, *TTN,* and *RYR1/RYR2* (8), all of which code for skeletal muscle antigens that are key autoantibody targets in TAMG, i.e., the α-subunit of the AChR, titin and ryanodine receptors, respectively (2). In spite of this progress, the underlying mechanisms leading to the above mentioned common features of MG-associated thymomas have remained largely enigmatic. Moreover, despite the molecular and morphological diversity among thymoma histotypes (8), the hypothesis has not been thoroughly addressed that the underlying mechanisms leading to TAMG might have histotype-specific facets. To test this, we re-analyzed the aforementioned TCGA data sets of thymomas (8) after stratification for thymoma histotype. Since the TCGA study did not reveal TAMG-associated "immune signatures" across the whole thymoma cohort (8), we here focused on histotype-specific enrichments of immunologically relevant pathways in association to TAMG.

## MATERIALS AND METHODS

### Access to the TCGA Thymoma Data Set

We analyzed the TCGA data set, "Thymoma, PanCancer Atlas," through the CBioPortal database (http://www.cbioportal.org/), following the final diagnoses submitted by Radovich et al. (8).

### Selection and Stratification of Thymomas in the TCGA Thymoma Data Set

To simplify the analysis of this highly heterogeneous thymoma cohort (8), we focused our stratification on the two most prevalent thymoma subtypes, type AB ($N = 47$) and B2 ($N = 25$) thymomas. This choice was also motivated by the fact that among the thymoma subtypes that are often accompanied by MG [i.e., AB, B1, B2, and B3 thymomas (1)], the differences between AB and B2 thymomas in terms of epithelial morphology, genotype, and gene expression signatures are highly significant, while the abundance of intratumorous, non-neoplastic immature T cells *on average* is comparable (8).

Still, the content of non-neoplastic, immature T cells can be quite variable among type AB thymomas as well as B2 thymomas and this variability may potentially obscure differences between the neoplastic epithelial cells of MG+ and MG- thymomas. Therefore, we divided each of the cohorts of type AB and B2 thymomas further into an immature T lymphocytes-high and immature T lymphocyte-low subgroup based on the mRNA expression levels of TdT (terminal deoxynucleotidyl transferase), i.e., a *bona fide* marker gene of immature T lymphocytes in the thymus. To this end, we first calculated the mean of the normalized counts (∼9,743) from all thymoma samples and then chose 10,000 as the cutoff for low (<10k) and high (>10k) subgroups. Among type AB thymomas, this strategy resulted in a TdT-high subgroup that contained 4 MG+ and 21 MG- cases, and a TdT-low subgroup that contained 4 MG+ and 18 MG- cases. In type B2 thymomas, the TdT-high subgroup contained

8 MG+ and 4 MG- cases, and the TdT-low subgroup contained 6 MG+ and 7 MG- cases (**Figure S1**).

## Differential Expression Analysis

An ANOVA was performed to identify differential expressed genes using a commercial software package SAS JMP11 Genomics, version 7, from SAS (SAS Institute, Cary, NC, USA). A false positive rate of a = 0.05 with FDR correction was taken as the level of significance. For the comparison of the gene expression levels of NEFL, NEFM, CHRNA1, RYR3, SLCO1A2, and PRAME between MG+ and MG- groups (**Figure S2**), we used the Wilcoxon test with JMP14 (SAS, Cary, North Carolina, USA). Differences at $P < 0.05$ were considered to be significant.

## Analysis of Functional Pathways

Based on the differentially expressed genes between the various MG+ and MG- subgroups of the cohorts of type AB and B2 thymomas, significantly upregulated or downregulated pathways in each MG+ subgroup were extracted, using the KEGG database (https://www.genome.jp/kegg/kegg_ja.html). Because of the particular immunological perspective of our analysis, the differentially expressed genes were also mapped on the extensive collection of inflammation-related pathways that was described by Shen and coworkers (9) after the publication of the TCGA thymoma paper (8). Then, Gene Set Enrichment Analysis (GSEA) was used to determine whether defined lists (or sets) of genes exhibit a statistically significant bias in their distribution within a ranked gene list using the *R* software-packages EnrichmentBrowser (10). The genes were ranked due to their *t*-value based on the comparison between MG+ vs. MG- subgroup. The study was performed under the approval of the Medical Ethics Committee II, Medical Faculty Mannheim, Heidelberg University.

# RESULTS

## Differentially Upregulated Functional Pathways in MG+ Compared to MG- Thymomas

According to the stratification of the thymoma cohorts described in Materials and Methods, the following MG+ and MG- thymoma subgroups were compared in terms of gene expression followed by the extraction of functional pathways: (1) all type AB thymomas, (2) all type B2 thymomas, (3) TdT-low type AB thymomas, (4) TdT-high type AB thymomas, (5) TdT-low type B2 thymomas, and (6) TdT-high type B2 thymomas. In both type AB and B2 thymomas, the number of genes that were differentially expressed between MG+ and MG- cases was higher in each of the TdT-low and TdT-high subgroups (subgroups 3–6) than in the non-stratified, i.e., total cohorts of AB and B2 thymomas (subgroups 1 and 2). The differences between MG+ and MG- cases were particularly obvious in both TdT-low subsets (**Figure 1**). The pathways that were significantly upregulated or downregulated (in the same manner) in both TdT-low and TdT-high subgroups are shown in **Table 1**. Then, we evaluated whether these pathways were also shared between the AB and B2 thymoma cohorts as shown next.

## Type AB and B2 Thymomas Upregulate Different Functional KEGG Pathways in Relation to their MG Status

When focusing on functional pathways derived from the KEGG database ($N = 310$), 19 functional pathways (6%) showed an MG association in AB thymomas: ten pathways, such as those related to Oxidative phosphorylation, Parkinson disease, and Alzheimer disease, were significantly upregulated, while nine pathways, such as those related to Adherens junction, AGE-RAGE signaling, and TGF-beta signaling, were significantly downregulated in MG+ compared to MG- type AB thymomas. In type B2 thymoma, only eight functional pathways showed an MG association: the pathway related to Olfactory transduction was significantly upregulated in MG+ cases, while seven pathways, such as Protein processing, Metabolism, and DNA replication, were downregulated in MG+ B2 thymomas (**Table 1**). The respective upregulated and downregulated pathways were not overlapping between type AB and B2 thymomas.

## Type AB and B2 Thymomas Are Differentially Enriched in Inflammation Related Pathways

Taking into account that inflammatory features of the thymoma microenvironment could be involve in the pathogenesis of TAMG, we next focused on pathways ($N = 70$) that are related to inflammation, including features of tumor-infiltrating immune cells and cytokines (9). We identified three pathways, TNF-alpha signaling, MacTh1 cluster, and Chemokine signaling that were significantly downregulated in MG+ type AB thymoma. No pathways were significantly upregulated in MG+ type AB thymoma. In contrast, five pathways, such as those related to T cell cluster, MacTh1 cluster, and LCK median, were significantly upregulated in MG+ type B2 thymoma (**Table 2**).

## Opposite Enrichment Status of Identical Functional Pathways in MG+ Type AB and B2 Thymomas

Surprisingly, some of the functional pathways that were significantly upregulated in MG+ type AB thymoma were significantly downregulated in MG+ type B2 thymoma: Among the pathways derived from the KEGG dataset, this pattern concerned the pathways of oxidative phosphorylation, Parkinson disease, and Huntington disease (**Figure 2** and **Table 1**). Vice versa, the Olfactory transduction pathway that was significantly upregulated in MG+ type B2 thymomas, was downregulated in MG+ type AB thymomas, although the difference was significant only in the TdT-low subset (and with a minor trend in the TdT-high subset) (**Figure 2** and **Table S1**). Among the "immune pathways," the "MacTH1 cluster" also followed this pattern (**Figure 2**, **Table 2**).

# DISCUSSION

When addressing the question, why some thymomas are accompanied by MG, while others are not, most previous studies have not considered possible pathogenetic differences

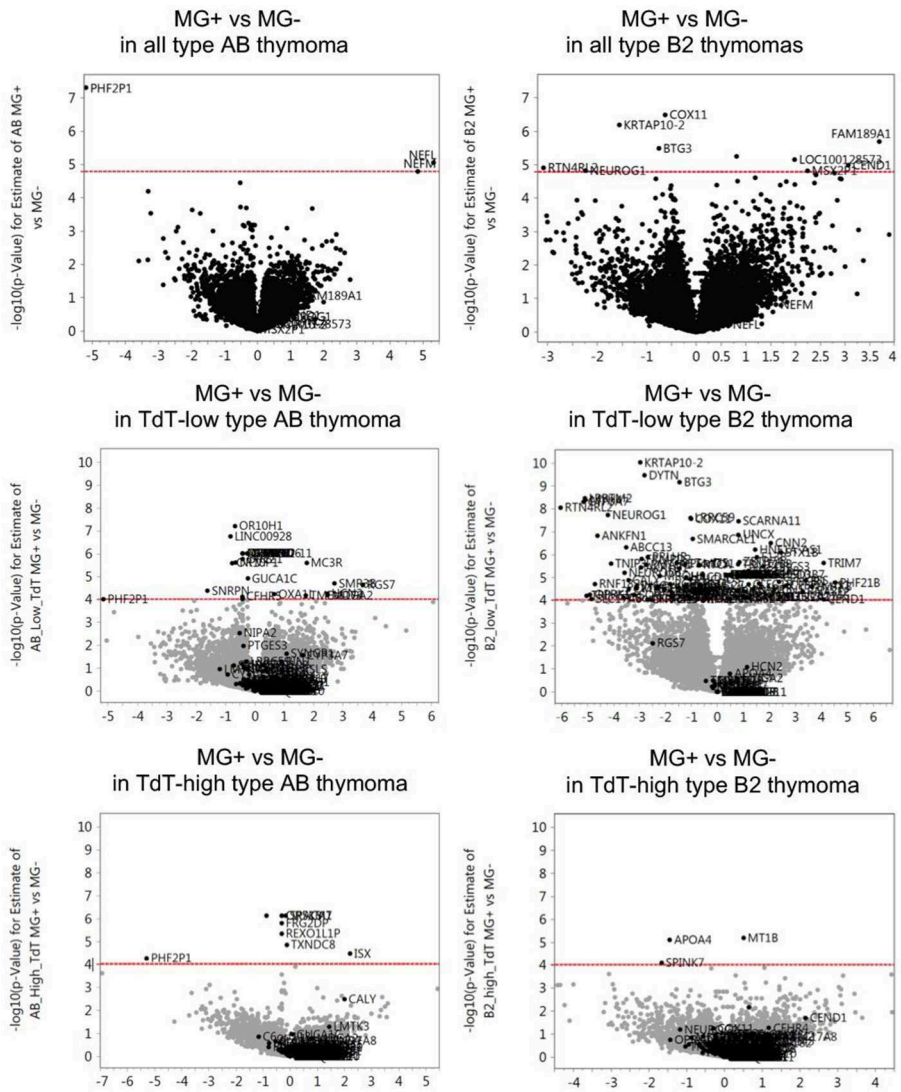



**FIGURE 1 |** Differentially expressed genes between MG+ and MG- thymomas in type AB and B2 thymomas. For each gene the -log10 (*p*-value) for the difference of gene expression levels between MG+ and MG- groups (vertical axis) is plotted against its log2(fold change) relative expression level of the MG+ group compared to the MG- group (horizontal axis). The dashed red line represents the statistical significance threshold ($P \leq 0.05$ after adjustment with False Discovery Rate). In both type AB and B2 thymomas, the number of genes that were differentially expressed between MG+ and MG- cases was higher in each of the TdT (terminal deoxynucleotidyl transferase, a *bona fide* marker gene of immature T lymphocytes)-low and TdT-high subgroups than in the non-stratified, i.e., total cohorts of AB and B2 thymomas. The differences between MG+ and MG- cases were particularly obvious in both TdT-low subsets.

between the various thymoma histological subtypes (3, 11)—with rare exceptions (12, 13). Here, we have addressed this question separately in type AB and B2 thymomas using the comprehensive and highly reliable TCGA data sets (8). We found that upregulated or downregulated pathways associated with MG were not only barely overlapping between the two subtypes, but for some pathways showed an oppositely enrichment status, i.e., MG-associated pathways that were upregulated in one histotype where downregulated in the other, and vice versa. These observations were more evident in the subgroups of type AB and B2 thymomas, which were poor in non-neoplastic immature, TdT+ T cells. Together, these findings suggest that the mechanisms underlying TAMG might be different in the various thymoma histotypes and mainly operative in neoplastic epithelial cells and/or non-neoplastic mature stromal cells, but not the quantitatively often overwhelming population of immature T cells.

The current study that is based on RNA expression profiles, does not allow to clarify, how the various differentially expressed genes and functional pathways identified here might contribute to TAMG in the two major thymoma histotypes, type AB and B2 thymomas. To truly understand and eventually prove the relevance of the identified pathways for the pathomechanisms leading to TAMG, detailed in situ analyses, the investigation

**TABLE 1 |** Upregulated or downregulated KEGG pathways in myasthenia gravis (MG).

| Name | Main_Category | Sub_Category | NES | P-value |
|---|---|---|---|---|
| **Upregulated pathways in MG(+) cases in type AB thymoma** | | | | |
| ***hsa00190_Oxidative_phosphorylation*** | 1. Metabolism | 1.2. Energy metabolism | 2,57 | 0,0048 |
| ***hsa05012_Parkinson_disease*** | 6. Human Diseases | 6.4. Neurodegenerative diseases | 2,45 | 0,0048 |
| ***hsa05010_Alzheimer_disease*** | 6. Human Diseases | 6.4. Neurodegenerative diseases | 1,985 | 0,0048 |
| hsa05016_Huntington_disease | 6. Human Diseases | 6.4. Neurodegenerative diseases | 1,985 | 0,0048 |
| hsa04932_Non-alcoholic_fatty_liver_disease_(NAFLD) | 6. Human Diseases | 6.7. Endocrine and metabolic diseases | 1,875 | 0,0048 |
| hsa04714_Thermogenesis | 5. Organismal Systems | 5.10. Environmental adaptation | 2,02 | 0,0050 |
| hsa03050_Proteasome | 2. Genetic Information Processing | 2.3. Folding, sorting and degradation | 2,03 | 0,0124 |
| hsa04723_Retrograde_endocannabinoid_signaling | 5. Organismal Systems | 5.6. Nervous system | 1,51 | 0,0221 |
| hsa03010_Ribosome | 2. Genetic Information Processing | 2.2. Translation | 2,47 | 0,0247 |
| hsa04260_Cardiac_muscle_contraction | 5. Organismal Systems | 5.3. Circulatory system | 1,7 | 0,0267 |
| **Downregulated pathways in MG(+) cases in type AB thymoma** | | | | |
| hsa04520_Adherens_junction | 4. Cellular Processes | 4.3. Cellular community - eukaryotes | −1,905 | 0,0048 |
| hsa04933_AGE-RAGE_signaling_pathway_in_diabetic_complications | 6. Human Diseases | 6.7. Endocrine and metabolic diseases | −1,84 | 0,0061 |
| hsa04350_TGF-beta_signaling_pathway | 3. Environmental Information Processing | 3.2. Signal transduction | −1,745 | 0,0065 |
| hsa05205_Proteoglycans_in_cancer | 6. Human Diseases | 6.1. Cancers: Overview | −1,65 | 0,0149 |
| hsa05140_Leishmaniasis | 6. Human Diseases | 6.10. Infectious diseases: Parasitic | −1,695 | 0,0178 |
| hsa05206_MicroRNAs_in_cancer | 6. Human Diseases | 6.1. Cancers: Overview | −1,58 | 0,0226 |
| hsa05145_Toxoplasmosis | 6. Human Diseases | 6.10. Infectious diseases: Parasitic | −1,66 | 0,0248 |
| hsa04062_Chemokine_signaling_pathway | 5. Organismal Systems | 5.1. Immune system | −1,485 | 0,0251 |
| hsa05200_Pathways_in_cancer | 6. Human Diseases | 6.1. Cancers: Overview | −1,445 | 0,0254 |
| **Upregulated pathways in MG(+) cases in type B2 thymoma** | | | | |
| hsa04740_Olfactory_transduction | 5. Organismal Systems | 5.7. Sensory system | 1,92 | 0,0130 |
| **Downregulated pathways in MG(+) cases in type B2 thymoma** | | | | |
| hsa04141_Protein_processing_in_endoplasmic_reticulum | 2. Genetic Information Processing | 2.3. Folding, sorting and degradation | −1,88 | 0,0119 |
| hsa01100_Metabolic_pathways | 1. Metabolism | 1.0 Global and overview maps | −1,36 | 0,0126 |
| hsa03030_DNA_replication | 2. Genetic Information Processing | 2.4. Replication and repair | −2,07 | 0,0137 |
| ***hsa00190_Oxidative_phosphorylation*** | 1. Metabolism | 1.2. Energy metabolism | −1,725 | 0,0148 |
| ***hsa05012_Parkinson_disease*** | 6. Human Diseases | 6.4. Neurodegenerative diseases | −1,66 | 0,0204 |
| ***hsa05016_Huntington_disease*** | 6. Human Diseases | 6.4. Neurodegenerative diseases | −1,56 | 0,0218 |
| hsa00240_Pyrimidine_metabolism | 1. Metabolism | 1.4. Nucleotide metabolism | −1,645 | 0,0246 |

*NES, normalized enrichment score; P-value, adjusted P-value; Both NES and P-value are mean score (or value) between that of TdT-low and -high groups.*

of isolated cell types sorted from fresh thymoma resection specimens and functional studies using *in vitro* or *in vivo* model systems would be necessary. Nevertheless, in face of the fact that TAMG is a neuromuscular disease, it is interesting that some of the identified TAMG-associated KEGG pathways are related to neurodegenerative diseases (Parkinson disease, Alzheimer disease, and Huntington disease). In line with this finding, it has been known for long that expression of neurofilaments in thymomas is associated with TAMG (8, 14), and we show here that this association is strongest in type AB thymomas (**Figure S2**). Furthermore, expression of the brain-type ryanodine receptor, *RYR3*, in thymomas has been shown to be associated with TAMG (8), but in this case we now find that the association is strongest in type B2 thymomas (**Figure S2**). Of note, pathways that play a role in the above mentioned neurodegenerative diseases, have been found enriched in a variety of immunobiological settings, including chronic infections, graft-vs.-host disease, cancer biology, cell death, and inflammation (15–17). Likewise, the "Adherens junction" pathway that was the most significantly downregulated TAMG-associated pathway in type AB thymomas (**Table 1**) has been linked to thymic hypoplasia and lymphopenia (18) and to various autoimmune diseases in conjunction with the leakiness of several blood-tissue and inter-epithelial barriers (19, 20). Considering the quite specific pathology of tumor vessels in the different thymoma subtypes (21), in depth analysis of the tumor vasculature in relation TAMG appears warranted.

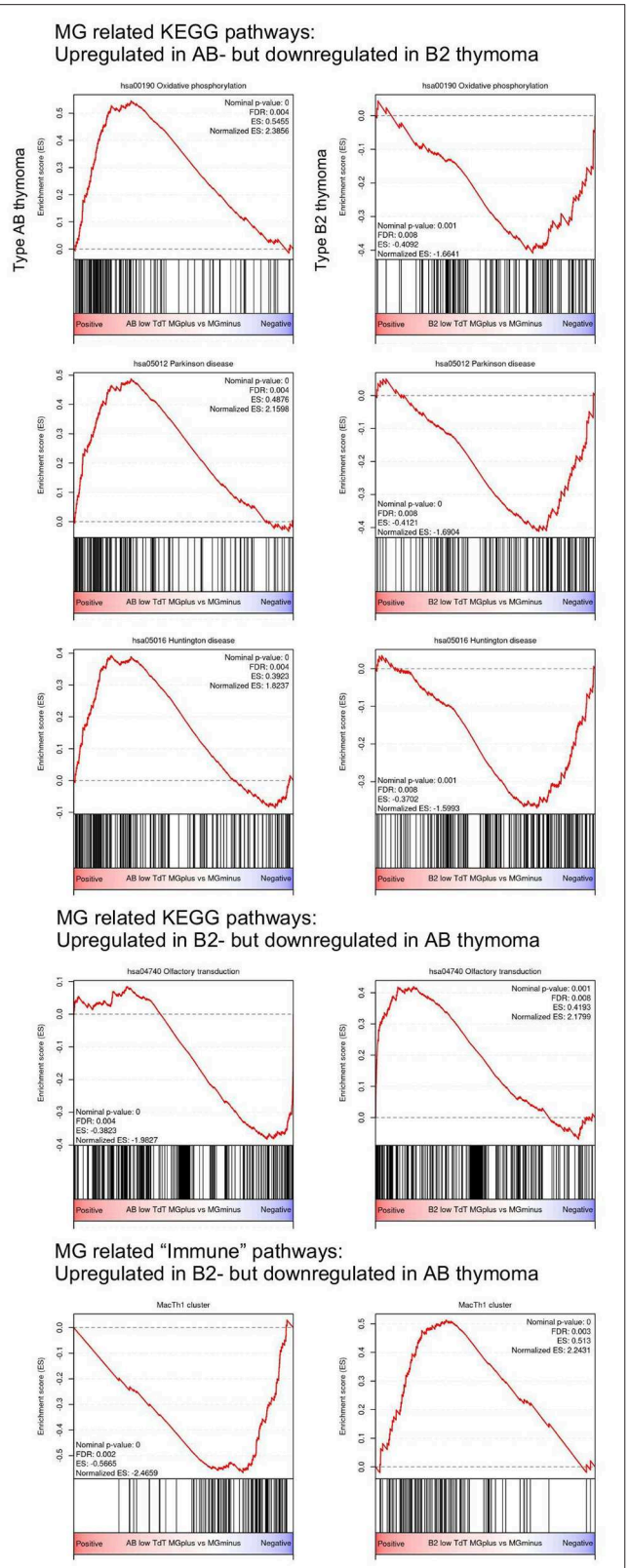



**FIGURE 3 |** Opposite upregulation/downregulation of MG-associated pathways in type AB and B2 thymomas based on Gene Set Enrichment Analysis (GSEA). Among the KEGG pathway, pathways related to "Oxidative phosphorylation," "Parkinson disease," and "Huntington disease" are significantly upregulated in MG+ type AB thymoma, but downregulated in MG+ B2 thymoma. On the other hand, the pathway related to "Olfactory transduction" is significantly downregulated in MG+ type AB thymoma, but upregulated in MG+ B2 thymoma. Among the "immune pathways" provided by Shen et al. (9), the "MacTh1 cluster" is significantly downregulated in type AB thymoma, but upregulated in type B2 thymoma.

*(Continued)*

**TABLE 2 |** Upregulated or downregulated "immune" pathways in myasthenia gravis (MG).

| Name | NES | *P*-value |
|---|---|---|
| **Suppressed pathways in MG(+) cases in type AB thymoma** | | |
| HALLMARK_TNFA_SIGNALING_VIA_NFKB | −2.09 | 0.0015 |
| ***MacTh1_cluster*** | −2.12 | 0.0020 |
| KEGG_CHEMOKINE_SIGNALING_PATHWAY | −1.535 | 0.0070 |
| **Activated pathways in MG(+) cases in type B2 thymoma** | | |
| T_Cell_cluster_Iglesia | 2.315 | 0.0031 |
| ***MacTh1_cluster*** | 2.24 | 0.0031 |
| LCK_Median | 2.07 | 0.0031 |
| UNC_MCD3_CD8 | 2.015 | 0.0050 |
| CD8_cluster | 2.145 | 0.0054 |

*NES, normalized enrichment score; P-value, adjustd P-value.*
*Both NES and P-value are mean score (or value) between that of TdT-low and -high groups.*

Other pathways that have not been linked previously to TAMG to the best of our knowledge are related to metabolism: Oxdative phosphorylation, Protein processing, Metabolic pathways, DNA replication, and Pyrimidine metabolism. Although the mechanisms that link these pathways to TAMG remain enigmatic, it is noteworthy that "Metabolic pathways" and the above mentioned "Alzheimer pathway" are significantly enriched KEGG pathways in Lupus nephritis (22), i.e., in an autoimmmue disease that is often associated with thymomas, though not as commonly as TAMG (23). Similarly, it is unclear how the upregulated "Olfactory transduction" pathway might be linked to the pathogenesis of TAMG in B2 thymomas, but it is interesting that this pathway has been found to be associated with rheumatoid arthritis (24), i.e., another autoimmune disease that occurs in thymoma patients (23).

Completely unexpected was the new finding that among the few identified MG associated pathways (<10% of more than 350 investigated pathways) there was a small subset of 4 pathways that were shared by AB and B2 thymomas, but with diametrically opposed enrichment status in the two tumor types. The detection of these shared but "counter-enriched" pathways and the lack of shared MG-associated "concordantly enriched" pathways among the two thymoma subtypes, lend support to the rational of our "stratification strategy," to increase the sensitivity of our search for MG-associated pathways by focusing on histologically homogeneous thymoma subtypes. Apart from the above pathways related to neurodegeneration (Parkinson and Huntington disease) and metabolism (Oxdative

phosphorylation) (bold/italics in **Table 1**), one of the eight identified MG-associated inflammatory pathways (9) showed the "counter-enrichment" pattern as well: The MacTh1 cluster-associated gene set was significantly upregulated in TAMG-associated B2 thymomas and downregulated in AB thymomas (bold/italics in **Table 2**). Imbalanced macrophage polarization is well-known to play an important role in T cell- and autoantibody-mediated autoimmune and allergic diseases (25–28), and can affect T cell apoptosis (29), i.e., a key feature of normal thymic tolerance induction and abnormal thymopoiesis inside TAMG-associated thymomas (30). Furthermore, analyses of MHC class II expression levels and the step-wise maturation of thymocytes inside different thymoma subtypes already gave strong hints that the mechanisms shaping the autoimmune CD4+ T cell repertoire are different in AB and B2 thymomas (31). Accordingly, it is not a priori unreasonable to hypothesize that identical pathways but with opposite enrichment status (e.g., differentially polarized macrophages) could contribute to the same, TAMG-prone phenotype in histologically different thymomas, namely generation of autoreactive CD4+ T cells in very different thymoma microenvironments. Therefore, we deem the MacTh1 cluster-associated gene set a highly promising and potentially informative candidate pathway that warrants in depth comparative analysis in AB and B2 thymomas to elucidate pathogenetic mechanisms leading to TAMG.

In summary, we have identified functional pathways with a significant association with TAMG and, thus, a potential role in its pathogenesis. The identified pathways appear to be mainly operative in cells other than the numerous, thymoma-associated immature T cells, are virtually non-overlapping between type AB and B2 thymomas, and the few shared pathways show diametrically opposite enrichments. These findings parallel the diverse morphology, genetics and global gene expression profiles of AB and B2 thymomas (8, 32). Most previously identified, TAMG-associated genes coded for highly TAMG-specific proteins, such as the acetylcholine receptor itself, or proteins sharing epitopes with TAMG-associated autoantibody targets, such as titin and the ryanodine receptors (8). By contrast, the TAMG-associated pathways detected here appear mostly non-specific, since they are apparently relevant in a variety of other autoimmune diseases (see above). Nevertheless, these pathways appear as promising candidates for future analysis to fill the wide gap of knowledge between the largely enigmatic microenvironments of the various thymoma histotypes and the stereotypic, TAMG-eliciting egress of autoreactive T cells from thymopoietically active, histologically diverse thymomas (31).

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The study was performed under the approval of the Medical Ethics Committee II, Medical Faculty Mannheim, Heidelberg University (approval # 2015-541N-MA).

## AUTHOR CONTRIBUTIONS

YY and AM designed the study and wrote the manuscript. CS performed the bioinformatic and statistical analyses. YY, CS, and AM analyzed the data. C-AW, JT, BS, and PS helped with the interpretation of the data under a tumor-biological and neurological perspective. All the authors contributed to the editing of the manuscript, and read and approved the final version before submission.

## FUNDING

This work was supported by Department of Diagnostic Pathology, Kyoto University Hospital through a visiting grant to YY.

## ACKNOWLEDGMENTS

Cancer research in the Marx lab is supported by the German Federal Ministry of Education and Research (BMBF).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00664/full#supplementary-material

## REFERENCES

1. Travis WD, Brambilla E, Burke AP, Marx A, Nicholson AG. *WHO Classification of Tumours of Lung, Pleura, Thymus and Heart*. Lyon: IARC. (2015).
2. Gilhus NE. Myasthenia Gravis. *N Engl J Med.* (2016) 375:2570–81. doi: 10.1056/NEJMra1602678
3. Ströbel P, Helmreich M, Menioudakis G, Lewin SR, Rüdiger T, Bauer A, et al. Paraneoplastic myasthenia gravis correlates with generation of mature naive CD4(+) T cells in thymomas. *Blood.* (2002) 100:159–66. doi: 10.1182/blood.v100.1.159
4. Chuang WY, Ströbel P, Gold R, Nix W, Schalke B, Kiefer R, et al. A CTLA4high genotype is associated with myasthenia gravis in thymoma patients. *Ann Neurol.* (2005) 58:644–8. doi: 10.1002/ana.20577
5. Ströbel P, Rosenwald A, Beyersdorf N, Kerkau T, Elert O, Murumägi A, et al. Selective loss of regulatory T cells in thymomas. *Ann Neurol.* (2004) 56:901–4. doi: 10.1002/ana.20340
6. Savino W, Manganella G, Verley JM, Wolff A, Berrih S, Levasseur P, et al. Thymoma epithelial cells secrete thymic hormone but do not express class II antigens of the major histocompatibility complex. *J Clin Invest.* (1985) 76:1140–6. doi: 10.1172/JCI112069
7. Ströbel P, Murumägi A, Klein R, Luster M, Lahti M, Krohn K, et al. Deficiency of the autoimmune regulator AIRE in thymomas is insufficient to elicit autoimmune polyendocrinopathy syndrome type 1 (APS-1). *J Pathol.* (2007) 211:563–71. doi: 10.1002/path.2141
8. Radovich M, Pickering CR, Felau I, Ha G, Zhang H, Jo H, et al. The integrated genomic landscape of thymic epithelial tumors. *Cancer Cell.* (2018) 33:244–58.e10. doi: 10.1016/j.ccell.2018.01.003

9. Shen H, Shih J, Hollern DP, Wang L, Bowlby R, Tickoo SK, et al. Integrated molecular characterization of testicular germ cell tumors. *Cell Rep.* (2018) 23:3392–406. doi: 10.1016/j.celrep.2018.05.039
10. Geistlinger L, Csaba G, Zimmer R. Bioconductor's enrichment browser: seamless navigation through combined results of set- & network-based enrichment analysis. *BMC Bioinform.* (2016) 17:45. doi: 10.1186/s12859-016-0884-1
11. Marx A, Porubsky S, Belharazem D, Saruhan-Direskeneli G, Schalke B, Ströbel P, et al. Thymoma related myasthenia gravis in humans and potential animal models. *Exp Neurol.* (2015) 270:55–65. doi: 10.1016/j.expneurol.2015.02.010
12. Wilisch A, Gutsche S, Hoffacker V, Schultz A, Tzartos S, Nix W, et al. Association of acetylcholine receptor alpha-subunit gene expression in mixed thymoma with myasthenia gravis. *Neurology.* (1999) 52:1460–6. doi: 10.1212/wnl.52.7.1460
13. Maclennan CA, Vincent A, Marx A, Willcox N, Gilhus NE, Newsom-Davis J, et al. Preferential expression of AChR epsilon-subunit in thymomas from patients with myasthenia gravis. *J Neuroimmunol.* (2008) 201-202:28–32. doi: 10.1016/j.jneuroim.2008.06.016
14. Schultz A, Hoffacker V, Wilisch A, Nix W, Gold R, Schalke B, et al. Neurofilament is an autoantigenic determinant in myasthenia gravis. *Ann Neurol.* (1999) 46:167–75. doi: 10.1002/1531-8249(199908)46:2<167::aid-ana5>3.0.co;2-3
15. Pillai JA, Maxwell S, Bena J, Bekris LM, Rao SM, Chance M, et al. Key inflammatory pathway activations in the MCI stage of Alzheimer's disease. *Ann Clin Transl Neurol.* (2019) 6:1248–62. doi: 10.1002/acn3.50827
16. Wang YG, Zheng DH, Shi M, Xu XM. T cell dysfunction in chronic hepatitis B infection and liver cancer: evidence from transcriptome analysis. *J Med Genet.* (2019) 56:22–8. doi: 10.1136/jmedgenet-2018-105570
17. Li P, Nie Y, Yu J. An effective method to identify shared pathways and common factors among neurodegenerative diseases. *PLoS ONE.* (2015) 10:e0143045. doi: 10.1371/journal.pone.0143045
18. Bernstock JD, Totten AH, Elkahloun AG, Johnson KR, Hurst AC, Goldman F, et al. Recurrent microdeletions at chromosome 2p11.2 are associated with thymic hypoplasia and features resembling DiGeorge syndrome. *J Allergy Clin Immunol.* (2019) 145:358–367.e2. doi: 10.1016/j.jaci.2019.09.020
19. Maes M, Sirivichayakul S, Kanchanatawan B, Vodjani A. Breakdown of the paracellular tight and adherens junctions in the gut and blood brain barrier and damage to the vascular barrier in patients with deficit schizophrenia. *Neurotox Res.* (2019) 36:306–22. doi: 10.1007/s12640-019-00054-6
20. Zhao M, Liu S, Luo S, Wu H, Tang M, Cheng W, et al. DNA methylation and mRNA and microRNA expression of SLE CD4+ T cells correlate with disease phenotype. *J Autoimmun.* (2014) 54:127–36. doi: 10.1016/j.jaut.2014.07.002
21. Pfister F, Hussain H, Belharazem D, Busch S, Simon-Keller K, Becker D, et al. Vascular architecture as a diagnostic marker for differentiation of World Health Organization thymoma subtypes and thymic carcinoma. *Histopathology.* (2017) 70:693–703. doi: 10.1111/his.13114
22. Luan S, Li P, Yi T. Series test of cluster and network analysis for lupus nephritis, before and after IFN-K-immunosuppressive therapy. *Nephrology (Carlton).* (2018) 23:997–1006. doi: 10.1111/nep.13159
23. Marx A, Willcox N, Leite MI, Chuang WY, Schalke B, Nix W, et al. Thymoma and paraneoplastic myasthenia gravis. *Autoimmunity.* (2010) 43:413–27. doi: 10.3109/08916930903555935
24. Li Y, Lai-Han Leung E, Pan H, Yao X, Huang Q, Wu M, et al. Identification of potential genetic causal variants for rheumatoid arthritis by whole-exome sequencing. *Oncotarget.* (2017) 8:111119–29. doi: 10.18632/oncotarget.22630
25. Ma C, Xia Y, Yang Q, Zhao Y. The contribution of macrophages to systemic lupus erythematosus. *Clin Immunol.* (2019) 207:1–9. doi: 10.1016/j.clim.2019.06.009
26. Veremeyko T, Yung AWY, Dukhinova M, Kuznetsova IS, Pomytkin I, Lyundup A, et al. Cyclic AMP pathway suppress autoimmune neuroinflammation by inhibiting functions of encephalitogenic CD4 T cells and enhancing M2 macrophage polarization at the site of inflammation. *Front Immunol.* (2018) 9:50. doi: 10.3389/fimmu.2018.00050
27. Wang Y, Zhu J, Zhang L, Zhang Z, He L, Mou Y, et al. Role of C/EBP homologous protein and endoplasmic reticulum stress in asthma exacerbation by regulating the IL-4/signal transducer and activator of transcription 6/transcription factor EC/IL-4 receptor α positive feedback loop in M2 macrophages. *J Allergy Clin Immunol.* (2017) 140:1550–61.e8. doi: 10.1016/j.jaci.2017.01.024
28. Weng Q, Wang J, Sattar F, Zhang Z, Zheng J, Xu Z, et al. Lenalidomide regulates CNS autoimmunity by promoting M2 macrophages polarization. *Cell Death Dis.* (2018) 9:251. doi: 10.1038/s41419-018-0290-x
29. Ghosh S, Mukherjee S, Choudhury S, Gupta P, Adhikary A, Baral R, et al. Reactive oxygen species in the tumor niche triggers altered activation of macrophages and immunosuppression: role of fluoxetine. *Cell Signal.* (2015) 27:1398–412. doi: 10.1016/j.cellsig.2015.03.013
30. Ströbel P, Preisshofen T, Helmreich M, Müller-Hermelink HK, Marx A. Pathomechanisms of paraneoplastic myasthenia gravis. *Clin Dev Immunol.* (2003) 10:7–12. doi: 10.1080/1044667031000159852 8
31. Ströbel P, Helmreich M, Kalbacher H, Müller-Hermelink HK, Marx A. Evidence for distinct mechanisms in the shaping of the CD4 T cell repertoire in histologically distinct myasthenia gravis-associated thymomas. *Dev Immunol.* (2001) 8:279–90. doi: 10.1155/2001/49127
32. Petrini I, Meltzer PS, Kim IK, Lucchi M, Park KS, Fontanini G, et al. A specific missense mutation in GTF2I occurs at high frequency in thymic epithelial tumors. *Nat Genet.* (2014) 46:844–9. doi: 10.1038/ng.3016


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 05 May 2020
doi: 10.3389/fimmu.2020.00613

# Long-Lasting Rituximab-Induced Reduction of Specific—But Not Total—IgG4 in MuSK-Positive Myasthenia Gravis

*Mariapaola Marino[1,2†], Umberto Basile[3†], Gregorio Spagni[4,5], Cecilia Napodano[6,7], Raffaele Iorio[5], Francesca Gulli[8], Laura Todi[1,2], Carlo Provenzano[1,2], Emanuela Bartoccioni[1,2*] and Amelia Evoli[4,5]*

[1] Istituto di Patologia Generale, Università Cattolica del Sacro Cuore, Rome, Italy, [2] Fondazione Policlinico Universitario "A. Gemelli" IRCCS, Rome, Italy, [3] Area Diagnostica di Laboratorio, Fondazione Policlinico Universitario "A. Gemelli" IRCCS, Rome, Italy, [4] Istituto di Neurologia, Università Cattolica del Sacro Cuore, Rome, Italy, [5] Dipartimento di Neuroscienze, Fondazione Policlinico Universitario "A. Gemelli" IRCCS, Rome, Italy, [6] Istituto di Medicina Interna, Università Cattolica del Sacro Cuore, Rome, Italy, [7] Area di Gastroenterologia e Oncologia Medica, Fondazione Policlinico Universitario "A. Gemelli" IRCCS, Rome, Italy, [8] Dipartimento di Medicina di Laboratorio, Ospedale Madre Giuseppina Vannini, Rome, Italy

OPEN ACCESS

**Edited by:**
*Linda L. Kusner, George Washington University, United States*

**Reviewed by:**
*Kevin C. O'Connor, Yale University, United States*
*Pilar Martinez, Maastricht University, Netherlands*
*Peter S. Linsley, Benaroya Research Institute, United States*

***Correspondence:**
*Emanuela Bartoccioni*
*emanuela.bartoccioni@unicatt.it*

*† These authors have contributed equally to this work*

**Specialty section:**
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

**Received:** *14 November 2019*
**Accepted:** *17 March 2020*
**Published:** *05 May 2020*

**Citation:**
*Marino M, Basile U, Spagni G, Napodano C, Iorio R, Gulli F, Todi L, Provenzano C, Bartoccioni E and Evoli A (2020) Long-Lasting Rituximab-Induced Reduction of Specific—But Not Total—IgG4 in MuSK-Positive Myasthenia Gravis. Front. Immunol. 11:613. doi: 10.3389/fimmu.2020.00613*

The use of rituximab (RTX), an anti-CD20 monoclonal antibody (Ab), in refractory myasthenia gravis (MG) is associated with a better response in patients with Abs to the muscle-specific tyrosine kinase (MuSK) than in other MG subgroups. Anti-MuSK Abs are mostly IgG4 with proven pathogenicity and positive correlation with clinical severity. The rapid and sustained response to RTX may be related to MuSK Ab production by short-lived Ab-secreting cells derived from specific $CD20^+$ B cells. Here, we investigated the long-term effects of RTX in nine refractory MuSK-MG patients with a follow-up ranging from 17 months to 13 years. In patients' sera, we titrated MuSK-specific IgG (MuSK-IgG) and MuSK-IgG4, along with total IgG and IgG4 levels. Optimal response to RTX was defined as the achievement and maintenance of the status of minimal manifestations (MM)-or-better together with a $\geq$ 50% steroid reduction, withdrawal of immunosuppressants, and no need for plasma-exchange or intravenous immunoglobulin. After a course of RTX, eight patients improved, with optimal response in six, while only one patient did not respond. At baseline, MuSK-IgG and MuSK-IgG4 serum titers were positive in all patients, ranging from 2.15 to 49.5 nmol/L and from 0.33 to 46.2 nmol/L, respectively. MuSK Abs mostly consisted of IgG4 (range 63.80–98.86%). RTX administration was followed by a marked reduction of MuSK Abs at 2–7 months and at 12–30 months ($p < 0.02$ for MuSK-IgG and $p < 0.01$ for MuSK-IgG4). In patients with a longer follow-up, MuSK Ab titers remained suppressed, paralleling clinical response. In the patient who achieved long-term complete remission, MuSK-IgG4 was no longer detectable within 2 years, while MuSK-IgG remained positive at very low titers up to 10 years after RTX. In the patient who did not respond, MuSK-IgG and MuSK-IgG4 remained unchanged. In this patient series, total IgG and IgG4 transiently decreased ($p < 0.05$) at 2–7 months after RTX. The different trends of

reduction between MuSK-IgG4 and total IgG4 after RTX support the view that short-lived Ab-secreting cells are the main producers of MuSK Abs. The ratio between short-lived Ab-secreting cells and long-lived plasma cells may influence the response to RTX, and B-cell severe depletion may reduce self-maintaining autoimmune reactivity.

**Keywords: rituximab, antibodies, MuSK, myasthenia gravis, IgG4, short-lived antibody-secreting cells, plasmablasts, plasma cells**

## INTRODUCTION

Rituximab (RTX) is a chimeric mouse/human monoclonal antibody (Ab) directed against the B-lymphocyte membrane protein CD20. RTX acts through complement-mediated cytotoxicity, Ab-dependent cell-mediated cytotoxicity, and induction of apoptosis, leading to a profound depletion of circulating naïve and memory B cells (1). Since plasma cells do not express CD20, RTX administration does not directly affect immunoglobulin (Ig) levels (2). Originally licensed for the treatment of non-Hodgkin B-cell lymphoma, RTX has been used in a broad range of autoimmune diseases. Its therapeutic effect is conceivably related to both the Ab-dependent and Ab-independent (antigen presentation and cytokine production) roles of B lymphocytes in the immune system (3). RTX has proved particularly effective in the treatment of IgG4-related disease, a multi-organ fibro-inflammatory condition (4), as well as in IgG4-mediated autoimmune conditions (5).

IgG4 develops after prolonged antigen exposure and constitutes the least represented IgG subclass. IgG4 Abs may exert a protective role in allergy and are generally considered non-pathogenic, owing to their inability to activate complement and to cross-link identical antigens after Fab-arm exchange (6). More recently, the capacity of IgG4 Abs to directly interfere with the antigen function has been recognized and associated with their causative role in disease (7).

Myasthenia gravis (MG) is a rare disease of the neuromuscular junction caused by Abs against postsynaptic membrane proteins, such as the acetylcholine receptor (AChR), the muscle-specific tyrosine kinase (MuSK), or the low-density receptor-related protein 4 (LRP4). MG with Abs to MuSK (MuSK-MG) was one of the first diseases in which IgG4 pathogenicity was demonstrated (8) and currently represents a prototype of IgG4-mediated autoimmunity.

MuSK Abs are present in 5–7% of MG patients in association with a phenotype dominated by bulbar and neck weakness, are predominantly of the IgG4 subclass (9), and correlate with disease severity (10). When changes in clinical status and MuSK-specific IgG (MuSK-IgG) subclasses were investigated, only IgG4 levels were related to disease severity (11), and the IgG4 fraction, purified from patients' serum, proved pathogenic *in vitro* (12, 13) and in passive transfer studies (8). MuSK Abs mostly bind the extracellular Ig1-like domain, which is crucial for MuSK–LRP4 interaction and AChR clustering (12). According to recent investigations, monovalent MuSK-IgG4, derived from Fab-arm exchange, are present *in vivo*, in patient serum (13). These bispecific Abs are chief players in the inhibition of agrin-induced MuSK activation, while monospecific MuSK Abs would rather have a protective effect by enhancing MuSK phosphorylation (14).

RTX-induced B-cell depletion was reported to be effective in patients with refractory MG, although with some variability in the degree of clinical response and reduction of pathogenic Abs (15–19). Data from clinical reports (15, 16, 19) and meta-analyses (20, 21) consistently showed a more pronounced and long-standing response in patients with MuSK Abs. In these cases, improvement in clinical status was paralleled by a prolonged reduction of MuSK-IgG4 (15).

The present study investigated the effect of RTX in patients with refractory MuSK-MG, together with MuSK-IgG and MuSK-IgG4 and total IgG and IgG4 serum concentrations. We found a correlation between variations in clinical status and changes in MuSK Ab titers. Treatment with RTX induced a marked reduction of MuSK-IgG4 while the serum levels of total IgG4 did not change significantly.

## PATIENTS AND METHODS

### Patients

We retrospectively evaluated nine MuSK-MG patients treated with RTX at Policlinico Gemelli, Università Cattolica. All patients were diagnosed with refractory MG by meeting one of the following criteria: (1) presence of disabling weakness or MG relapses despite adequate treatment (prednisone plus immunosuppressants); (2) inability to reduce steroid dosage because of disease worsening on prednisone tapering, requiring repeated courses of plasma-exchange or intravenous immunoglobulin (IVIg) (at least three per year) and serious side effects from treatment (22).

In these subjects, maximum disease severity ranged from classes IIIb to V according to the Myasthenia Gravis Foundation of America (MGFA) classification (23); two patients had focal muscle atrophy; all were intolerant of pyridostigmine. All patients had received prednisone as initial treatment at a daily dose $\geq$1 mg/kg of body weight, followed by chronic administration every other day, when possible. Because of symptom relapse on prednisone tapering, immunosuppressive agents and emergency treatment (24), mostly with plasma-exchange, were required in all cases. Seven patients had tried two or more immunosuppressants (azathioprine, AZA; mycophenolate mofetil, MMF; and cyclosporine A, CyA); two patients had received AZA for at least 1 year with no response, when we decided to administer RTX on account of disabling

weakness, short-term benefit from plasma-exchange, and serious side effects from steroids (diabetes and osteoporosis in both cases). One of these patients (#5 in **Table 1**) had received a course of RTX 6 years previously in another hospital, with reported benefit; from 1994 to 2000, before MuSK-MG onset, she had suffered from relapsing thrombotic thrombocytopenic purpura treated with high-dose steroids, IVIg, and splenectomy.

RTX was administered according to the non-Hodgkin lymphoma regimen adopted in our institution. A RTX course consisted of an infusion of 375 mg/m$^2$ once a week for four consecutive weeks, plus a single dose of 375 mg/m$^2$ after 3 months. Infusion was preceded by intravenous methylprednisolone and oral antihistamine medication to minimize acute reactions.

Before treatment, patients were screened for active infections including viral hepatitis (B and C) and tuberculosis (25) and for significant arrhythmias and ischemic heart disease (26).

We classified response to RTX considering changes in both clinical status and treatment. Optimal response was defined by the achievement and maintenance of the status of minimal manifestations (MM) or better (23), together with a $\geq$ 50% reduction of steroid dose, withdrawal of immunosuppressants, no need for plasma-exchange, or IVIg. Patients had a partial response when, although improved, they failed to achieve the status of MM-or-better or when prednisone reduction was $<$50% of pretreatment dosage, with or without immunosuppressant withdrawal, no need for plasma-exchange, or IVIg. No response corresponded to nochange in clinical status together with minimal or no variations in the required treatment.

Clinical changes were assessed as post-intervention status (PIS) (23).

## Laboratory Assays

Fifty-two serum samples from the nine patients included in the study (four to seven samples per patient) were collected before and after RTX treatment. Samples were stored at −20°C until the analysis was performed.

MuSK-IgG titration was performed using human recombinant $^{125}$I-labeled MuSK (RSR Limited, Cardiff, United Kingdom; cutoff $\geq$ 0.05 nmol/L), according to the manufacturer's instruction, with minor modifications. In brief, 50 µl of a 10X serum dilution (in assay buffer) was incubated overnightwith 50 µlof $^{125}$I-MuSK. The antigen/Ab complexes were precipitated by the addition of 50 µl of goat anti-human IgG and separated by centrifugation. Serum samples with saturating titers were further analyzed at different dilutions with normal human serum (NHS, from 10X to100X) (10).

MuSK-IgG4 Abs were determined as described by Tsiamalos and coworkers with minor modifications (27). Briefly, 20 µl of $^{125}$I-MuSK solution [from the aforementioned radioimmunoassay (RIA)] was incubated with 0.2 µl of patients' serum for 8–12 h at 4°C (brought to a final volume of 20 µl with assay buffer). Serum samples were previously diluted with NHS to maintain the antigen/Ab binding in the linear zone of the slope. After adding5 µl of sheep anti-human IgG4 (Binding Site, Birmingham, United Kingdom), samples were incubated overnight at 4°C. Then, 15 µl of anti-sheep IgG anti-serum (Binding Site, Birmingham, United Kingdom) was added for 4 h at room temperature to precipitate MuSK-IgG4Abs complexed to $^{125}$I-MuSK. After final washes, the radioactivity was measured in a gammacounter. Results were expressed as $^{125}$I-MuSK nanomoles per liter. All samples from the same patient were titrated in the same batch to limit inter-assay variability.

Total IgG and IgG4 serum levels were measured by turbidimetry (human IgG and IgG subclass liquid reagent kits, The Binding Site) on the Optilite instrument according to the manufacturer's recommendations. Normal range is 8–18 g/L for total IgG and 0.04–0.86 g/L for the IgG4 subclass. Serological analysis was performed blind of clinical data.

B lymphocytes in peripheral blood were evaluated as routine assessment in patients treated with RTX, by standard flowcytometry in our institution's central laboratories. CD19$^+$ cell normal values ranged from 100 to 400 cells/mm$^3$ (7–16% of lymphocyte count).

## Statistical Analysis

For the statistical analysis, we converted actual serological titers into percentage values considering the baseline levels as 100%. We employed one-way ANOVA among three groups (baseline *vs* 2–7 months *vs* 12–30 months); Student's*t* test was performed between two groups. A $p$-value $< 0.05$ was considered significant (**Supplementary Table**).

## Ethical Consideration

The ethic committee of our institution approved the study. All patients gave written informed consent to off-label treatment with RTX and to the use of their clinical and serological data in this study.

# RESULTS

## Clinical Response

The study population included nine patients (eight females) with refractory MuSK-MG, treated with RTX between July 2006 and October 2018. The patients' demographics, baseline and post-treatment clinical data, and follow-up duration are shown in **Table 1**.

Age at infusion ranged 38–73 years (mean 50.4 $\pm$ 12.8). The interval between disease onset and RTX administration varied between 3 and 21 years (mean 11 $\pm$ 6.04). At the time of treatment, MG severity ranged from IIb to IIIb according tothe MGFA classification (23), and all patients were taking prednisone plus one immunosuppressant; five of nine patients were on maintenance treatment with plasma-exchange.

Optimal response to RTX was recorded in six of nine patients (66.6%). Prednisone was tapered off in two patients (#2 and #7) and was reduced by 75% to 87.5% of the pretreatment dosage (actual doses of 5–10 mg every other day) in the others. After a single course of RTX, patient #7 achieved electromyography (EMG)-confirmed complete stable remission (CSR), persisting at the last follow-up, 13 years after RTX administration and 5 years from immunosuppression withdrawal. In the other patients, optimal response to RTX (PIS ranging from CSR to MM) lasted

**TABLE 1 |** Rituximab in refractory MuSK-MG patients.

| Pt # gender | Age at onset (years) | Age at RTX | Max MGFA | MGFA at RTX | Therapy at RTX (daily dosage) | Post-RTX best PIS | Response | Response duration (months) | RTX cycles | Follow-up after RTX (months) |
|---|---|---|---|---|---|---|---|---|---|---|
| #1. F | 61 | 64 | V | IIIb | P (25 mg), AZA (150 mg), PE | MM | optimal | 30, ongoing | 1 | 30 |
| #2. F | 46 | 62 | V | IIb | P (25 mg), MMF (1.5 g), PE | CSR | optimal | 42 | 2* | 46 |
| #3. F | 30 | 44 | V | IIIb | P (20 mg), MMF (2 g), PE | MM | optimal | 28 | 1 | 31 |
| #4. M | 38 | 47 | IIIb | IIIb | P (30 mg), MMF (2 g), PE | U | no | - | 2** | 53 |
| #5. F | 23 | 38 | V | IIIb | P (37.5 mg), MMF (2 g) | I | partial | 24 | 1 | 48 |
| #6. F | 29 | 40 | V | IIb | P (40 mg), CyA (175 mg) | PR | optimal | 71, ongoing | 1 | 71 |
| #7. F | 42 | 48 | IVb | IIIb | P (30 mg), MMF (2 g), PE | CSR | optimal | 144, ongoing | 1 | 144 |
| #8. F | 17 | 38 | IIIb | IIIb | P (30 mg), MMF (2 g) | MM | optimal | 20, ongoing | 1 | 20 |
| #9 F | 69 | 73 | V | IIIb | P (20 mg), AZA (150 mg) | I | partial | 23, ongoing | 1 | 23 |

*MGFA: Myasthenia Gravis Foundation America clinical status; P: prednisone; AZA: azathioprine; PE: plasma-exchange; MMF: mycophenolate mofetil; CyA: cyclosporine A.PIS: post-intervention status; CSR: complete stable remission; PR: pharmacological remission; MM: minimal manifestations; I: improved; U: unchanged. *Pt #2 received a second RTX cycle on Sep 2019. **Pt #4 received a second RTX cycle on Oct 2018 with improvement. Follow-up after RTX = at last visit.*

28–42 months in patients #2 and #3, and is still ongoing 20 to 71 months after RTX, in patients #1, #6, and #8 (see **Table 1**). A second course of RTX has just been completed in patient #2 and has been planned for patient #3.

Two patients showed a partial response since they did not achieve MM status, although prednisone and immunosuppressive therapy were reduced by 50%. In patient #9 who had developed a myopathic face, weakness of facial muscles remained unchanged, while dysarthria and neck weakness markedly improved. Patient #4, suffering from tongue wasting and severe bulbar weakness, did not improve after a first cycle of RTX, while he responded to a second course with improvement of dysarthria and partial reversal of tongue atrophy. RTX was well tolerated with no infusion reactions or long-term side effects.

## MuSK Abs and MuSK-IgG Profile After Treatment

The results of serological assays, together with PIS and changes in B-cell count, are reported in **Table 2**. Changes from baseline to 60 months after RTX are shown in **Figure 1** (panels: A for MuSK IgG, B for MuSK IgG4, C for total IgG, D for total IgG4). At baseline, MuSK-IgG and MuSK-IgG4 serum titers were positive in all patients, ranging from 2.15 to 49.5 nmol/L and from 0.33 to 46.2 nmol/L, respectively. MuSK Abs mostly consisted of IgG4 (range 63.80–98.86%) in all patients but one (#5, not included in the range), in whom the MuSK-IgG4/MuSK-IgG ratio was 15.34%.

MuSK-IgG and MuSK-IgG4 titers did not change after a course of RTX in the only patient (#4) who did not respond (**Figure 2A**), while, in the other patients, they decreased to a different extent, paralleling clinical improvement (**Figure 2B**). In patient #7, who achieved CSR, MuSK-IgG and MuSK-IgG4 were greatly reduced and became negative after RTX. However, while MuSK-IgG was still detectable at very low levels up to 10 years from treatment, MuSK-IgG4 was undetectable 2 years after RTX and remained negative in subsequent assays (**Figure 2C**).

For statistical analysis, we compared MuSK-IgG and MuSK-IgG4 baseline levels with those at 2–7 months and at 12–30 months after RTX in eight patients (samples from patient #5 were not available). We found a significant reduction at both time points ($p < 0.02$ for MuSK-IgG and $p < 0.01$ for MuSK-IgG4, by ANOVA), while the MuSK-IgG4/MuSK-IgG ratio was reduced significantly only at 2–7 months after RTX ($p < 0.05$, by Student's $t$ test).

In patients who experienced prolonged benefit from RTX, MuSK Ab titers remained suppressed, regardless of B-cell count normalization (see **Table 2**).

At baseline, in two of nine patients,total IgG serum levels were lower than those in normal controls. The relative proportions of the IgG subclasses were within the normal range in eight of nine patients (data not shown). Patient #7 had a higher IgG4/IgG ratio (7.3%) that persisted (after a temporary decrease in the first 2 years after RTX) in the long-term follow-up, when MuSK-IgG4 was no longer detectable.

Total IgG and IgG4 did not significantly change at 2–7 and 12–30 months after RTX when compared to the baseline levels ($p = 0.15$ for total IgG; $p = 0.17$ for IgG4, by ANOVA). However, we found a significant reduction when comparing baseline levels with those at 2–7 months ($p < 0.05$ for total IgG and IgG4, by Student's $t$ test); afterward, total IgG and IgG4 returned to pretreatment levels. The total IgG4/IgG ratio was unchanged at both time points.

# DISCUSSION

In line with previous reports (15, 18–21, 28, 29), our data confirm that, in patients with MuSK-MG, RTX is safe and induces long-term benefit associated with a strong steroid- and immunosuppressant-sparing effect. As MuSK-MG is often a life-threatening disease with a high proportion of patients refractory to conventional therapy (30, 31), RTX has been proposed as an early therapeutic option in patients unresponsive to first-line immunosuppression (32).

The rapid and sustained response to RTX suggests that MuSK Abs are mostly produced by short-lived Ab-secreting cells (33, 34), a cell pool that needs to be constantly refilled from the B-cell compartment (35). In contrast, bone marrow long-lived plasma cells, which are crucial for maintaining

**TABLE 2 |** Serological and clinical profiles in MuSK-MG patients after treatment with rituximab.

| Pt | Months from RTX | anti-MuSK IgG (nmol/L) | anti-MuSK IgG4 (nmol/L) | IgG (g/L) | IgG4 (g/L) | MGFA class/PIS | CD19+ n°. x $mm^3$ (% lymphocytes) |
|---|---|---|---|---|---|---|---|
| **1** | 0 | 11.85 | 11.7 | 14.39 | 0.25 | IIIb | 292 (10.6%) |
| | 4 | 5.4 | 4.25 | 6.95 | 0.20 | Improved | 13 (0.9%) |
| | 6 | 6.6 | 4.71 | 7.31 | 0.20 | Improved | 36 (1.6%) |
| | 10 | 2.7 | 1.84 | 6.39 | 0.20 | MM | 32 (0.1%) |
| | 24 | 7.3 | 6.1 | 6.86 | 0.19 | MM | 21 (1.5%) |
| **2** | 0 | 32.4 | 25.3 | 8.56 | 0.23 | IIb | 200 (9.4%) |
| | 2 | 24.5 | 20.8 | 7.20 | 0.22 | Improved | n.d. |
| | 6 | 17.7 | 10.1 | 9.74 | 0.25 | MM | 9 (0.4%) |
| | 12 | 13 | 6.1 | 10.71 | 0.21 | PR | 18 (0.8%) |
| | 30 | 10.6 | 8 | 9.76 | 0.28 | CSR | 352 (15.8%) |
| | 41 | 12.1 | 11.4 | 12.39 | 0.28 | CSR | 341 (18.1% |
| | 46 | 17.9 | 16 | 11.56 | 0.31 | IIb* | n.d. |
| **3** | 0 | 7.57 | 7.17 | 22.04 | 0.19 | IIIb | 118 (11%) |
| | 5 | 3.44 | 2.44 | 13.32 | 0.11 | improved | 16 (0.5%) |
| | 7 | 2.14 | 2.03 | 9.09 | 0.07 | MM | 9 (0.3%) |
| | 26 | 2.3 | 0.6 | 8.68 | 0.05 | MM | 47 (2.5%) |
| | 31 | 2.4 | 0.3 | 8.43 | 0.04 | IIb | 181 (6.4%) |
| **4** | 0 | 10.57 | 9.79 | 8.6 | 0.21 | IIIb | 138 (15%) |
| | 2 | 14.9 | 11.2 | 7.55 | 0.23 | Unchanged | 15 (1%) |
| | 4 | 10 | 7.7 | 9.93 | 0.27 | Unchanged | n.d. |
| | 24 | 19.8 | 16.6 | 7.51 | 0.20 | Unchanged | n.d |
| | 34 | 17 | 13 | 5.55 | 0.11 | Unchanged | n.d |
| | 53 | 2.6 | 2.6 | 5.73 | 0.13 | Improved** | n.d |
| **5** | 0 | 2.15 | 0.33 | 6.33 | 0.20 | IIIb | 193 (4%) |
| | 1 | 1.15 | 0.22 | 6.65 | 0.09 | Unchanged | 9 (0.2%) |
| | 14 | 0.34 | 0.07 | 5.33 | 0.15 | Improved | 11 (0.4%) |
| | 18 | n.d. | 0.07 | 5.77 | 0.10 | Improved | 11 (0.3%) |
| **6** | 0 | 18.07 | 11.53 | 7.30 | 0.36 | IIb | 100 (9.1%) |
| | 4 | 5.2 | 3.8 | 7.11 | 0.29 | PR | 2 (0.1%) |
| | 15 | 8.49 | 5.84 | 6.54 | 0.33 | PR | 0 |
| | 24 | 9.4 | 5.65 | 6.83 | 0.37 | PR | 29 (2.6%) |
| | 36 | 3.38 | 1.84 | 7.42 | 0.47 | PR | n.d. |
| | 48 | 2.6 | 2.57 | 8.56 | 0.57 | MM | 100 (9.6%) |
| | 60 | 2.7 | 1.6 | 9.61 | 0.68 | MM | 142 (10.5%) |
| | 71 | 2.7 | 2.4 | 10.36 | 0.81 | MM | n.d. |
| **7** | 0 | 12.24 | 12.1 | 14.59 | 1.07 | IIIb | n.d. |
| | 4 | 7.34 | 4.87 | 12.22 | 0.26 | Improved | n.d. |
| | 24 | 0.21 | 0.01 | 12.23 | 0.49 | MM | n.d. |
| | 80 | 0.18 | 0.01 | 11.78 | 1.07 | CSR | n.d. |
| | 120 | 0.21 | 0.01 | 11.96 | 1.15 | CSR | n.d. |
| | 132 | 0 | 0 | 12.23 | 1.19 | CSR | n.d. |
| **8** | 0 | 13 | 11.4 | 11.46 | 0.58 | IIIb | 298 (12.3%) |
| | 1 | 10.3 | 8.5 | 11.58 | 0.56 | Unchanged | 0 |
| | 3 | 7 | 5.5 | 11.06 | 0.56 | MM | 0 |
| | 6 | 5.7 | 2.4 | 9.53 | 0.56 | MM | 7 (0.67%) |
| | 10 | 3.8 | 1.6 | 11.64 | 0.60 | MM | N/D |
| | 15 | 1.1 | 1.1 | 12.70 | 0.72 | MM | N/D |
| | 20 | 0.94 | 0.9 | 13.59 | 0.82 | MM | N/D |
| **9** | 0 | 49.5 | 46.2 | 10.99 | 0.34 | IIIb | 107 (11.8%) |
| | 4 | 37.3 | 34.3 | 9.47 | 0.22 | Improved | 0 |
| | 16 | 8.1 | 7 | 10.81 | 0.29 | Improved | N/D |
| | 23 | 11.8 | 11.8 | 13.96 | 0.40 | Improved | 157 (17%) |

*Pt: patient; MGFA: Myasthenia Gravis Foundation America clinical status; PIS: post interventional status; MM: minimal manifestations; PR: pharmacological remission, CSR: complete stable remission. Normal range for IgG: 8-18 g/L. Normal range for IgG4 subclass: 0.04–0.86 g/L. *Pt #2 received a second RTX cycle on Sep, 2019. **Pt #4 received a second RTX cycle on Oct, 2018 with improvement.*

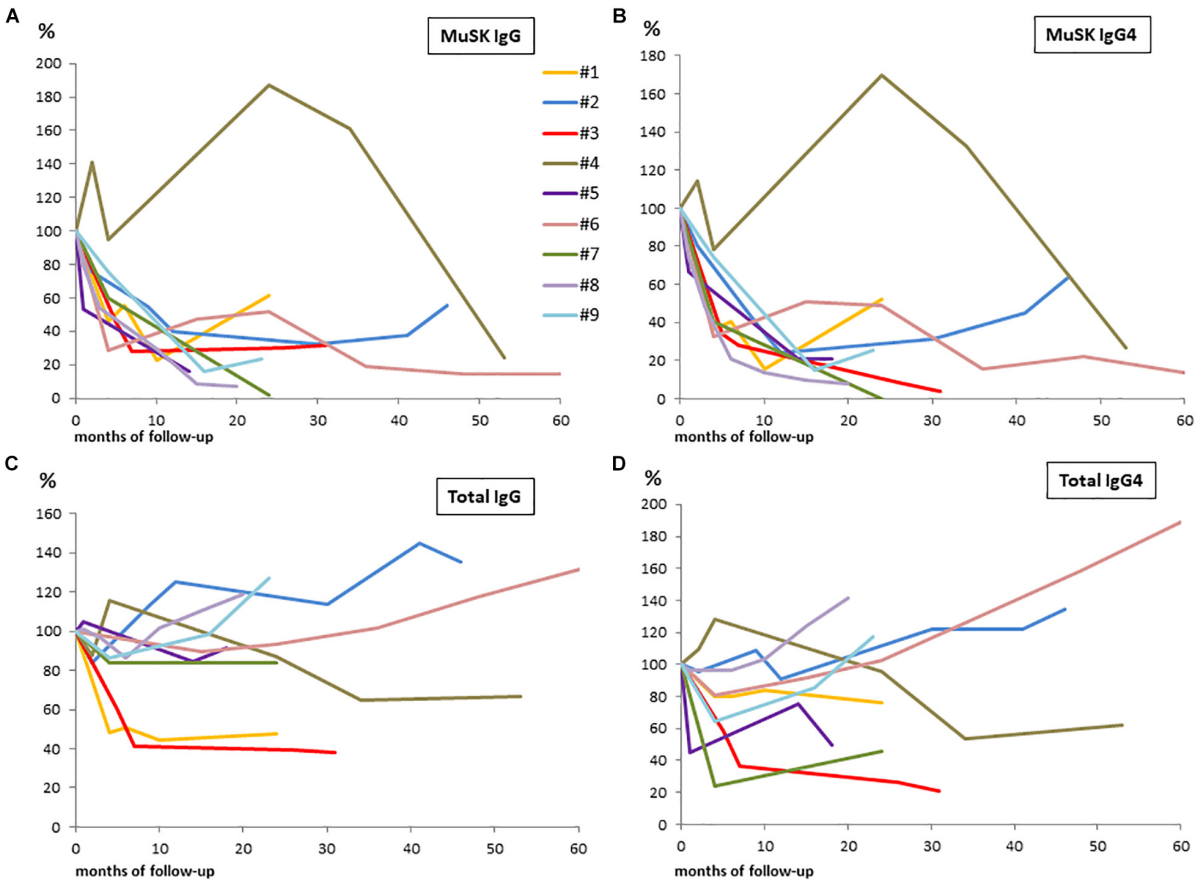



**FIGURE 1 |** Changes from baseline to 60 months after RTX in patients serum samples are shown in panels: **(A)** for MuSK-IgG; **(B)** for MuSK-IgG4; **(C)** for total IgG; and **(D)** for total IgG4. We converted actual serological titers into percentage values considering the baseline levels as 100%.

serum IgG concentration, are known to be scarcely affected by RTX (3). Recent studies, in MuSK-MG patients, showed that auto-Ab-expressing $CD27^+$ B cells are present in the peripheral blood during disease relapses after RTX and that circulating $CD20^-CD27^{high}$ $CD38^+$plasmablasts contribute to MuSK Ab production (36). In this model, supported by consistent findings in other IgG4-mediated diseases (37, 38), the therapeutic effect of RTX would be mostly related to depletion of plasmablast precursors (36, 39).

A comparison of our results with other studies is limited by differences in MG severity and therapy at baseline, follow-up duration, and treatment protocol. Our decision to repeat RTX was entirely clinical, based on reappearance of disabling symptoms. The majority of our patients received a single course of RTX that granted most of them a status of MM-or-better, including one case of long-standing CSR with MuSK Ab negativization. Clinical response lasted 24–42 months in patients who relapsed after RTX. It is still ongoing in the other patients after a clinical observation of up to 71 months. These findings confirm earlier reports of a long-standing effect of RTX in MuSK-MG (15, 18, 19, 29, 40). On the other hand, three (33.3%) of our patients had a less satisfactory outcome than usually reported in this MG subtype. It is worth mentioning that two of these subjects had developed focal muscle atrophy. As, in this series, a second RTX course brought about a clear benefit in a previously unresponsive patient, timely re-treatment may be required in these cases.

Our results confirm the correlation between MuSK Abs and clinical status (11, 15), as in all RTX-responsive patients, MuSK-IgG and MuSK-IgG4 serum titers were significantly reduced and, in most of these cases, remained suppressed regardless of B-cell count normalization(patients #2–3–6–9 in **Table 2**).

In our population, IgG4 was the main MuSK Ab isotype in all our patients but one, in whom IgG4 accounted for 15.3% of MuSK Ab titer. This finding might be related to the patient's medical history (splenectomy for thrombotic thrombocytopenic purpura and previous treatment with RTX). In patient #2, an increase in serum MuSK Abs, and above all, in MuSK-IgG4 heralded clinical deterioration. Conversely, in patient #7, who achieved long-standing CSR, MuSK-IgG4 was no longer detectable several years before MuSK-IgG negativization. Overall, these data agree with the view that MuSK-IgG4 is pathogenic and is mostly produced by short-lived Ab-secreting cells.

There is no established protocol for RTX administration in MG, although the non-Hodgkin lymphoma regimen has been the most common induction treatment (21). Recently, in a

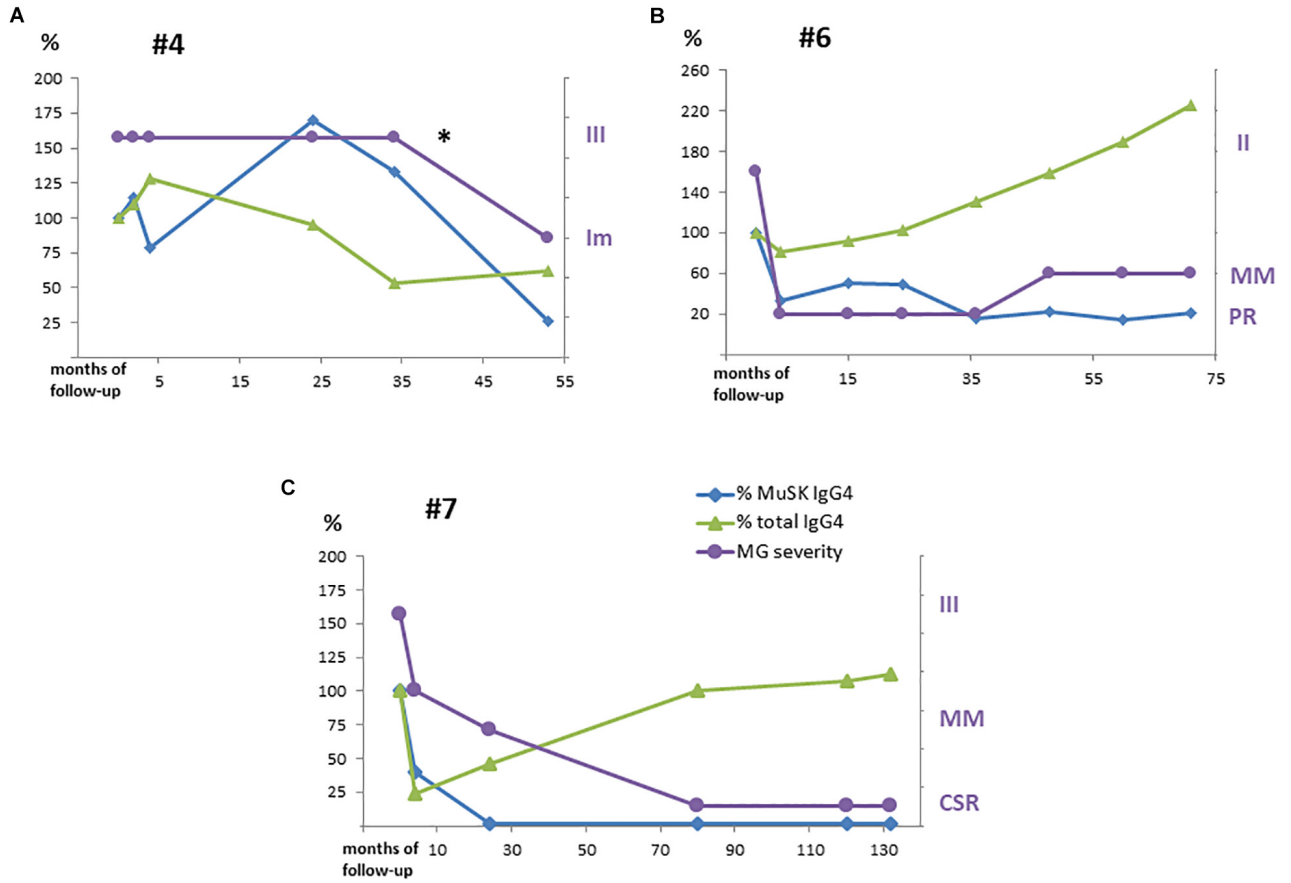



**FIGURE 2 |** MuSK-IgG4 and total IgG4 subclass levels after RTX treatment along with variations in clinical severity are shown in panels: **(A)** for #4, **(B)** for #6, and **(C)** for #7. Serological titers were converted into percentage values considering the baseline levels as 100%. MG severity was referred to as MGFA/PIS as reported in **Table 2**. *Pt #4 received a second RTX with improvement (Im).

multicenter study investigating the response to different RTX dosages, the protocol of 375 mg/m$^2$/week for 4 weeks plus an infusion monthly for the next 2 months, which is similar to that adopted in our study, was associated with the lowest relapse rate in MuSK-MG (41). RTX cycles were repeated based on clinical relapses in some studies (15, 16, 29) or per protocol in others (17, 19, 28, 42), with an overall positive correlation between number of treatment courses and clinical outcome (18). Considering MuSK-MG pathogenesis, reemerging CD27$^+$ B cells and plasmablasts in peripheral blood can serve as a valuable reference for timing re-treatment. As an increase in MuSK-IgG (and MuSK-IgG4) can herald a clinical relapse, changes in Ab titers may be used to monitor clinical response.

It is well known that RTX is highly effective in IgG4-associated diseases and can deplete short-lived Ab-secreting cells without affecting long-lived plasma cells [for a review, see (43)]. The effect of RTX on the longitudinal levels of total serum IgG4, in MuSK-MG, has not been investigated. In our population, RTX administration did not affect total IgG4 to a greater extent than other IgG subclasses, as total IgG and IgG4 serum levels both decreased in the first months after treatment and then returned to normal.From our data, total IgG and IgG4 appear to be mostly produced by long-lived plasma cells, even though short-lived Ab-secreting cells, may contribute. On the other hand, the long-lasting decline of MuSK-Abs, particularly of IgG4 isotype, supports the view that short-lived Ab-secreting cells are the main producers of MuSK Abs.

Overall, our data show that the therapeutic effects of RTX can persist for several years after treatment, suggesting that, by depleting autoreactive B-cell clones, RTX may significantly disrupt the immunopathogenic circuit responsible for disease maintenance. It is well known that B-cell activity depends on T–B lymphocyte cross talk and cooperation. Future studies should investigate how RTX affects such an interaction, particularly regarding specific T- and B-cell repertoires.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario "A. Gemelli" – I.R.C.C.S. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

MM, EB, and AE wrote the manuscript. MM, UB, and EB designed the experiments. GS, RI, and AE collected the serum samples and clinical data. UB, CN, FG, LT, and CP performed the experiments. EB and AE analyzed the data.

## FUNDING

This research and its publication have been funded from Universitá Cattolica del Sacro Cuore Fondazione Policlinico Universitario Agostino Gemelli I.R.C.C.S. as a part of its programs on promotion and dissemination of scientific research (Linea D1 to MM). Also the support of AFM-Telethon with the project "Effect of Rituximab therapy on T cell repertoire in MuSK positive Myasthenia Gravis" (20819 to EB) is gratefully acknowledged.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00613/full#supplementary-material

## REFERENCES

1. Stübgen J-P. B cell-targeted therapy with rituximab and autoimmune neuromuscular disorders. *J Neuroimmunol.* (2008) 204:1–12. doi: 10.1016/j.jneuroim.2008.07.019
2. Marco H, Smith RM, Jones RB, Guerry MJ, Catapano F, Burns S, et al. The effect of rituximab therapy on immunoglobulin levels in patients with multisystem autoimmune disease. *BMC Musculoskelet Disord.* (2014) 15:178. doi: 10.1186/1471-2474-15-178
3. Hofmann K, Clauder AK, Manz RA. Targeting B cells and plasma cells in autoimmune diseases. *Front Immunol.* (2018) 9:835. doi: 10.3389/fimmu.2018.00835
4. Ebbo M, Grados A, Samson M, Groh M, Loundou A, Rigolet A, et al. Long-term efficacy and safety of rituximab in IgG4-related disease: data from a French nationwide study of thirty-three patients. *PLoS One* (2017) 12:e0183844. doi: 10.1371/journal.pone.0183844
5. Huijbers MG, Plomp JJ, van der Maarel SM, Verschuuren JJ. IgG4-mediated autoimmune diseases: a niche of antibody-mediated disorders. *Ann NY Acad Sci.* (2018) 1413:92–103. doi: 10.1111/nyas.13561
6. Trampert DC, Hubers LM, van de Graaf SFJ, Beuers U. On the role of IgG4 in inflammatory conditions: lessons for IgG4-related disease. *Biochim Biophys Acta Mol Basis Dis.* (2018) 1864:1401–9. doi: 10.1016/j.bbadis.2017.07.038
7. Huijbers MG, Zhangc W, Kloosterb R, Niksa EH, Friesec MB, Straasheijmb KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc Natl Acad Sci USA.* (2013) 110:20783–8.
8. Klooster R, Plomp JJ, Huijbers MG, Niks EH, Straasheijm KR, Detmers FJ, et al. Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. *Brain.* (2012) 135:1081–101.
9. McConville J, Farrugia ME, Beeson D, Kishore U, Metcalfe R, Newsom-Davis J, et al. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. *Ann Neurol.* (2004) 55:580–4. doi: 10.1002/ana.20061
10. Bartoccioni E, Scuderi F, Minicuci GM, Marino M, Ciaraffa F, Evoli A. Anti-MuSK antibodies: correlation with myasthenia gravis severity. *Neurology.* (2006) 67:505–7. doi: 10.1212/01.wnl.0000228225.23349.5d
11. Niks EH, van Leeuwen Y, Leite MI, Dekker FW, Wintzen AR, Wirtz PW, et al. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. *J Neuroimmunol.* (2008) 195:151–6. doi: 10.1016/j.jneuroim.2008.01.013
12. Huijbers MG, Zhang W, Klooster R, Niks EH, Friese MB, Straasheijm KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc Natl Acad Sci USA.* (2013) 110:20783–8.
13. Koneczny I, Stevens JA, De Rosa A, Huda S, Huijbers MG, Saxena A, et al. IgG4 autoantibodies against muscle-specific kinase undergo Fab-arm exchange in myasthenia gravis patients. *J Autoimmun.* (2017) 77:104–7. doi: 10.1016/j.jaut.2016.11.005
14. Huijbers MG, Vergoossen DL, Fillié-Grijpma YE, van Es IE, Koning MT, Slot LM, et al. MuSK myasthenia gravis monoclonal antibodies. Valency dictates pathogenicity. *Neurol Neuroimmunol Neuroinflamm.* (2019) 6:e547. doi: 10.1212/NXI.0000000000000547
15. Díaz-Manera J, Martínez-Hernández E, Querol L, Klooster R, Rojas-García R, Suárez-Calvet X, et al. Long-lasting treatment effect of rituximab in MuSK myasthenia. *Neurology.* (2012) 78:189–93. doi: 10.1212/WNL.0b013e3182407982
16. Anderson D, Phan C, Johnston WS, Siddiqi ZA. Rituximab in refractory myasthenia gravis: a prospective, open-label study with long-term follow-up. *Ann. Clin. Transl. Neurol.* (2016) 3:552–5. doi: 10.1002/acn3.314
17. Robeson KR, Kumar A, Keung B, DiCapua DB, Grodinsky E, Patwa HS, et al. Durability of the rituximab response in acetylcholine receptor autoantibody-positive myasthenia gravis. *JAMA Neurol.* (2017) 74:60–6. doi: 10.1001/jamaneurol.2016.4190
18. Hehir MK, Hobson-Webb LD, Benatar M, Barnett C, Silvestri NJ, Howard JFJr., et al. Rituximab as treatment for anti-MuSK myasthenia gravis: Multicenter blinded prospective review. *Neurology.* (2017) 89:1069–77.
19. Topakian R, Zimprich F, Iglseder S, Embacher N, Guger M, Stieglbauer K, et al. High efficacy of rituximab for myasthenia gravis: a comprehensive nationwide study in Austria. *J Neurol.* (2019) 266:699–706. doi: 10.1007/s00415-019-09191-6
20. Iorio R, Damato V, Alboini PE, Evoli A. Efficacy and safety of rituximab for myasthenia gravis: a systematic review and meta-analysis. *J Neurol.* (2015) 262:1115–9. doi: 10.1007/s00415-014-7532-3
21. Tandan R, Hehir MKII, Waheed W, Howard DB. Rituximab treatment of myasthenia gravis: A systematic review. *Muscle Nerve.* (2017) 56:185–96. doi: 10.1002/mus.25597
22. Drachman DB, Adams RN, Hu R, Jones RJ, Brodsky RA. Rebooting the immune system with high-dose cyclophosphamide for treatment of refractory myasthenia gravis. *Ann N Y Acad Sci.* (2008) 1132:305–14. doi: 10.1196/annals.1405.033
23. Jaretzki AIII, Barohn RJ, Ernstoff RM, Kaminski HJ, Keesey JC, Penn AS, et al. Myasthenia gravis: recommendations for clinical research standards. task force of the medical scientific advisory board of the myasthenia gravis foundation of America. *Neurology.* (2000) 55:16–23. doi: 10.1212/WNL.55.1.16
24. Sanders DB, Evoli A. Immunosuppressive therapies in myasthenia gravis. *Autoimmunity.* (2010) 43:428–35. doi: 10.3109/08916930903518107
25. Buch MH, Smolen JS, Betteridge N, Breedveld FC, Burmester G, Dörner T, et al. Updated consensus statement on the use of rituximab in patients with rheumatoid arthritis. *Ann Rheum Dis.* (2011) 70:909–20. doi: 10.1136/ard.2010.144998

26. Rituxan. *Rituxan-FDA Prescribing Information, Side Effects and Uses*. (2020). Available online at: http://www.drugs.com/pro/rituxan.html (January 1, 2020).
27. Tsiamalos P, Kordasa G, Poulasa K, Tzartos SJ. Epidemiological and immunological profile of muscle-specific kinase myasthenia gravis in Greece. *Eur Neurol.* (2009) 16:925–30. doi: 10.1111/j.1468-1331.2009.02624.x
28. Keung J, Robeson KR, Di Capua DB, Rosen JB, O'Connor KC, Goldstein JM. Long-term benefit of rituximab in MuSK autoantibody myasthenia gravis patients. *Neurol Neurosurg Psychiatry.* (2013) 84:1407–9. doi: 10.1136/jnnp-2012-303664
29. Beecher G, Anderson D, Siddiqi ZA. Rituximab in refractory myasthenia gravis: extended prospective study results. *Muscle Nerve.* (2018) 58:452–5. doi: 10.1002/mus.26156
30. Evoli A, Alboini PE, Bisonni A, Mastrorosa A, Bartoccioni E. Management challenges in muscle-specific tyrosine kinase myasthenia gravis. *Ann N Y Acad Sci.* (2012) 1274:86–91. doi: 10.1111/j.1749-6632.2012.06781.x
31. Suh J, Goldstein JM, Nowak RJ. Clinical characteristics of refractory myasthenia gravis patients. *Yale J Biol Med.* (2013) 86:255–60.
32. Sanders DB, Wolfe GI, Benatar M, Evoli A, Gilhus NE, Illa I, et al. International consensus guidance for management of myasthenia gravis: executive summary. *Neurology*. (2016) 87:419–25.
33. Takata K, Stathopoulos P, Cao M, Mané-Damas M, Fichtner ML, Benotti ES, et al. Characterization of pathogenic monoclonal autoantibodies derived from muscle-specific kinase myasthenia gravis patients. *JCI Insight.* (2019) 4:e127167. doi: 10.1172/jci.insight.127167
34. Yi JS, Guptill JT, Stathopoulos P, Nowak RJ, O'Connor KC. B cells in the pathophysiology of myasthenia gravis. *Muscle Nerve.* (2018) 57:172–84. doi: 10.1002/mus.25973
35. Bortnick A, Allman D. What is and what should always have been: long-lived plasma cells induced by T-cell independent antigens. *J. Immunol.* (2013) 190:5913–8. doi: 10.4049/jimmunol.1300161
36. Stathopoulos P, Kumar A, Nowak RJ, O'Connor KC. Autoantibody-producing plasmablasts after B cell depletion identified in muscle-specific kinase myasthenia gravis. *JCI Insight.* (2017) 2:94263. doi: 10.1172/jci.insight.94263
37. Khosroshahi A, Bloch DB, Deshpande V, Stone JH. Rituximab therapy leads to rapid decline of serum IgG4 levels and prompt clinical improvement in IgG4−related systemic disease. *Arthritis Rheum.* (2010) 62:1755–62. doi: 10.1002/art.27435
38. Khosroshahi A, Carruthers MN, Deshpande V, Unizony S, Bloch DB, Stone JH. Rituximab for the treatment of IgG4-related disease: lessons from 10 consecutive patients. *Medicine (Baltimore).* (2012) 91:57–66. doi: 10.1097/MD.0b013e3182431ef6
39. Stathopoulos P, Kumar A, Heiden JAV, Pascual-Goñi E, Nowak RJ, O'Connor KC. Mechanisms underlying B cell immune dysregulation and autoantibody production in MuSK myasthenia gravis. *Ann. N Y Acad Sci.* (2018) 1412:154–65. doi: 10.1111/nyas.13535
40. Topakian, R., Zimprich, F., Iglseder, S., Embacher, N., Guger, M., Stieglbauer, K., et al. High efficacy of rituximab for myasthenia gravis: a comprehensive nationwide study in Austria. *J. Neurol.* (2019) 266:699–706. doi: 10.1007/s00415-019-09191-6
41. Cortés-Vicente E, Rojas-Garcia R, Díaz-Manera J, Querol L, Casasnovas C, Guerrero-Sola A, et al. The impact of rituximab infusion protocol on the long-term outcome in anti-MuSK myasthenia gravis. *Ann Clin Transl Neurol.* (2018) 5:710–6. doi: 10.1002/acn3.564
42. Nowak RJ, Dicapua DB, Zebardast N, Goldstein JM. Response of patients with refractory myasthenia gravis to rituximab: a retrospective study. *Ther Adv Neurol Disord.* (2011) 4:259–66. doi: 10.1177/1756285611411503
43. Marino M, Bartoccioni E, Alboini PE, Evoli A. Rituximab in myasthenia gravis: a "to be or not to be" inhibitor of T cell function. *Ann N Y Acad Sci.* (2018) 1413:41–8. doi: 10.1111/nyas.13562


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 06 May 2020
doi: 10.3389/fimmu.2020.00782

# Comparative Analysis of Thymic and Blood Treg in Myasthenia Gravis: Thymic Epithelial Cells Contribute to Thymic Immunoregulatory Defects

*Frédérique Truffault[1†], Dani Nazzal[1†], Julien Verdier[1†], Angeline Gradolatto[1†], Elie Fadel[2], Régine Roussin[2], Bruno Eymard[3], Rozen Le Panse[1*] and Sonia Berrih-Aknin[1]*

[1] Sorbonne Université, INSERM, Institut de Myologie, Centre de Recherche en Myologie, Paris, France, [2] Marie Lannelongue Hospital, Le Plessis-Robinson, France, [3] AP-HP, Referral Center for Neuromuscular Disorders, Pitié-Salpêtrière Hospital, Institute of Myology, Paris, France

OPEN ACCESS

**Edited by:**
*Carlo Riccardi, University of Perugia, Italy*

**Reviewed by:**
*Socrates J. Tzartos, Pasteur Hellenic Institute, Greece*
*Alexander Marx, University of Heidelberg, Germany*

***Correspondence:**
*Rozen Le Panse*
*rozen.lepanse@upmc.fr*

*[†] These authors have contributed equally to this work*

**Specialty section:**
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

**Received:** *16 January 2020*
**Accepted:** *06 April 2020*
**Published:** *06 May 2020*

**Citation:**
*Truffault F, Nazzal D, Verdier J, Gradolatto A, Fadel E, Roussin R, Eymard B, Le Panse R and Berrih-Aknin S (2020) Comparative Analysis of Thymic and Blood Treg in Myasthenia Gravis: Thymic Epithelial Cells Contribute to Thymic Immunoregulatory Defects. Front. Immunol. 11:782. doi: 10.3389/fimmu.2020.00782*

The thymus is involved in autoimmune Myasthenia gravis (MG) associated with anti-acetylcholine (AChR) antibodies. In MG, thymic regulatory T cells (Treg) are not efficiently suppressive, and conventional T cells (Tconv) are resistant to suppression. To better understand the specific role of the thymus in MG, we compared the phenotype and function of peripheral and thymic Treg and Tconv from controls and MG patients. Suppression assays with thymic or peripheral CD4 + T cells showed that the functional impairment in MG was more pronounced in the thymus than in the periphery. Phenotypic analysis of Treg showed a significant reduction of resting and effector Treg in the thymus but not in the periphery of MG patients. CD31, a marker lost with excessive immunoreactivity, was significantly reduced in thymic but not blood resting Treg. These results suggest that an altered thymic environment may explain Treg differences between MG patients and controls. Since thymic epithelial cells (TECs) play a major role in the generation of Treg, we co-cultured healthy thymic CD4 + T cells with control or MG TECs and tested their suppressive function. Co-culture with MG TECs consistently hampers regulatory activity, as compared with control TECs, suggesting that MG TECs contribute to the immune regulation defects of MG CD4 + T cells. MG TECs produced significantly higher thymic stromal lymphopoietin (TSLP) than control TECs, and a neutralizing anti-TSLP antibody partially restored the suppressive capacity of Treg derived from co-cultures with MG TECs, suggesting that TSLP contributed to the defect of thymic Treg in MG patients. Finally, a co-culture of MG CD4 + T cells with control TECs restored numbers and function of MG Treg, demonstrating that a favorable environment could correct the immune regulation defects of T cells in MG. Altogether, our data suggest that the severe defect of thymic Treg is at least partially due to MG TECs that overproduce TSLP. The Treg defects could be corrected by replacing dysfunctional TECs by healthy TECs. These findings highlight the role of the tissue environment on the immune regulation.

**Keywords: myasthenia gravis, thymus, PBMC, thymic epithelial cells, Treg, CD31, TSLP, immune regulation**

## INTRODUCTION

Myasthenia gravis (MG) is a chronic autoimmune disorder caused, in most patients, by anti-acetylcholine receptors (AChR) antibodies, which mainly destroy AChR at the neuromuscular junction, leading to muscle weakness and fatigability (1). Accumulating arguments strongly support that the thymus plays a role in the pathology of MG (2). Indeed, thymectomy has favorable clinical effects, especially in young patients (3). In addition, functional and morphological abnormalities of the thymus occur very frequently in MG patients: about 50% of them present thymus hyperplasia with the development of lymphoid follicles, and 10 to 15% have an epithelial tumor of the thymus (4). Thymic hyperplasia is particularly common in young women with a high level of anti-AChR antibodies (5), that decreases after thymectomy in association with clinical improvement (6). Thus the thymus seems to play a key role in anti-AChR antibody production.

Several signs of activation and inflammation can be found in the hyperplastic thymus of MG patients. B cells spontaneously produce anti-AChR antibodies (7), and CD4 + T cells express higher levels of Fas/CD95 (8) and proliferate more in response to recombinant interleukin (IL)-2 than CD4 + T cells from control individuals (9). In addition, suppression by CD4 + CD25 + thymic regulatory T cells (Treg) is severely reduced in MG patients compared with controls (10), and CD4 + CD25- thymic conventional cells (Tconv) exhibit resistance to the suppressive activity (11). Taken together, these findings suggest that the thymus is chronically activated in MG.

Using microarray experiments, we showed that type I- and type II-interferon (IFN)-regulated genes are highly expressed in MG thymus in comparison with control thymus (12). Although the origin of the thymic inflammation is still unclear, we showed that molecules mimicking a viral infection increase the expression of the autoantigen (AChR) as well as that of chemokines (13) through the production of IFN-β that appears to play a central role in MG thymic changes (14).

Despite evidence of a dysregulated immune response in the thymus of MG patients, the role of thymic epithelial cells (TECs) has been only scarcely considered. It was shown that TECs from MG patients overproduce IL-1, IL-6, and RANTES in comparison with TECs from healthy subjects (9, 15). Recently, we showed that the overproduction of IL-17 in MG thymuses is sustained by a higher secretion of IL-23 by MG TECs (16).

In control thymuses, medullary TECs (mTECs) promote the generation of thymic Treg and favor their function, an effect mainly due to IL-2 overproduction by thymic CD4 + conventional T cells (Tconv) (17). Treg were first defined by their high expression of CD25 (18, 19), then by the expression of FoxP3, a master control gene for Treg development and function (20). In humans, however, FoxP3 is not exclusively expressed in Treg as it can be transiently induced in TCR-stimulated naïve CD4 + T cells without conferring any suppressive activity (21). The absence of CD127 is also a hallmark of Treg, yet some CD25hiCD127low CD4 + T cells also contain non-Treg (22, 23). In addition, the combination of FoxP3 and CD45RA can separate three phenotypically and functionally distinct subpopulations: CD45RA + FoxP3lo resting Treg (rTreg) and CD45RA-FoxP3hi effector Treg (eTreg) that are both suppressive *in vitro*, while cytokine-secreting CD45RA-FoxP3lo cells (Fraction III, FIII) are non-suppressive (24). Finally, the co-expression of chemokine receptors in Treg identifies specific functional subsets. Treg expressing chemokine receptors travel to the sites of inflammation to deliver a suppressive activity. As an example, CD4 + FoxP3 + CXCR5 + cells defined as follicular regulatory T cells control follicular effector T cells (CXCR5 + FoxP3neg) in lymphoid follicles (25).

In order to better understand the specific role of the thymus in MG, we compared peripheral and thymic Tconv and Treg from controls and MG patients by combinations of markers, and we addressed whether TECs from control and MG patients had distinct immune imprinting properties. We showed that the phenotypic and functional defects in MG were more pronounced in the thymus than in the periphery and that TECs from MG patients induced immune regulation defects in control CD4 + T cells, thus demonstrating that mTECs in MG patients contribute to the immunoregulatory defects in MG.

## MATERIALS AND METHODS

### Thymuses and Blood Samples

Thymuses were obtained from MG patients (12–41 year-old) undergoing thymectomy at the "Hôpital Marie Lannelongue" or "Centre Hospitalier Universitaire de Strasbourg." The clinical details of the 44 patients included in the study are described in **Table 1**. Normal thymuses were obtained from infants (4 days to 11 years) and adults (13–35 years) undergoing cardiac surgery at the "Hôpital Marie Lannelongue."

Thymocytes were isolated from thymuses by mechanical dissociation of fresh thymic tissue, as previously described (26).

**TABLE 1 |** Clinical characteristics of MG patients included in the study.

| | | |
|---|---|---|
| Age (median) | 29 years | |
| Sex | 39 (89%) | Female |
| | 5 (11%) | Male |
| Antibody status | 40 (91) | Anti-AChR positive |
| | 4 (9%) | Seronegative |
| | 1 (2%) | Anti-MuSK positive |
| Therapy at time of analysis | 43 (98%) | Acetylcholinesterase inhibitors |
| | 2 (5%) | Plus corticosteroids |
| | 1 (2%) | Plus immunosuppression |
| MG clinical subtype | 31 (70%) | Generalized MG |
| | 12 (27%) | Ocular MG |
| | 1 (2%) | Not defined |
| Thymectomy | 41 (93%) | Yes |
| | 3 (7%) | No |
| Thymic histology | 31 (70%) | Hyperplasia |
| | 1 (2%) | Thymoma |
| | 7 (18%) | No abnormality detected |
| | 2 (5%) | Not known |

The cells were filtered through cell strainer device to remove thymic tissues and washed once with HBSS.

Blood was obtained from MG patients (19–45 years old) just before thymectomy or during follow–up consultation, and from sex-matched control donors (20–64 years old) from the "Etablissement Français du Sang" (EFS). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (Eurobio, Les Ulis, France).

These investigations were approved by the local Ethics Committee ("Comité Consultatif de Protection des Personnes"), Ile de France VII (Kremlin Bicêtre, France). The relevant authorization numbers are ID RCB 2006-A00164-47 and 2010-A00250-39.

## Isolation of CD4+ T Cells

CD4 + T cells were obtained from fresh thymic tissue or from fresh PBMCs, as previously described (10). Total CD4 +, CD4 + CD25 +, or CD4 + CD25- cells were purified using magnetic separation according to the manufacturer's instructions (Dynabeads CD4 + CD25 + Treg Separation Kit, Life Technologies, Saint Aubin, France and CD4 + CD25 + regulatory T cell Isolation Kit, Miltenyi, Paris, France). For the isolation of CD4 + thymic cells, we added an anti-CD8 antibody (AbSerotec, Düsseldorf, Germany) to eliminate double-positive cells expressing both CD4 and CD8.

## Flow Cytometry

Staining was performed on fresh total and frozen CD4 + thymocytes, as well as on frozen PBMCs. The antibodies used in this study are described in **Table 2**. Two different combinations of antibodies were used and are described in **Table 3**. Data were acquired on a BD FACS CALIBUR then analyzed using Cellquest software (United States) for the combination of 4 colors, and on BD FACS Verse [with FACSuite software then analyzed using FlowJo Software (United States)] for the combination of 6 colors. Gating strategies to identify subpopulation of Treg are shown in **Supplementary Figure 1**.

## Cell Culture

Cell culture products (Hank's balanced salt solution (HBSS), RPMI 1640 Glutamax I medium, Penicillin, Streptomycin, Trypsin) were obtained from Invitrogen (Cergy-Pontoise, France) while sera were obtained from Eurobio (Les Ulis, France) and Ultroser-G from PALL-Biosepra (Cergy-Pontoise, France).

Primary TEC cultures were obtained from mechanically minced fresh human thymus tissue and seeded onto cell culture flasks, as previously detailed (27). These culture conditions result mainly in medullary TECs (mTECS) that remain functional (27). Indeed, mTECs in primo-cultures keep their ability to express key molecules involved in immune tolerance processes such as autoimmune regulator, tissue-specific antigens, chemokines, and cytokines (27). After 8 to 12 days of primary culture, the confluent monolayers were trypsinized and used in co-culture experiments, or frozen for further use.

**TABLE 2 |** List of antibodies used in cytometry experiments.

| Specificity | Label | Type | Clone | Producer |
|---|---|---|---|---|
| CD4 | FITC | Mouse IgG1, κ | MT 310 | DAKO |
| CD4 | PE | Mouse IgG1, κ | MT 310 | DAKO |
| CD4 | APC | Mouse IgG1, κ | MT 310 | DAKO |
| CD4 | PerCp-Cy5.5 | Mouse IgG1, κ | RPA-T4 | BECTON DICKINSON |
| CD8 | APC | Mouse IgG1, κ | DK25 | DAKO |
| CD8 | PE-Cy7 | Mouse IgG1, κ | RPA-T8 | BECTON DICKINSON |
| CD25 | PE-Cy7 | Mouse IgG1, κ | 2A3 | BECTON DICKINSON |
| CD25 | PE | Mouse IgG1, κ | ACT-1 | DAKO |
| CD31 | PerCp-eFluor 710 | Mouse IgG1, κ | WM-59 | eBioscience |
| CD45RA | FITC | Mouse IgG2b | H1100 | BECTON DICKINSON |
| CD95 | PE | Mouse IgG1, κ | DX2 | BECTON DICKINSON |
| CD95 | APC | Mouse IgG1, κ | DX2 | BECTON DICKINSON |
| CD127 | Brilliant Violet 421 | Mouse IgG1, κ | HIL-7R-M21 | BECTON DICKINSON |
| CXCR3 | FITC | Mouse IgG1 | 49801 | R&D Systems |
| CCR4 | PE | Mouse IgG2b | 205410 | R&D Systems |
| CCR4 | FITC | Mouse IgG2b | 205410 | R&D Systems |
| CXCR5 | Biotin | Mouse IgG2b | 51505 | R&D Systems |
| CXCR5 | Alexa Fluor 488 | Rat IgG2b | RF8B2 | R&D Systems |
| CCR7 | FITC | Mouse IgG2a | 150503 | R&D Systems |
| CCr7 | APC | Mouse IgG2a | 150503 | R&D Systems |
| Foxp3 | eFluor 660 | Mouse IgG1, κ | 236A/E7 | eBiosciences |

## Cocultures

Freshly purified CD4 + thymocytes ($5 \times 10^5$ cells/well) were cultured alone, or cocultured with mTECs, in 24-well plates in RPMI 1640 Glutamax I medium supplemented with 10% fetal calf serum as previously described (17). For staining experiments, CD4 + cells were cocultured with $2 \times 10^5$ mTECs during 24 h. For immunosuppression assay, the CD4 + were cocultured with $1 \times 10^5$ mTECs for 3 days.

## Suppressive Assay

The suppressive activity of CD4 + T cells cultured alone or with mTECs was evaluated by tritiated thymidine incorporation, as previously described (10). The suppressive capacity of Treg was normalized as the percent of proliferative response of phytohaemagglutinin-activated Tconv alone. In some experiments, blocking anti-TSLP (R&D Systems, Lille, France) antibody was used at 0.1 μ g/ml.

## Enzyme-Linked Immunosorbent Assay

Thymic stromal lymphopoietin (TSLP) concentrations were measured in duplicate from supernatants of cultures of TECs, using commercially available enzyme-linked immunosorbent assays (Peprotech, France) according to the manufacturer's instructions. Measurements were performed on 100 μl of cell culture supernatant previously frozen and analyzed on a MRX

**TABLE 3 |** Combination of antibodies used in flow cytometry experiments.

**A. Combinations of 4 antibodies**

| Antibody 1 | Antibody 2 | Antibody 3 | Antibody 4 |
|---|---|---|---|
| CD4 FITC | CD95 PE | CD25 PE-Cy7 | CD8 APC |
| CD4 FITC | CCR4 PE | CD25 PE-Cy7 | CD8 APC |
| CD4 FITC | CD25 PE | CXCR5 biotin + Streptavidin PE-CY7 | CD8 APC |
| CXCR3 FITC | CD4 PE | CD25 PE-Cy7 | CD8 APC |
| CCR7 FITC | CD4 PE | CD25 PE-Cy7 | CD8 APC |

**B. Combinations of 5 or 6 antibodies**

| Antibody 1 | Antibody 2 | Antibody 3 | Antibody 4 | Antibody 5 | Antibody 6 |
|---|---|---|---|---|---|
| CD4 APC | CD8 PE-Cy7 | CD25 PE | CD127 BV 421 | CD45RA FITC | CD31 PerCp-eF 710 |
| CD4 PerCp-Cy5.5 | CD8 PE-Cy7 | CD25 PE | CD127 BV 421 | CD45RA FITC | FOXP3 eF660 |
| CD4 PerCp-Cy5.5 | CD8 PE-Cy7 | CD25 PE | CD127 BV 421 | CXCR3 FITC | |
| CD4 PerCp-Cy5.5 | CD8 PE-Cy7 | CD25 PE | CD127 BV 421 | CXCR5 Alexa Fluor 488 | |
| CD4 PerCp-Cy5.5 | CD8 PE-Cy7 | CD25 PE | CD127 BV 421 | CCR4 FITC | CCR7 APC |
| CD4 PerCp-Cy5.5 | CD8 PE-Cy7 | CD25 PE | CD127 BV 421 | CD95 APC | |

Revelation microplate reader from Dynex (Thermolab System, Chantilly, VA, United States).

## Statistical Analyses

Differences between groups were evaluated using parametric or non-parametric *t*-tests for paired or unpaired data (Graph Pad Software, San Diego, CA, United States), with the significance level set to $p < 0.05$. Each figure legend mentions the statistical test used. Other statistical tests have been used and are indicated in the text (One-way anova to compare 3 groups and Spearman non-parametric correlation).

# RESULTS

## Functional Characterization of Thymic and Peripheral Regulatory Cells

The suppression function of Treg is profoundly impaired in the MG thymus (10). In order to investigate whether peripheral Treg behave similarly, we compared the suppressive function of Treg isolated from the thymus or from peripheral blood cells from MG and control donors.

In both compartments, the suppressive function was impaired in MG patients. In the thymus, the average proliferation was 23.0% for controls and 81.1% for MG patients (**Figure 1A**, $p < 0.001$). In PBMCs, the average proliferation was 29.4% for controls and 60.8% for MG patients (**Figure 1B**, $p < 0.04$). While in the thymus, there was no overlap between MG and control values, it was not the case for PBMC results (**Figures 1A,B**).

The more impaired suppressive activity of thymic Treg compared to peripheral Treg suggests that Treg from these two compartments might present phenotypic differences.

## Phenotypic Characterization of Thymic and Peripheral Regulatory Cells

To analyze the phenotype of Treg, we used several combinations of markers. We first analyzed the level of CD25 expression.

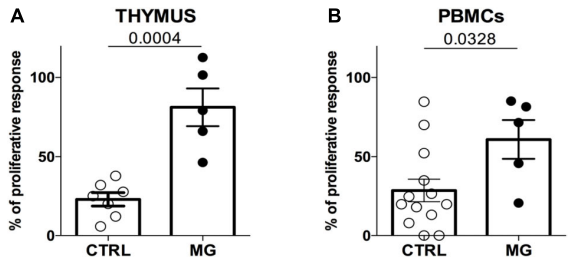



**FIGURE 1 |** The suppression function is more impaired in the thymus than in the periphery in MG patients. Percentages of proliferation of Tconv in co-culture with Treg (ratio 1:1) from control individuals (CTRL) or patients with myasthenia gravis (MG) using cells derived from the thymus **(A)** or from PBMCs **(B)**. Data represent the mean ± standard error of the mean. Statistical test: Two-tailed *t*-test.

We then investigated the combination of CD25 with CD127 since CD25 + CD127neg cells are defined as regulatory cells (22). Finally, to further define most precisely the subtypes of Treg, we used the CD45RA marker together with CD25 and FoxP3 to differentiate resting (CD45RApos CD25lo FoxP3lo) (rTReg), effector Treg (CD45RAneg CD25hi FoxP3hi) (eTReg) and CD45RAneg CD25lo FoxP3lo (CD25lo non-Treg, defined as FIII) cells as previously described (24). The gating strategy is shown in **Supplementary Figure 1**.

In MG patients, the percentages of Treg were reduced in the thymus but not in the periphery regardless of the gating strategy to define Treg (**Figure 2A**). Fractioning Treg subsets based on CD45RA expression revealed that percentages of both resting and effector Treg were reduced in the thymus of MG patients but not in the periphery (**Figure 2A**). As a consequence of Treg reduction, the percentage of CD25neg cells was increased in the thymus (**Figure 2A**, left panel). In PBMCs, the percentage of CD25neg was decreased together with an increase in CD25lo cells (**Figure 2A**, right panel). These results show a very dissimilar phenotype of Treg in the thymus and PBMCs.

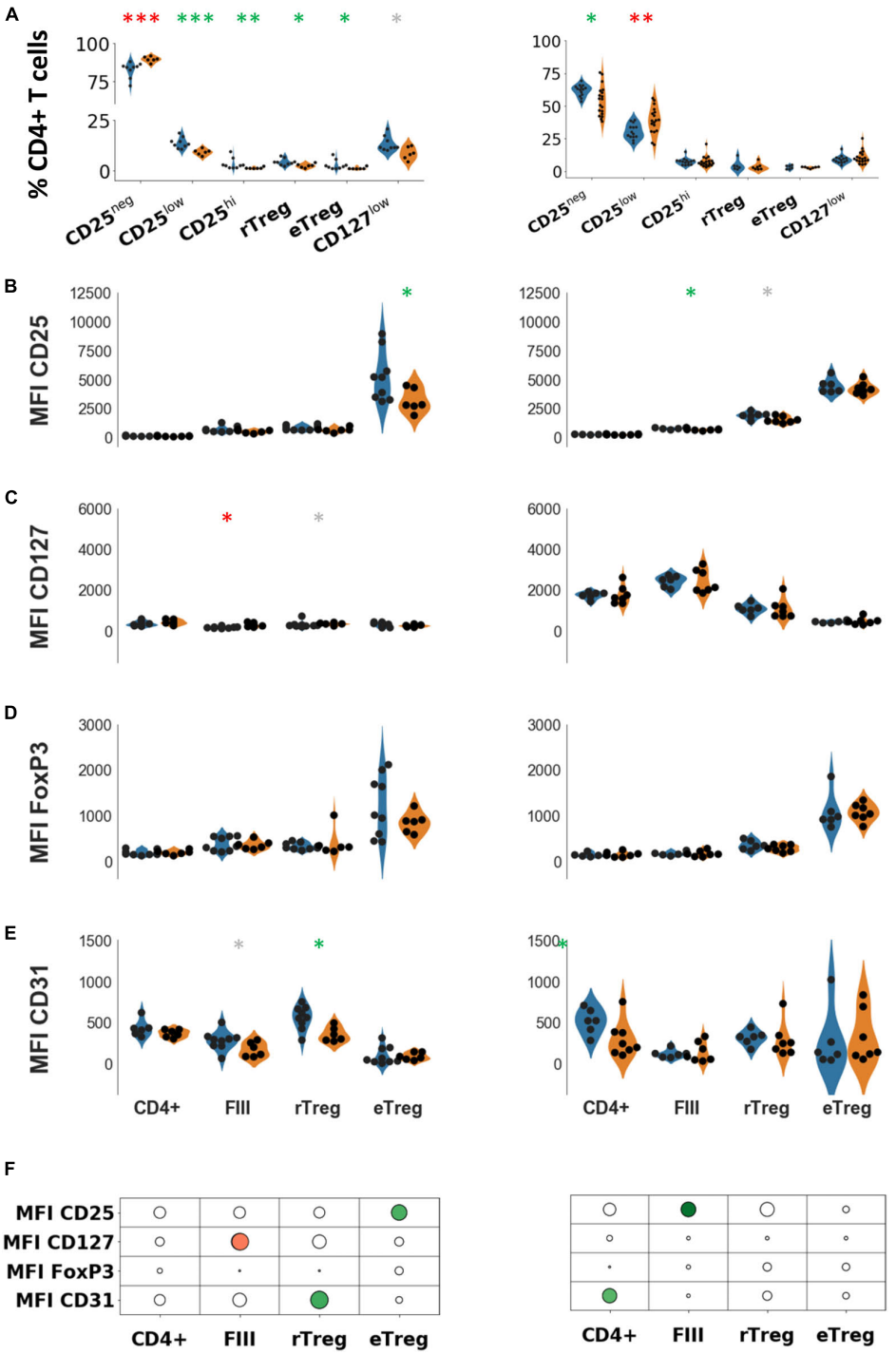



**FIGURE 2 |** Phenotypic characterization of thymic and peripheral regulatory cells. **(A)** Percentages of indicated cell populations among total CD4 + T cells in healthy individuals (blue) or in myasthenic patients (orange) in the thymus (left panel) or in PBMCs (right panel). Mean fluorescent intensities of CD25 **(B)**, FoxP3 **(C)**, CD127 **(D)**, CD31 **(E)** in healthy individuals (blue) or in myasthenic patients (orange) in the 3 subsets defined by the combination of CD45RA and CD25 in thymus (left panel) or in PBMCs (right panel). Statistical test: two-tailed Mann-Whitney (*$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.005$, gray star corresponds to $p < 0.1$). **(F)** Statistical summary of the data shown in **(B–E)**. Colorized bubbles correspond to significant differences. Size is proportional to the statistical significance and color represents fold change between mean values of control individuals and MG patients (green, lower value in MG; red, higher value in MG).

To characterize more precisely the phenotypic changes of the subsets defined with CD45RA (rTReg, eTreg FIII) in the thymus and PBMCs, we determined mean fluorescent intensities of markers associated with Treg function. In the thymus of MG patients, CD25 expression was significantly reduced in eTreg but not in the other subsets while in PBMCs a decrease in CD25 expression was observed in FIII (**Figure 2B**) but not in the other subsets. The level of CD127 was higher in FIII ($p < 0.02$) and in rTreg without reaching statistical significance ($p = 0.066$) in the MG thymus but not in PBMCs (**Figure 2C**).

CD127 expression is expected to be low in the two Treg subsets (rTreg and eTreg) and high in FIII. This was the case in the periphery ($p < 0.001$, one-way ANOVA for controls and MG) where an inverse correlation between CD25 and CD127 was observed ($r^2 = -0.77$ for MG patients and $r^2 = 0.87$ for controls, $p < 0.0001$, Spearman correlation test), but not in the thymus. CD127 expression was much lower in the thymus as compared with PBMCs (4 to 5 fold, $p < 0.0001$ for both MG and control groups, *t*-test) and the level in the thymus was similar in the different subpopulations (One-way ANOVA test).

As expected, the level of FoxP3 was higher in eTreg compared to the other subsets in both thymus and PBMCs (**Figure 2D**), but there was no change at all in MG patients compared to controls, both in the thymus and in PBMCs (**Figure 2D**).

We also investigated the expression of CD31 (PECAM-1), a marker of recent thymic emigrants that is lost with excessive immunoreactivity (28). The expression of CD31 in thymic CD4 + cells from MG patients was significantly reduced in rTreg while it was unchanged in eTreg, and a trend for a decrease in FIII was observed (**Figure 2E**, left panel). In PBMCs, the level of CD31 was unchanged in MG in the three different subsets although a decrease in CD31 expression was observed in the whole CD4 population (**Figure 2E**, right panel). A decrease in CD31 is consistent with an increased proliferation and activation of these cells (29). CD31 expression was reported to be higher in circulating FoxP3 + rTreg than in eTreg (30) (albeit in the absence of statistical testing), we observed this profile more clearly in the thymus than in the blood.

The results are summarized in the **Figure 2F** showing that the alterations of CD4 cells are dissimilar in the thymus and PBMCs. In the thymus, alterations were observed in all cell subsets, while in PBMCs, Treg subsets (rTreg and eTreg) did not appear to be affected.

## Expression of CD95 and Chemokine Receptors in Treg Subsets

In healthy subjects, the percentage of CD95 + cells increased with the level of CD25 on CD4 + T cells but more moderately in the thymus ($p = 0.054$, Spearman correlation test) than in the periphery ($p < 0.0001$, Spearman correlation test) (**Figure 3A**). Confirming what we previously showed (10), the percentage of CD95 + cells was significantly higher in thymocytes from MG patients compared with controls in all subpopulations (CD25neg, CD25lo, and CD25hi) (**Figure 3A**, left panel). In PBMCs, higher percentages of CD95 + cells were also observed in CD25neg and CD25low populations, but not in the CD25hi cells (**Figure 3A**, right panel).

Trafficking receptors expressed on Treg undergo changes according to their stages of activation and differentiation (31). CXCR3 and CXCR5 levels are increased in CD4 + T cells in the thymus and PBMCs of MG patients (32, 33). The thymic overexpression of CXCR3 and CXCR5 on CD4 + T cells in MG patients was independent of CD25 expression (**Figures 3B,D**, left panel). In PBMCs, the overexpression of CXCR3 and CXCR5 on CD4 + T cells was observed in CD25neg and CD25lo cells but not in CD25hi cells (**Figures 3B,D**, right panel).

In the thymus, the levels of CCR4 were high (> 60% in all CD4 + T cell subsets) (**Figure 3C**, left panel) while CCR7 was hardly detectable (**Figure 3E**, left panel). In PBMCs, the level of CD25 expression on CD4 + T cells was correlated with the level of CCR4 ($p < 0.0001$, Spearman correlation test) and inversely correlated with the level of CCR7 ($p < 0.001$, Spearman correlation test). Nevertheless, levels of CCR7 and CCR4 on CD4 + T cells were unaltered in MG both in the thymus (**Figures 3C,E**, left panel), and in PBMCs (**Figures 3C,E**, right panel).

Altogether, immune dysregulations were more pronounced in the thymus than periphery although some modifications were also found in the periphery such as CD95 and chemokine receptors (**Figure 3F**). Changes in the CD25hi subset was only observed in the thymus. These results suggest that the altered thymic environment in MG may directly contribute to the phenotypic differences we observed between MG patients and controls.

## Association Between Immune Defects and Clinical Features

To address whether clinical aspects of the disease affected the observed differences, we confronted the results shown in **Figures 2**, **3** with available clinical data. Although some analyses were not conclusive due to a low number of samples, several interesting observations were made.

### Thymus Pathology

We observed that the expression of several markers was positively correlated with the germinal center grade in the thymus: CD95 in CD25hi ($p < 0.05$) and CXCR3 in CD25lo ($p < 0.005$), suggesting that the changes in thymic cell subsets involve CD25 positive cells (including activated and regulatory cells) (**Supplementary Figures 2A,B**). Besides, we observed some changes in PBMCs: the percentage of CD25 + CD127- cells increase with the thymic germinal center grade (**Supplementary Figure 2C**) suggesting that thymic events could impact Treg changes in the periphery.

### Duration of the Disease

We did not observe any correlation between the duration of the disease and any biological parameter in the thymus, but the number of samples in each subgroup was small. In PBMCs, we found that patients with a long disease duration tended to have higher expression of CD25 and a higher percentage of CD25loCD45RAneg (FIII) (**Supplementary Figures 2D,E**).

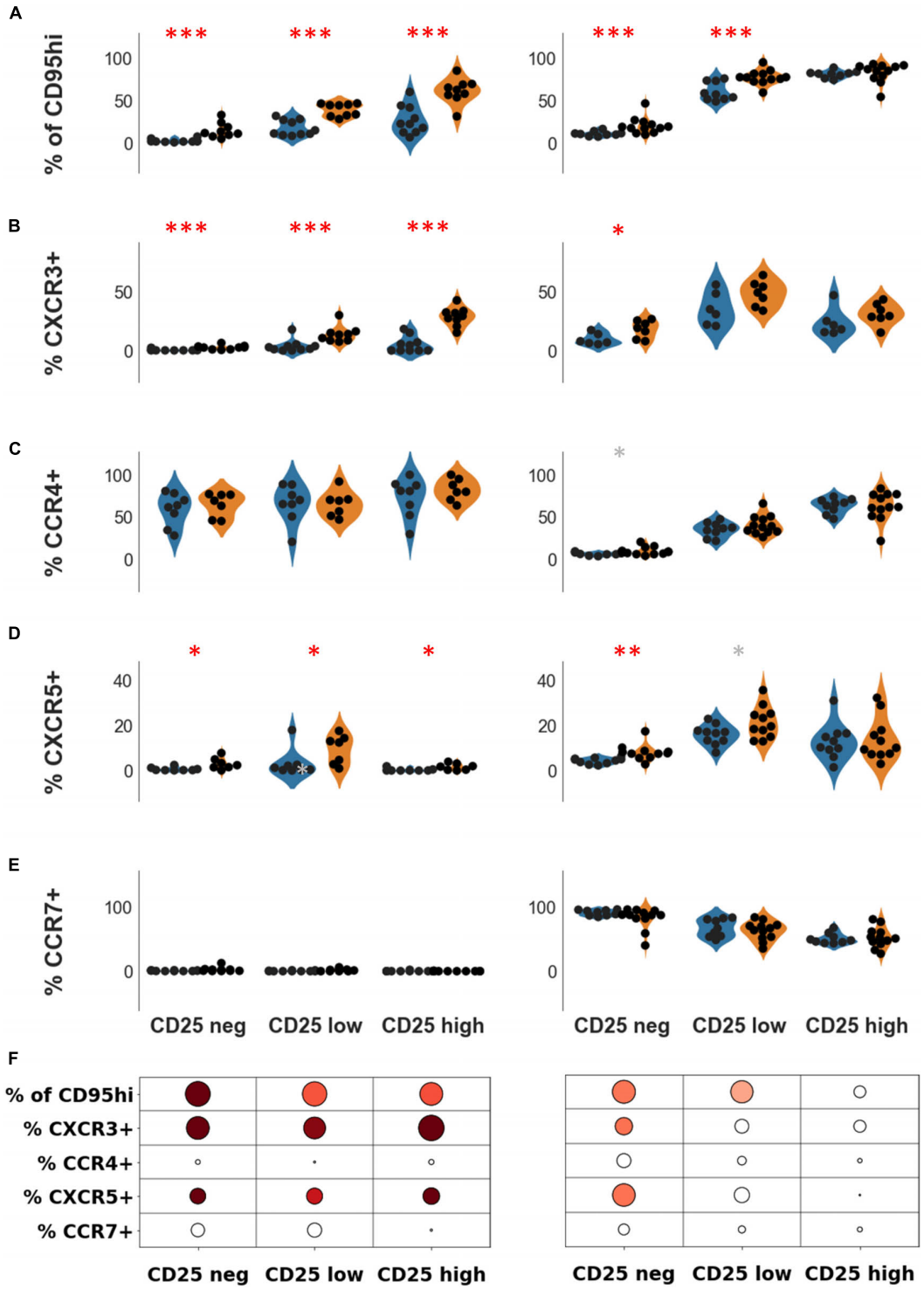



**FIGURE 3 |** Expression of CD95 and chemokine receptors in Treg subsets. Percentages of CD95hi **(A)** CXCR3 **(B)**, CCR4 **(C)**, CXCR5 **(D)**, and CCR7 **(E)** according to CD25 expression, in healthy individuals (blue) or in myasthenic patients (orange) in the thymus (left panel) or in PBMCs (right panel). Statistical test: two-tailed Mann-Whitney (*$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.005$, gray star corresponds to $p < 0.1$). **(F)** Statistical summary of the data shown in this figure. Colorized bubbles correspond to significant differences. Size is proportional to the statistical significance and color represents fold change between mean values of control individuals and MG patients (green, lower value in MG; red, higher value in MG).

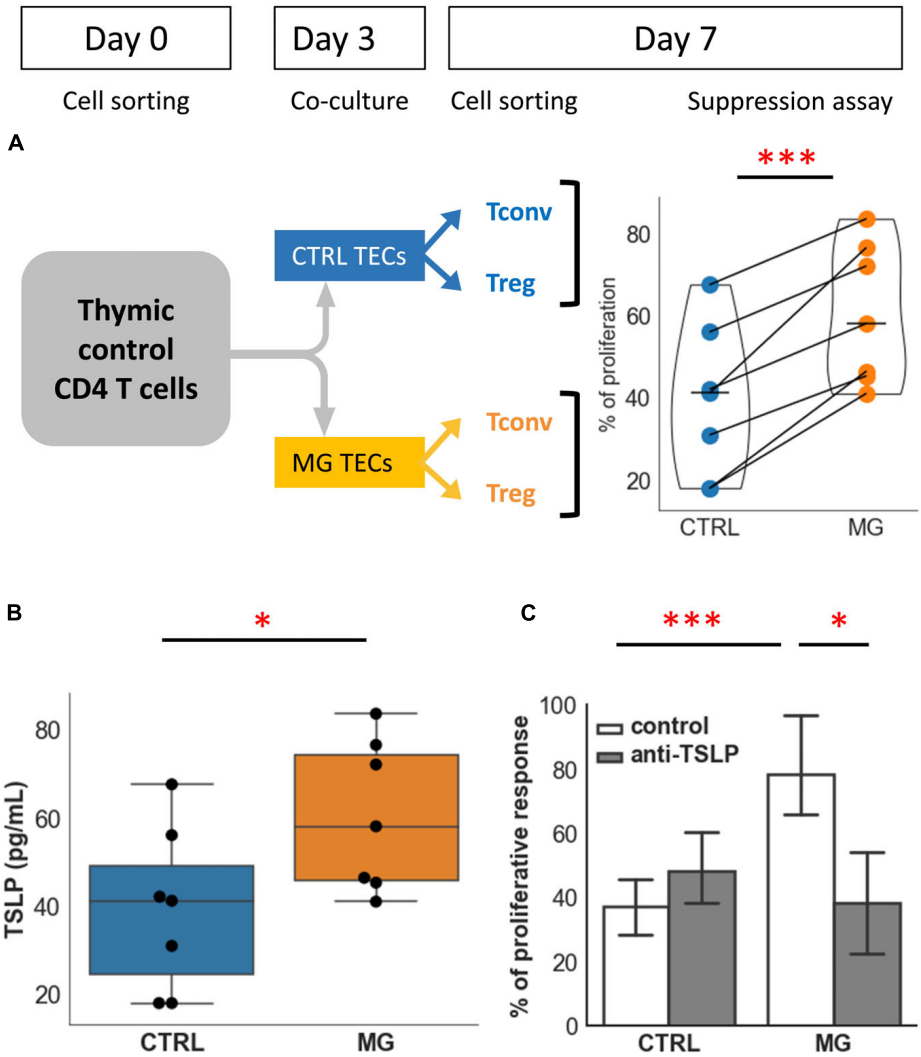



**FIGURE 4 |** TECs contribute to the functional defect of Treg: role of TSLP. **(A)** Percentages of proliferation of T conv in co-culture with Treg following co-culture of CD4 + cells from control individuals with TECs either from control individuals (CTRL, blue) or from MG patients (MG, orange). The experimental protocol is explained in the top left panel. **(B)** Production of TSLP measured in supernatants of cultured TEC from control individuals (CTRL, blue) or MG patients (MG, orange). **(C)** Percentage of proliferation of Tconv in co-culture with Treg following co-culture with TEC from control individuals (CTRL) or MG patients as described in **(A)**, in the absence or presence of a TSLP neutralizing antibody. Statistical tests: two tailed paired *t*-test in **(A,C)**, two-tailed Mann-Whitney in **(B)** (*$p \leq 0.05$; ***$p \leq 0.005$).

### Antibodies

Because the number of seronegative patients was low, it was not possible to determine whether the absence of auto-antibodies was related to the measured parameters. However, we did not observe any convincing correlation between measured parameters and levels of anti-AChR antibodies.

### Clinical Grade

We observed interesting correlations between the clinical grade and Tconv/Treg balance in PBMCs. Percentages of CD25 + Treg and of CD25lo were higher in severely affected patients compared with mild patients, while it was the opposite for CD25neg Tconv cells (**Supplementary Figures 3A–C**). The percentage of CD25 + CD127- cells was also higher in patients with more severe disease (**Figure 3D**). Finally, the level of CCR4 in CD25lo cells was correlated with the clinical grade (**Supplementary Figure 3E**); the severely affected patients have a lower level of CCR4, which is a marker associated with immune regulation.

## TECs Contribute to the Functional Defect of Treg: Role of TSLP

We previously showed that the thymic epithelium plays a major role in the generation of Treg (17). We wondered whether regulatory function defects in MG could be explained by alterations of MG TECs.

To answer this question, we co-cultured healthy thymic CD4 + T cells with control or MG TECs for 3 days. Then Teff and Treg were purified, and we tested the suppressive function in a proliferation assay. We observed a consistent loss of regulatory

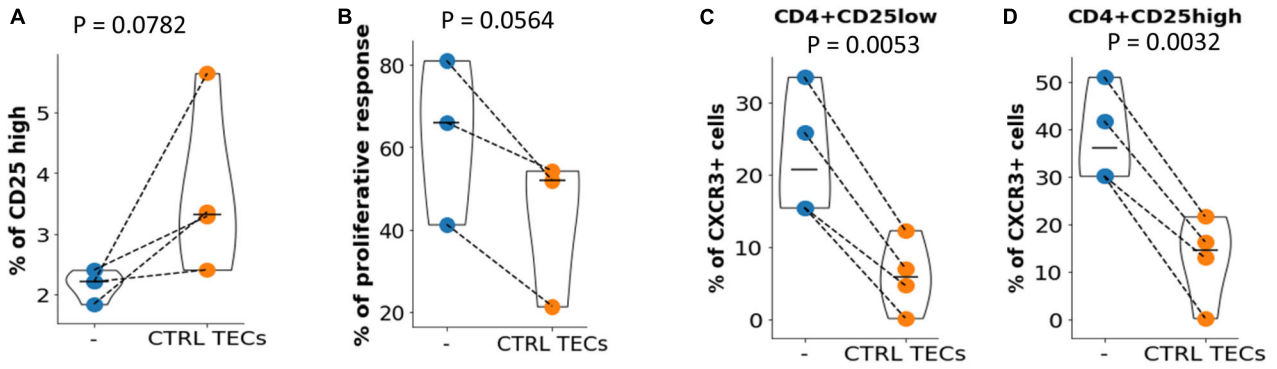



**FIGURE 5 |** Immune regulation in MG CD4 + cells could be restored. **(A)** Percentages of CD25 high cells in CD4 + T cells from MG patients before (–) or after (CTRL TECs) co-culture with TEC from control individuals. **(B)** Proliferation of Tconv in co-culture with Treg before (–) or after (CTRL TECs) co-culture of thymic CD4 + cells from MG patients with TEC from control individuals (CTRL, blue). Percentage of CXCR3 + cells among CD4 + CD25low **(C)** or CD4 + CD25high **(D)** thymic CD4 + cells from MG patients before (–) or after (CTRL TECs) co-culture with TECs from control individuals. Statistical tests: two-tailed paired *t*-test.

activity in all experiments ($n = 7$) (**Figure 4A**). Indeed, co-culture with control TECs resulted in a mean percentage of proliferation that was 39% while co-culture with MG TECs resulted in a mean percentage of proliferation that was approximately 60%. The mean of differences between the 2 culture conditions was 21.3%. These results support the idea that MG TECs contribute to the immune regulation defects of MG T cells.

We then hypothesized that soluble factors produced by TECs and having an effect on Treg could mediate the pathogenic mechanism due to MG TECs. We investigated IL-6 and TSLP that are known factors influencing regulatory function (34, 35).

Supernatants produced by TECs from MG patients produced significantly higher levels of TSLP than that from controls (**Figure 4B**). In our co-culture experiments, a neutralizing anti-TSLP antibody partially restored the suppressive capacity of Treg derived from co-cultures with MG TECs (**Figure 4C**). This suggested that TSLP was one of the factors contributing to the defect of thymic Treg in MG patient. By contrast, anti-IL-6 antibody had no effect on the suppressive capacity of Treg (not shown).

To analyze whether the level of TSLP is also increased in the whole thymus, we performed real-time PCR in thymic samples, and observed a significant increase in TSLP in MG thymus compared with age-matched controls, although this global analysis does not allow to determine which cell type over produce TSLP (**Supplementary Figure 4**).

Altogether, these results show that TECs played a significant role in the defect of Treg in MG patients, and this effect was at least partially mediated by TSLP that was overproduced by MG TECs.

## Immune Regulation in MG CD4+ Cells Could Be Restored

The experiments described above indicated that thymic CD4 + T cells from MG patients present numerous defects affecting Treg. Since TECs appear to play a key role in these defects, we asked if it was possible to normalize the number and function of MG Treg by a coculture with control TECs. The coculture of thymic CD4 + T cells from MG patients in presence of control TECs induced an increase in the CD25high cell number in the 4 experiments (**Figure 5A**). To evaluate the suppressive function, CD4 + thymic MG cells were incubated with control TECs for 3 days and Treg and Tconv were purified for a suppression assay. The suppression defect was reduced in all experiments (3 different MG patients tested) (**Figure 5B**). Finally, normal TECs decreased the expression of CXCR3 in CD25lo and CD25hi cells (**Figures 5C,D**), but had no effect on CXCR5 (not shown).

Collectively, these results show that the immune regulation defects of T cells could be corrected when MG T cells were pre-incubated with control TECs.

# DISCUSSION

This work presents a detailed comparative phenotyping analysis of thymic and blood Treg in MG and pinpoints a potential role of TECs in the thymic immunoregulatory defects. The main results of the study are: (1) a significant difference in the phenotype of thymic CD4 cells compared to peripheral cells in MG patients. Patients' thymic cells present alterations more striking than peripheral cells; (2) a significant impact of TECs is shown; (3) a potential participation of TSLP in the pathogenic processes is proposed and has never been described.

## Thymic and Peripheral Treg in Myasthenia Gravis

Published data are quite inconsistent for Treg numbers in MG. Several studies reported that percentages of Treg were unchanged in MG (10, 36, 37), while others reported some changes (38, 39). Previous studies investigated either cells extracted from the thymic tissue or blood, and the majority found that Treg from MG patients have impaired immunosuppressive functions in both tissues (10, 36, 40). This study is the first study to compare the function and phenotype of Treg from the inflammatory organ, the thymus, and from peripheral blood in control individuals and MG patients. We observed lower percentages

of thymic, but not circulating, Treg in MG patients, regardless of whether we defined them as CD25hi or CD25 + CD127-. Treg fractioning based on CD45RA expression revealed that both rTreg and eTreg were reduced in the thymus, but not in the blood, of MG patients. These results suggest that thymic specific factors could have impacted the phenotype of thymic Treg in MG. Surprisingly, FoxP3 expression was unaltered in MG, neither in the thymus nor in the periphery, consistent with the publication by Kohler et al. (39), but not with that of Thirrupathi et al. (36). To explain these apparent contradictions, we propose several explanations: (1) even if some reports indicate changes in the numbers of Treg, it is a mild one that could be easily missed in other cohorts due to patients heterogeneity. Disease duration or corticosteroid treatment could also impact the number of Treg. In multiple sclerosis, while both circulating rTreg and eTreg have an impaired suppressive function in patients with a disease duration lower than 10 years, eTreg appeared to have a restored function in patients with a longer disease duration (41). It will be interesting to determine if the differences we observed are affected by the disease duration. (2) it is also possible that these differences are dependent on experimental conditions. For example, using fresh total cells, or purified frozen cells could lead to different results as the cell subsets analyzed are not strictly the same ones; in addition, the methods used (PCR versus flow cytometry) and the nature of the antibody clone could also induce differences in the results.

Altogether, our results indicate that percentages of thymic Treg are decreased in MG, confirm the immunosuppressive impairment of Treg in MG patients and show that thymic Treg are phenotypically different from the peripheral Treg. These results are in line with previous data showing that changes in the percentage of cells expressing high level of CD95 is much more pronounced in the thymus than in the periphery (8).

## What Could Be the Role of the Thymic Epithelium in Myasthenia Gravis?

We previously showed that the inflammatory environment in the MG thymus could play an important role in the pathogenesis of MG by generating T cells that are out of control (11), but the thymic component responsible for this impairment was not identified in this work. Previous publications including ours, demonstrated that the thymic epithelium in MG presents some functional alterations. TECs from MG patients produce excessive amounts of cytokines (IL-6 and IL-1β), chemokines (CXCL13 and RANTES), as well as kinases (p38 and ERK1/2 MAPKs) (15, 42– 44). We recently showed that MG TECs overproduce IL-23 and TGF-β3 (16). Together with the increase in IL-6, and IL-1β, MG-TECs provide a pro-inflammatory context that contributes to the differentiation and activation of pathogenic Th17 cells (16). In addition, IFN-β that is overexpressed in MG thymuses (13) increases the expression of the autoantigen (α-AChR) and the production of CXCL13 and BAFF by mTECs (14). The current study provides a straightforward evidence that the thymic epithelium plays a role in the functional alterations of Treg in MG. We were able to show that control thymic CD4 + T cells co-cultured with MG TECs lost a part of their ability to suppress Tconv proliferation.

Interestingly, the epithelium has also been implicated in other autoimmune pathologies, such as Sjogren's syndrome (SS) and primary biliary cholangitis (PBC). In SS patients, the functional impairment of salivary glands comes as a result of an immune attack on epithelial cells of the affected organs, which has been named autoimmune epithelitis (45). Similar to MG, the epithelial cells overproduce cytokines and chemokines. Thus, we hypothesize that a defect in the epithelium mediates the pathogenesis in MG. How could this be possible? The epithelium is frequently an entry point for microorganisms and is constituted of one or several layers of epithelial tissue in many organs. The thymus is not an exception since a monolayer of epithelial cells surrounds each thymic lobule. In some autoimmune pathologies, the epithelial tissue undergoes increased apoptosis, which could lead to a presentation of many epithelial antigens. Although, this has not been shown in *ex vivo* experiments, indirect arguments favor such a possibility since IFN-β induces the TECs death *in vitro* and the uptake of TECs proteins by dendritic cells (14). The presence of viruses in the epithelium could lead to the production of IFN-I that will favor the recruitment of inflammatory cells and activate them to induce an immune response. Besides, the epithelial cells could play the role of antigen-presenting cells as they express HLA class II antigens and are capable of expressing co-stimulatory molecules.

In the current study, we showed that excessive TSLP production by MG TECs reduced the suppressive function of Treg.

The relationship between TSLP, Treg, and human thymus was described for the first time in 2005 by Watanabe et al. who showed that Hassall's corpuscles induce the proliferation and differentiation of Tconv into Treg via the production of TSLP that activate thymic dendritic cells (46). Other findings show that TSLP plays an important role in the expansion and survival of CD4 + T cells (47). In addition, in the asthmatic allergy model, it was shown that TSLP directly and selectively impairs IL-10 production of Treg and inhibits their suppressive activity (48). Together these findings show that TSLP has a complex role and could either promote the differentiation of Treg or impair its production. The likely explanation is the existence of two variants for TSLP in human tissues. The main isoform (short form) is expressed in steady-state and plays a homeostatic role, while the long form is upregulated in inflammatory conditions (49). In our study, it is likely that the increased expression of TSLP by mTEC is the inflammatory long-form. Although we did not analyze IL-10 production, we showed that inhibition by the anti-TSLP antibody could restore the defective suppressive function of patients' CD4 + T cells. Interestingly, viral stimuli could enhance TSLP expression by pulmonary epithelium (50); thus, the increased production of TSLP by MG TECs could be due to a viral stimulus in the thymus, a hypothesis previously discussed (4). In favor of this hypothesis, we showed that control TECs stimulated with Poly(I:C) or with IFN-I, increase their production of TSLP (data not shown). Interestingly, in human esophageal epithelial cells or in lung fibroblasts, the long isoform

but not the short one is regulated by poly(I:C) or TNF-α, respectively (51, 52).

### SIgnificance of CD31 Decrease in MG CD4+ T Cell Subsets

The CD31 molecule, also called PECAM-1, is expressed on the surface of many cells, including cells of the immune system. In CD4 + T cells, CD31 is often regarded as a marker for naive cells, although some memory cells also express CD31. The expression of CD31 is lost after *in vitro* stimulation and expansion cycles (42). It, therefore, appears that CD31 is a differentiation antigen of CD4 + T cells, which is lost during maturation and proliferation. CD31 helps to control T-cell activation, and in its absence, T cells have a higher propensity to become activated, resulting in increased susceptibility to becoming apoptotic (43). Also, expression of PECAM-1 is important for dampening levels of multiple pro-inflammatory cytokines on the cellular and whole animal level (45).

Expression of CD31 is found on blood T cells that had recently emigrated from the thymus (53). This cell subset has an enhanced proliferative capacity, and it has been proposed that these cells enhance susceptibility to autoimmune disease (44). Our study revealed that in the thymus, rTreg have a decrease in CD31, suggesting that this particular population was probably activated and stimulated without acquiring the CD45RO marker memory marker. Interestingly in the periphery, CD31 is not altered in Treg, which underlines the difference with thymic Treg. However, CD31 expression is decreased in the overall CD4 population, indicating that CD31 is decreased in Tconv. The significance of this finding is probably increased activation and stimulation of these cells. These results show that although the Treg are not altered in the blood of MG patients, other pathogenic mechanisms might take place in the periphery. In a general context, the number of CD31 + cells was found to be diminished in several pathologies. Reduced CD31 + T cells is a hallmark of atherosclerotic plaque thrombosis (54). Multiple sclerosis is associated with lower percentages of circulating rTreg expressing CD31 (13).

In conclusion, this work highlights the role of the thymus in MG, as it shows for the first time that alterations in CD4 cells are more pronounced in the thymus than in the blood. We here explain the causes for thymic CD4 alterations; we demonstrate that TECs play a major role in Treg thymic defects and that TSLP overproduced by MG TECs is one of the soluble mediators that contributes to the thymic immune defects. Finally, replacing MG TECs by normal TECs allowed restoring the immunosuppressive abilities of MG Treg. These findings demonstrate that in a favorable environment, it is possible to reestablish the regulatory function of Treg in MG patients.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethics Committee ("Comité Consultatif de Protection des Personnes"), Ile de France VII (Kremlin Bicêtre, France). The relevant authorization numbers are ID RCB 2006-A00164-47 and 2010-A00250-39. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

FT, DN, and AG performed and analyzed the experiments. FT collected the samples. BE provided the blood samples. EF and RR provided the thymic samples. JV, RL, and SB-A read and revised the manuscript. FT, JV, and SB-A analyzed the experiments and wrote the manuscript.

## FUNDING

This work was supported by grants from the European Community (FIGHT-MG/HEALTH-2009-242-210) and the "Association Française contre les Myopathies" (AFM).

## ACKNOWLEDGMENTS

We thank Perrine Cufi and Odessa-Maud Fayet for technical assistance on TSLP RT-PCR, Dr. Vincent de Montpreville from the anatomy and histology department from the Marie Lannelongue Surgical Center.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00782/full#supplementary-material

## REFERENCES

1. Gilhus NE, Tzartos S, Evoli A, Palace J, Burns TM, Verschuuren JJGM. Myasthenia gravis. *Nat Rev Dis Prim*. (2019) 5:30. doi: 10.1038/s41572-019-0079-y
2. Berrih-Aknin S. Myasthenia gravis: paradox versus paradigm in autoimmunity. *J Autoimmun*. (2014) 52:1–28. doi: 10.1016/j.jaut.2014.05.001
3. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo H-C, Marx A, et al. Randomized trial of thymectomy in Myasthenia gravis. *N Engl J Med*. (2016) 375:511–22. doi: 10.1056/NEJMoa1602489
4. Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J Autoimmun*. (2014) 52:90–100. doi: 10.1016/j.jaut.2013.12.011

5. Truffault F, de Montpreville V, Eymard B, Sharshar T, Le Panse R, Berrih-Aknin S. Thymic germinal centers and corticosteroids in Myasthenia gravis: an immunopathological study in 1035 cases and a critical review. *Clin Rev Allergy Immunol*. (2017) 52:108–24. doi: 10.1007/s12016-016-8558-3
6. Kuks JB, Oosterhuis HJ, Limburg PC, The TH. Anti-acetylcholine receptor antibodies decrease after thymectomy in patients with Myasthenia gravis clinical correlations. *J Autoimmun*. (1991) 4:197–211. doi: 10.1016/0896-8411(91)90018-8
7. Leprince C, Cohen-Kaminsky S, Berrih-Aknin S, Vernet-Der Garabedian B, Treton D, Galanaud P, et al. Thymic B cells from Myasthenia gravis patients are activated B cells. Phenotypic and functional analysis. *J Immunol*. (1990) 145:2115–22.
8. Moulian N, Bidault J, Truffault F, Yamamoto AM, Levasseur P, Berrih-Aknin S. Thymocyte Fas expression is dysregulated in Myasthenia gravis patients with anti-acetylcholine receptor antibody. *Blood*. (1997) 89:3287–95.
9. Cohen-Kaminsky S, Levasseur P, Binet JP, Berrih-Aknin S. Evidence of enhanced recombinant interleukin-2 sensitivity in thymic lymphocytes from patients with myasthenia gravis: possible role in autoimmune pathogenesis. *J Neuroimmunol*. (1989) 24:75–85.
10. Balandina A, Lécart S, Dartevelle P, Saoudi A, Berrih-Aknin S. Functional defect of regulatory CD4(+)CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis. *Blood*. (2005) 105:735–41.
11. Gradolatto A, Nazzal D, Truffault F, Bismuth J, Fadel E, Foti M, et al. Both Treg cells and Tconv cells are defective in the Myasthenia gravis thymus: roles of IL-17 and TNF-α. *J Autoimmun*. (2014) 52:53–63. doi: 10.1016/j.jaut.2013.12.015
12. Poea-Guyon S, Christadoss P, Le Panse R, Guyon T, De Baets M, Wakkach A, et al. Effects of cytokines on acetylcholine receptor expression: implications for myasthenia gravis. *J Immunol*. (2005) 174:5941–9.
13. Cufi P, Dragin N, Weiss JM, Martinez-Martinez P, De Baets MH, Roussin R, et al. Implication of double-stranded RNA signaling in the etiology of autoimmune myasthenia gravis. *Ann Neurol*. (2013) 73:281–93. doi: 10.1002/ana.23791
14. Cufi P, Dragin N, Ruhlmann N, Weiss JM, Fadel E, Serraf A, et al. Central role of interferon-beta in thymic events leading to myasthenia gravis. *J Autoimmun*. (2014) 52:44–52. doi: 10.1016/j.jaut.2013.12.016
15. Colombara M, Antonini V, Riviera AP, Mainiero F, Strippoli R, Merola M, et al. Constitutive activation of p38 and ERK1/2 MAPKs in epithelial Cells of myasthenic thymus leads to IL-6 and RANTES overexpression: effects on survival and migration of peripheral T and B Cells. *J Immunol*. (2005) 175:7021–8. doi: 10.4049/jimmunol.175.10.7021
16. Villegas JA, Bayer AC, Ider K, Bismuth J, Truffault F, Roussin R, et al. Il-23/Th17 cell pathway: a promising target to alleviate thymic inflammation maintenance in myasthenia gravis. *J Autoimmun*. (2019) 98:59–73. doi: 10.1016/j.jaut.2018.11.005
17. Nazzal D, Gradolatto A, Truffault F, Bismuth J, Berrih-Aknin S. Human thymus medullary epithelial cells promote regulatory T-cell generation by stimulating interleukin-2 production via ICOS ligand. *Cell Death Dis*. (2014) 5:e1420. doi: 10.1038/cddis.2014.377
18. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4 + CD25 high regulatory cells in human peripheral blood. *J Immunol*. (2001) 167:1245–53. doi: 10.4049/jimmunol.167.3.1245
19. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood. *J Exp Med*. (2001) 193:1303–10. doi: 10.1084/jem.193.11.1303
20. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. (2003) 4:330–6. doi: 10.1038/ni904
21. Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci USA*. (2006) 103:6659–64. doi: 10.1073/pnas.0509484103
22. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. (2006) 203:1693–700. doi: 10.1084/jem.20060468
23. Liu W, Putnam AL, Xu-yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. (2006) 203:1701–11. doi: 10.1084/jem.20060772
24. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019
25. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med*. (2011) 17:975–82. doi: 10.1038/nm.2425
26. Moulian N, Renvoize C, Desodt C, Serraf A, Berrih-Aknin S. Functional Fas expression in human thymic epithelial cells. *Blood*. (1999) 93:2660–70.
27. Villegas JA, Gradolatto A, Truffault F, Roussin R, Berrih-Aknin S, Le Panse R, et al. Cultured human thymic-derived cells display medullary thymic epithelial cell phenotype and functionality. *Front Immunol*. (2018) 9:1663. doi: 10.3389/fimmu.2018.01663
28. Marelli-Berg FM, Clement M, Mauro C, Caligiuri G. An immunologist's guide to CD31 function in T-cells. *J Cell Sci*. (2013) 126:2343–52. doi: 10.1242/jcs.124099
29. Demeure CE, Byun DG, Yang LP, Vezzio N, Delespesse G. CD31 (PECAM-1) is a differentiation antigen lost during human CD4 T-cell maturation into Thl or Th2 effector cells. *Immunology*. (1996) 88:110–5.
30. Booth NJ, McQuaid AJ, Sobande T, Kissane S, Agius E, Jackson SE, et al. Different proliferative potential and migratory characteristics of human CD4 + regulatory T cells that express either CD45RA or CD45RO. *J Immunol*. (2010) 184:4317–26. doi: 10.4049/jimmunol.0903781
31. Lim HW, Broxmeyer HE, Kim CH. Regulation of trafficking receptor expression in human forkhead box P3 + regulatory T cells. *J Immunol*. (2006) 177:840–51. doi: 10.4049/jimmunol.177.2.840
32. Feferman T, Maiti PK, Berrih-Aknin S, Bismuth J, Bidault J, Fuchs S, et al. Overexpression of IFN-induced protein 10 and its receptor CXCR3 in myasthenia gravis. *J Immunol*. (2005) 174:5324–31.
33. Wen Y, Yang B, Lu J, Zhang J, Yang H, Li J. Imbalance of circulating CD4(+)CXCR5(+)FOXP3(+) Tfr-like cells and CD4(+)CXCR5(+)FOXP3(-) Tfh-like cells in myasthenia gravis. *Neurosci Lett*. (2016) 630:176–82. doi: 10.1016/j.neulet.2016.07.049
34. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol*. (2010) 40:1830–5. doi: 10.1002/eji.201040391
35. Toomer KH, Malek TR. Cytokine signaling in the development and homeostasis of regulatory T cells. *Cold Spring Harb Perspect Biol*. (2018) 10:a028597. doi: 10.1101/cshperspect.a028597
36. Thiruppathi M, Rowin J, Ganesh B, Sheng JR, Prabhakar BS, Meriggioli MN. Impaired regulatory function in circulating CD4(+)CD25(high)CD127(low/-) T cells in patients with myasthenia gravis. *Clin Immunol*. (2012) 145:209–23. doi: 10.1016/j.clim.2012.09.012
37. Zhang H-L, Wu J, Ni F-M, Kaji R, Matsui N, Takahama Y, et al. Undiminished regulatory T cells in the thymus of patients with Myasthenia gravis. *Neurology*. (2010) 75:1121. doi: 10.1212/WNL.0b013e3181f46fa5
38. Xu WH, Zhang AM, Ren MS, Zhang XD, Wang F, Xu XC, et al. Changes of Treg-associated molecules on CD4+CD25 +Treg cells in myasthenia gravis and effects of immunosuppressants. *J Clin Immunol*. (2012) 32:975–83. doi: 10.1007/s10875-012-9685-0
39. Kohler S, Keil TOP, Hoffmann S, Swierzy M, Ismail M, Rückert JC, et al. CD4+ FoxP3+ T regulatory cell subsets in myasthenia gravis patients. *Clin Immunol*. (2017) 179:40–6. doi: 10.1016/j.clim.2017.03.003
40. Luther C, Adamopoulou E, Stoeckle C, Brucklacher-Waldert V, Rosenkranz D, Stoltze L, et al. Prednisolone treatment induces tolerogenic dendritic cells and a regulatory milieu in Myasthenia gravis patients. *J Immunol*. (2009) 183:841–8. doi: 10.4049/jimmunol.0802046
41. Venken K, Hellings N, Broekmans T, Hensen K, Rummens J-L, Stinissen P. Natural naive CD4 + CD25 + CD127 low regulatory T Cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory treg homeostasis during disease progression. *J Immunol*. (2008) 180:6411–20. doi: 10.4049/jimmunol.180.9.6411
42. Aime C, Cohen-Kaminsky S, Berrih-Aknin S. In vitro interleukin-1 (IL-1) production in thymic hyperplasia and thymoma from patients with myasthenia gravis. *J Clin Immunol*. (1991) 11:268–78. doi: 10.1007/BF00918185

43. Cohen-Kaminsky S, Delattre RM, Devergne O, Klingel-Schmitt I, Emilie D, Galanaud P, et al. High IL-6 gene expression and production by cultured human thymic epithelial cells from patients with Myasthenia gravis. *Ann N Y Acad Sci*. (1993) 681:97–9.
44. Meraouna A, Cizeron-Clairac G, Panse RL, Bismuth J, Truffault F, Tallaksen C, et al. The chemokine CXCL13 is a key molecule in autoimmune myasthenia gravis. *Blood*. (2006) 108:432–40. doi: 10.1182/blood-2005-06-2383
45. Selmi C, Luigi P, Gershwin ME. Primary biliary cirrhosis and Sjögren ' s syndrome: autoimmune epithelitis. *J Autoimmun*. (2012) 39:34–42. doi: 10.1016/j.jaut.2011.11.005
46. Watanabe N, Wang YH, Lee HK, Ito T, Wang YH, Cao W, et al. Hassall's corpuscles instruct dendritic cells to induce CD4 +CD25+ regulatory T cells in human thymus. *Nature*. (2005) 436:1181–5. doi: 10.1038/nature03886
47. Al-Shami A, Spolski R, Kelly J, Fry T, Schwartzberg PL, Pandey A, et al. A role for thymic stromal lymphopoietin in CD4+ T cell development. *J Exp Med*. (2004) 200:159–68. doi: 10.1084/jem.20031975
48. Nguyen KD, Vanichsarn C, Nadeau KC. TSLP directly impairs pulmonary Treg function: association with aberrant tolerogenic immunity in asthmatic airway. *Allergy Asthma Clin Immunol*. (2010) 6:4. doi: 10.1186/1710-1492-6-4
49. Tsilingiri K, Fornasa G, Rescigno M. Thymic stromal lymphopoietin: to cut a long story short. *Cell Mol Gastroenterol Hepatol*. (2017) 3:174–82. doi: 10.1016/j.jcmgh.2017.01.005
50. Miazgowicz MM, Elliott MS, Debley JS, Ziegler SF. Respiratory syncytial virus induces functional thymic stromal lymphopoietin receptor in airway epithelial cells. *J Inflamm Res*. (2013) 6:53–61. doi: 10.2147/JIR.S42381
51. Chandramouleeswaran PM, Shen D, Lee AJ, Benitez A, Dods K, Gambanga F, et al. Preferential secretion of thymic stromal lymphopoietin (TSLP) by terminally differentiated esophageal epithelial cells: relevance to eosinophilic esophagitis (EoE). *PLoS One*. (2016) 11:e0150968. doi: 10.1371/journal.pone.0150968
52. Datta A, Alexander R, Sulikowski MG, Nicholson AG, Maher TM, Scotton CJ, et al. Evidence for a functional thymic stromal lymphopoietin signaling axis in fibrotic lung disease. *J Immunol*. (2013) 191:4867–79. doi: 10.4049/jimmunol.1300588
53. Kilpatrick RD, Rickabaugh T, Hultin LE, Hultin P, Hausner MA, Detels R, et al. Homeostasis of the Naive CD4 $^{+}$ T cell compartment during aging. *J Immunol*. (2008) 180:1499–507. doi: 10.4049/jimmunol.180.3.1499
54. Huang L, Zheng Y, Yuan X, Ma Y, Xie G, Wang W, et al. Decreased frequencies and impaired functions of the CD31+ subpopulation in Treg cells associated with decreased FoxP3 expression and enhanced Treg cell defects in patients with coronary heart disease. *Clin Exp Immunol*. (2017) 187:441–54. doi: 10.1111/cei.12897


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 08 May 2020
doi: 10.3389/fimmu.2020.00707

# Muscle-Specific Kinase Myasthenia Gravis

*Lucia S. Borges and David P. Richman**

*Department of Neurology, University of California, Davis, Davis, CA, United States*

Thirty to fifty percent of patients with acetylcholine receptor (AChR) antibody (Ab)-negative myasthenia gravis (MG) have Abs to muscle specific kinase (MuSK) and are referred to as having MuSK-MG. MuSK is a 100 kD single-pass post-synaptic transmembrane receptor tyrosine kinase crucial to the development and maintenance of the neuromuscular junction. The Abs in MuSK-MG are predominantly of the IgG4 immunoglobulin subclass. MuSK-MG differs from AChR-MG, in exhibiting more focal muscle involvement, including neck, shoulder, facial and bulbar-innervated muscles, as well as wasting of the involved muscles. MuSK-MG is highly associated with the HLA DR14-DQ5 haplotype and occurs predominantly in females with onset in the fourth decade of life. Some of the standard treatments of AChR-MG have been found to have limited effectiveness in MuSK-MG, including thymectomy and cholinesterase inhibitors. Therefore, current treatment involves immunosuppression, primarily by corticosteroids. In addition, patients respond especially well to B cell depletion agents, e.g., rituximab, with long-term remissions. Future treatments will likely derive from the ongoing analysis of the pathogenic mechanisms underlying this disease, including histologic and physiologic studies of the neuromuscular junction in patients as well as information derived from the development and study of animal models of the disease.

**Keywords: myasthenia gravis, muscle specific kinase, neuromuscular junction, pathogenesis, treatment, animal models, review**

OPEN ACCESS

***Edited by:***
*Anna Rostedt Punga,*
*Uppsala University, Sweden*

***Reviewed by:***
*Ruksana Huda,*
*The University of Texas Medical Branch at Galveston, United States*
*Inga Koneczny,*
*Medical University of Vienna, Austria*

***Correspondence:**
*David P. Richman*
*dprichman@ucdavis.edu*

***Specialty section:***
*This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology*

***Received:*** *11 November 2019*
***Accepted:*** *30 March 2020*
***Published:*** *08 May 2020*

***Citation:***
*Borges LS and Richman DP (2020) Muscle-Specific Kinase Myasthenia Gravis. Front. Immunol. 11:707. doi: 10.3389/fimmu.2020.00707*

## INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction synapse (NMJ) characterized by weakness that worsens with continued muscle work and improves with resting of the involved muscle(s). Non-immune genetic diseases of this synapse, referred to as congenital myasthenic syndromes, produce similar symptoms (1, 2). For MG, the distribution of weakness is distinctive, involving primarily the extraocular muscles. In ocular MG, involvement is limited to these muscles. In more severe cases (generalized MG), the pontine- and bulbar-innervated muscles and the respiratory muscles are commonly also affected. Least frequently involved are the extremity muscles.

Most MG patients have circulating antibodies (Abs) to the NMJ postsynaptic neurotransmitter receptor, nicotinic acetylcholine receptor (AChR), AChR-MG (3, 4). The pathogenic role of these Abs has been demonstrated by induction of MG in experimental animals by both passive transfer of MG serum Abs (5) or anti-AChR monoclonal Abs (mAbs) (6–8) and by active immunization with purified AChR (9). For both AChR-MG and its experimental models, the Abs induce a destructive inflammatory attack on the AChR-containing postsynaptic membrane (10–13). In generalized MG, AChR Abs are present in 90 percent of patients. The remaining cases were initially designated as seronegative MG.

The earliest studies of these AChR Ab-negative MG cases failed to identify clinical or electrophysiologic features that distinguished it from AChR-MG. In 2001, Hoch and coworkers identified Abs to a different postsynaptic membrane protein, muscle-specific kinase (MuSK), present in the sera of 30–50 percent of seronegative MG patients (14). Once this group of MuSK-Ab-positive MG patients (MuSK-MG) was identified, clinical characteristics of MuSK-MG were discerned that distinguish it from AChR-MG, suggesting that MuSK-MG is a distinct autoimmune disease. Most striking is muscle wasting in many of the affected muscles. Although MuSK-MG is also an Ab-mediated disease, inflammatory damage to the NMJ does not occur. In fact, the majority of the Abs are of the IgG4 immunoglobulin subclass, which is characterized partly by inability to activate complement or bind to Fc receptors. The proposed mode of action of these auto-Abs is blockade of the normal function of MuSK.

Many of the standard treatments of AChR-MG are of limited effectiveness in MuSK-MG, including thymectomy and cholinesterase inhibitors. Therefore, current treatment involves immunosuppression, primarily by corticosteroids or B cell depletion agents.

Since the initial identification of MuSK-MG, a number of experimental animal models of this disease have been developed. As is the case in AChR-MG, careful analyses of both the human disease and the animal models have led to the determination of the pathogenic mechanisms underlying this disease. Such information has the potential for the development of improved treatments of MuSK-MG and similar diseases.

## MuSK AND THE NEUROMUSCULAR JUNCTION

Muscle specific kinase was identified as a postsynaptic integral membrane protein playing a pivotal role in the development of the NMJ (15–19). This synapse begins to form when the axon growth cone of a developing motor neuron encounters a developing myotube and begins to secrete agrin, a glycoprotein with a laminin-binding domain that anchors it to the extracellular matrix (20–25). Prior to the arrival of the axon, AChRs, which initially are spread diffusely along the myotube, begin to cluster in the central region of the myotube (26, 27). When the axon growth cone eventually encounters this region and secretes agrin (**Figure 1**) (9, 10), the agrin induces more extensive dense clustering of the AChRs in the postsynaptic endplate membrane, which is the first step in the elaboration of this structure into its adult architecture (**Figure 2**), including a pretzel-like topographic profile (**Figure 3A**) and marked folding and specialization of that membrane at the ultrastructural level (**Figure 3B**) (20–25, 28–34).

Both the initial spontaneous AChR clustering and the agrin-induced effects require the presence of MuSK (23, 35, 36). The paradoxical observation that agrin and MuSK do not bind *in vitro* led to a search for a third (intermediary) protein required for their interaction, which was eventually found and identified as the postsynaptic transmembrane protein low density lipoprotein receptor-related protein 4 (lrp4) (37–39).

The agrin-lrp4-MuSK interaction leads first to MuSK dimerization and then self-phosphorylation. The latter effect initiates a series of intracellular protein phosphorylations mediated through a downstream signal transduction pathway beginning with Dok7 and ending with rapsyn and the β subunit of AChR (40–43). Activation of this pathway results in dense AChR clustering, the first step in the elaboration of the postsynaptic components of the synapse (**Figure 2**) (44, 45). The AChR clustering also includes MuSK and lrp4 and the other components of the MuSK-associated signaling pathway (21, 46).

Activation of the agrin/lrp4/MuSK pathway leads, as well, to increased expression/synthesis of the components of the pathway and other endplate-specific proteins (by subsynaptic muscle nuclei) (22, 47–49). The induced AChR clustering, and the eventual elaboration of the entire adult postsynaptic endplate structure, involves polymerization of actin leading to the production of an intracellular scaffolding, comprised of a number of proteins, upon which the mature structure of the muscle endplate is formed. This process results in tight packing of the phosphorylated AChRs on the peaks of the synaptic folds opposite the specialized nerve terminal (**Figure 3B**) (44, 45, 50). This actin/cytoskeletal remodeling is contributed to by a number of other proteins in the MuSK signaling pathway, most prominently cortactin, which when phosphorylated directly enhances further actin polymerization (44, 51). Extracellularly, ColQ, the collagen-like portion of the NMJ enzyme acetylcholinesterase, binds to the extracellular portion of concentrated (clustered) MuSK (52, 53) and also to the extracellular matrix protein perlecan, leading to anchoring of the enzyme to the extracellular matrix at the clustering sites (53).

The agrin/lrp4-induced activation (phosphorylation) of MuSK is also associated with development of the presynaptic portion of the NMJ. MuSK activation initiates a separate (less well understood) retrograde pathway, resulting first in a stop signal terminating the travels of the motor axon (**Figure 1**) (54, 55). The increased concentration (clustering) of lrp4 at the developing NMJ induced by activation of the MuSK transduction pathway is required for the further development of the axon growth cone into the adult specialized presynaptic nerve terminal. The concentrated lrp4 binds the nerve terminal, but the presynaptic "receptor" for lrp4 and the subsequent developmental steps have not yet been identified (56) (21).

The further maturation of the NMJ and, in particular, the mechanisms involved in the maintenance of the mature NMJ, are even less well understood (33, 55, 57, 58). Maintenance of the NMJ does appear to require MuSK functionality, as demonstrated by the dissolution of the synapse in adult animals (in the absence of inflammation) both in (1) experimental MuSK-MG induced by either passive or active immunization with MuSK (59–63) and (2) in adult animals in which MuSK has been inactivated or knocked down (64, 65).

## MuSK MOLECULAR STRUCTURE

Muscle specific kinase is a 100 kD single-pass transmembrane receptor tyrosine kinase with an N-terminal extracellular domain

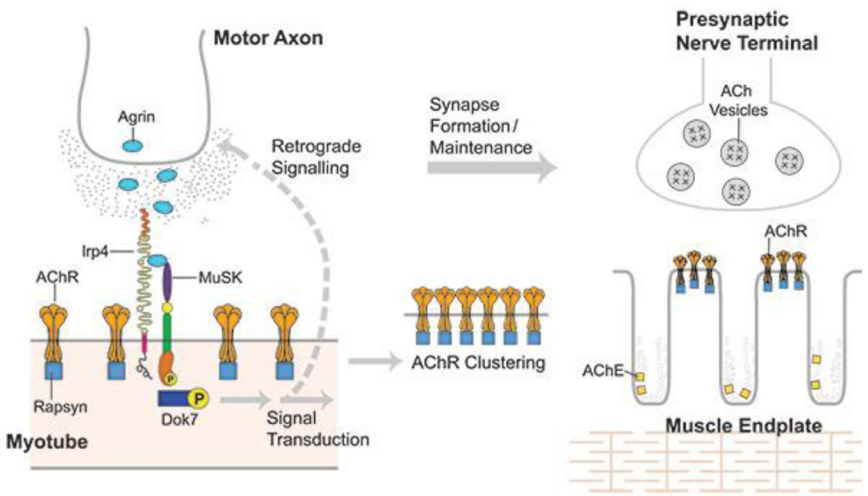



**FIGURE 1 |** Developing NMJ: The motor axon growth cone releases agrin into the intercellular matrix when it reaches a developing myotube. Agrin binds lrp4 and the complex binds MuSK resulting in activation of MuSK, which self-phosphorylates and then initiates a series of phosphorylations beginning with Dok7 and ending with rapsyn and 8 subunit of AChR. This process induces dense AChR clustering, the first step in the development of both the postsynaptic and presynaptic portions of the mature NMJ. From Richman (66) with permission.

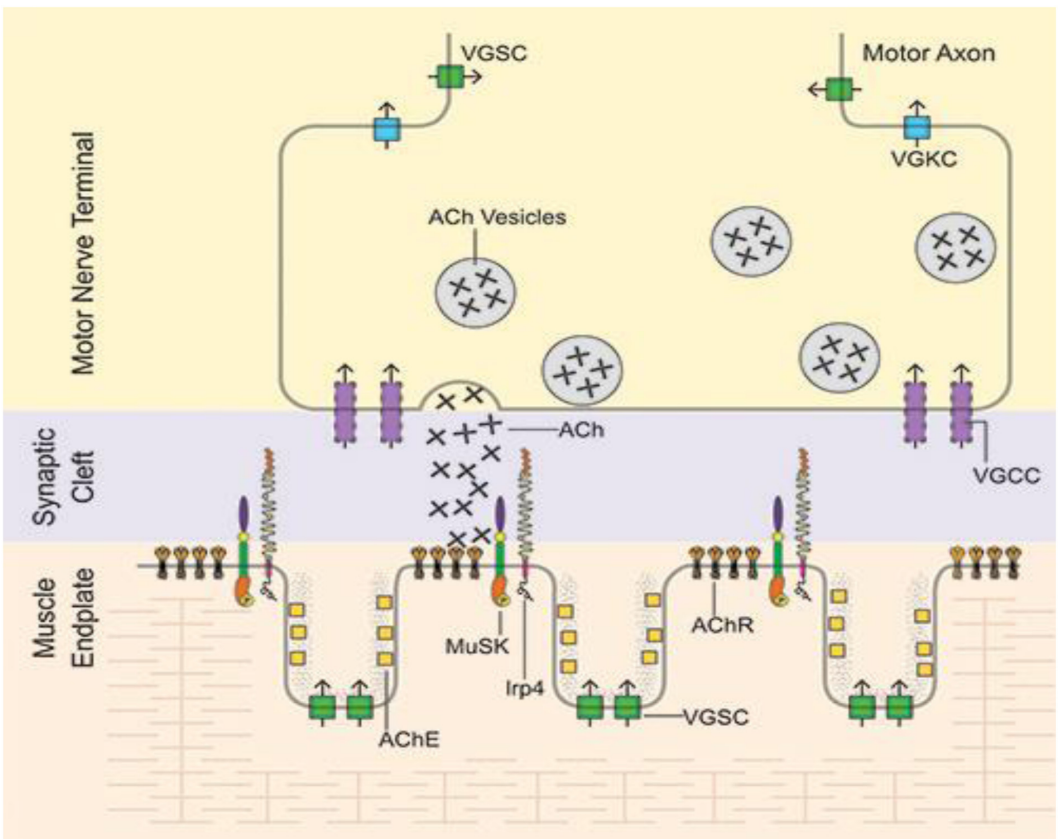



**FIGURE 2 |** Mature NMJ: Motor axon action potentials reach the motor nerve terminal leading to release of vesicles of acetylcholine (ACh), which diffuses across the synaptic clef to bind to the tightly packed acetylcholine receptors (AChR) located on the peaks of the folds of the endplate membrane. After AChR activation, ACh is then hydrolyzed by acetylcholinesterase (AChE) in the muscle basal lamina. VGSC, voltage-gated sodium channels; VGKC, voltage-gated potassium channels; VGCC, voltage-gated calcium channels. From Richman (66) with permission.

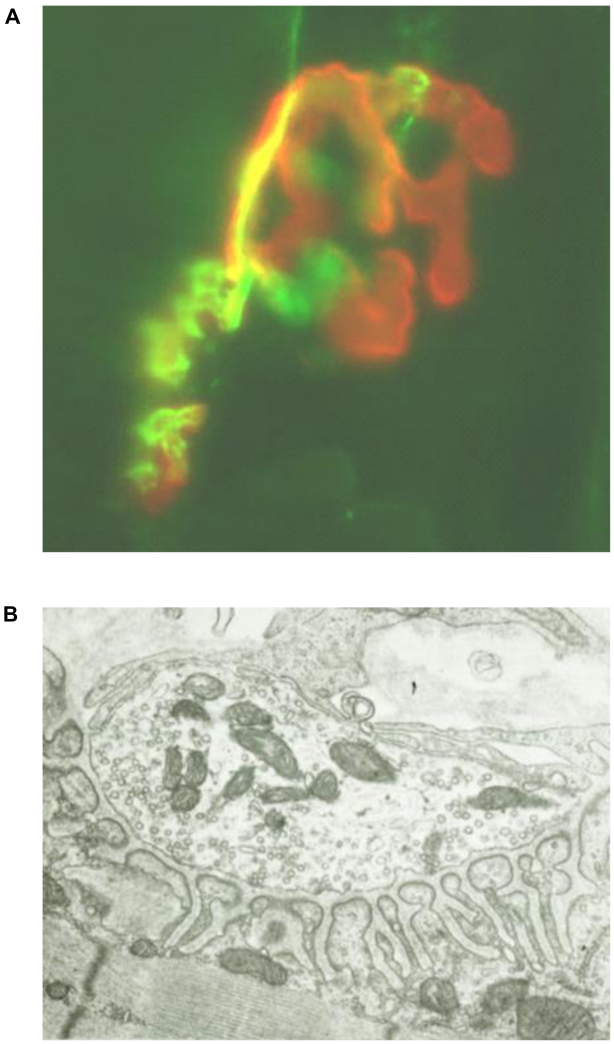



**FIGURE 3 | (A)** Photomicrograph (x80) of longitudinal frozen section of diaphragm muscle stained immunohistochemically with alpha-bungarotoxin to label AChR (red) and anti-synapsin plus anti-neurofilament Abs to label presynaptic nerve terminals and axons (green), demonstrating pretzel appearance of endplate membrane. **(B)** Electron micrograph (×5000) of transverse section of diaphragm muscle neuromuscular junction demonstrating highly folded endplate membrane. [Modified from (63) with permission].

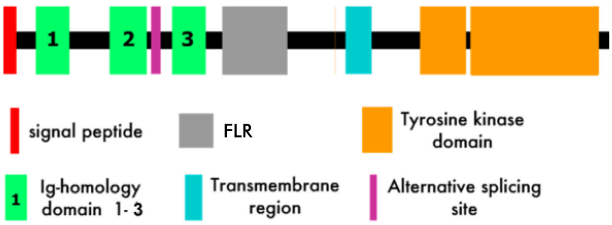



**FIGURE 4 |** MuSK Structure (Modified from 15). FLR, Frizzled-like region.

followed by a short transmembrane domain and then a C-terminal cytoplasmic domain (**Figure 4**) (15, 16, 18, 19). The extracellular domain of MuSK, which is required for interaction with agrin and lrp4, comprises three immunoglobulin (Ig)-like domains (37, 39, 67) followed by a cysteine-rich frizzled-like region (labeled C6-box in **Figure 4**) (15, 16, 18, 45). The cytoplasmic domain contains the kinase activity and signaling components of the molecule that lead to the development of the postsynaptic apparatus (see above) (45).

The first two extracellular Ig-like domains, which are rigidly joined in a linear array (67), appear to play a dual role in activation of MuSK signaling. First, Ig-1 is crucial for binding to the MuSK ligand, i.e., agrin-associated lrp4 (68). Second, it is the substrate for the dimerization of two MuSK molecules (67). Dimerization is required for MuSK (*trans*) autophosphorylation, the first step in the activation of the MuSK-associated signaling pathway (69, 70). Autophosphorylation, along with binding of Dok7, an intracellular MuSK target, results in full activation of the MuSK kinase activity (71, 72). It is the combination of ligand (agrin/lrp4) binding and the full establishment of its kinase activity that results in the sequence of protein phosphorylations by MuSK that comprise the MuSK-associated signaling pathway that leads first to AChR clustering and subsequently to formation of the mature NMJ. The role of the frizzled-like region, which functions as a receptor for the wnt family of intracellular signaling proteins, is not yet well understood (73–76).

Early studies employing both rat and human MuSK have determined that it is only the extracellular domain of the molecule that is the target of the MuSK Abs in MuSK-MG (see below) (14, 77).

## ANTI-MuSK MYASTHENIA

### Disease Characteristics

The MuSK Ab-positive subgroup of "seronegative patients," anti-MuSK MG, does have clinical similarities to AChR-MG but tends to differ significantly in exhibiting more focal involvement than AChR-MG, frequently with severe involvement of neck, shoulder, facial and bulbar-innervated muscles, although there is considerable variability from patient to patient (78–82). When the extremities are involved, proximal muscles are more affected than distal ones (83). Unlike AChR-MG, many patients have wasting of these muscles (78, 79, 81, 84–86), and data suggest that this represents a direct myopathy and is not the result of denervation, a point that remains somewhat controversial (86–91).

The demographic characteristics of MuSK-MG differ from AChR MG. In the latter disease, the age/incidence curve is bimodal with a peak in the early 20's, which is majority female, and with a second peak in the 60's and 70's, which is majority male (92–94). In contrast, MuSK-MG tends to occur in the 30's with very strong female predominance (95). Also, MuSK-MG is highly associated with the HLA DR14-DQ5 haplotype (96).

The restricted HLA (MHC) class II association in MuSK-MG suggests a role for T helper cells in this Ab-mediated disease. A recent study observed antigen (MuSK)-specific T cell responses

in cultures of circulating mononuclear cells (MNC) from MuSK-MG patients. These anti-MuSK responses utilized a somewhat limited number of T cell receptor variable region genes (97–99), consistent with a genetically influenced disease-specific T cell response. Also, in contrast to AChR-MG, only rare MuSK MG patients have been found to have thymic lymphoid hyperplasia (97–101).

Remarkably, data concerning NMJ histology and microphysiology in MuSK-MG are very limited. In the three histologic studies available (102–104), only relatively mild abnormalities of NMJ morphology/function were observed. The changes were all postsynaptic, including partially denervated postsynaptic membrane and moderate degeneration of postsynaptic folds (102). One of two microelectrode studies found only postsynaptic abnormalities, marked decrease in miniature endplate potential (MEPP) amplitudes (103). However, the other study found both postsynaptic abnormalities, mild decrease in MEPP amplitudes, and presynaptic abnormalities, reduced levels of presynaptic acetylcholine release (102).

## Anti-MuSK Antibodies in MuSK-MG

Anti-MuSK Abs are detected in 1–10% of patients with MG, 40% of the AChR Ab-negative patients (14, 77, 78, 80, 81, 105). Most of the anti-MuSK Abs belong to the IgG4 immunoglobulin subclass (77, 106), which is unable to either activate complement or induce antigenic modulation (107). However, passive transfer of the IgG4 component of MuSK-MG serum is especially effective in inducing the experimental disease (108).

Abs of the IgG4 subclass behave as if they are functionally monovalent (109). Because of single amino acid differences in the IgG4 heavy chain constant region, the inter-heavy chain disulfide bonds that join the two halves of the immunoglobulin molecule are markedly weakened, leading to frequent separation of the two heavy chains. The resultant "half molecules," consisting of a single light chain covalently bound to a single heavy chain, can readily bind to a half molecule from another IgG4 Ab to reform a complete Ab molecule, so called "Fab arm exchange" (109, 110). The new Ab molecule is now bispecific, that is each Ab arm binds a different antigen. Such Abs, including IgG4 MuSK Abs, cannot crosslink single antigens and, therefore cannot induce antigenic modulation (which requires antigen crosslinking), a mechanism important in AChR-MG. Also as noted above, these IgG4 Abs are minimally interactive with the innate immune system, in that they are deficient in complement activation and in binding to cell surface Fc receptors (107). Anti-MuSK Abs of the IgG1 subclass, a subclass capable of engaging these components of the innate immune system, are also present in most MuSK-MG patients, but at much lower levels than the IgG4 Abs (111, 112). The role the IgG1 Abs play in MuSK-MG is not known.

Hence, the pathogenic mode of action of the auto-Abs in MuSK-MG differs from that of the AChR Abs in AChR-MG. Rather than inducing destructive damage to the NMJ or antigenic modulation, the anti-MuSK Abs mask the binding sites on MuSK that interact with its binding proteins (ligands), including lrp4/agrin and ColQ, thereby blocking MuSK function (106, 111). Blockade of MuSK ligand binding leads to a reduced postsynaptic density of AChRs and impairs their alignment in the postsynaptic membrane (60).

Most anti-MuSK Abs bind to the Ig-like domains of the extracellular portion of MuSK (**Figure 4**) (14, 77, 106, 113, 114). In one study of 53 MuSK-MG patients, all had Abs to Ig-like domain 1 and about 50 percent also had Abs to Ig-like domain 2. For female patients, it was rare to have Ab reactivity to domains other than Ig-like domain 1 (113). However, Abs to the frizzled-like domain have been observed in MuSK MG (115).

# ANIMAL MODELS OF ANTI-MuSK MG

As noted above, few human studies have addressed directly the pathogenesis of MuSK-MG (102–104). None have observed complement-mediated injury or cellular infiltration of the NMJ. In fact, initially there was controversy concerning the role of anti-MuSK Abs in MuSK-MG pathogenesis (103, 116, 117), in spite of the ability of these Abs to act as MuSK antagonists *in vitro*. On the other hand, experimental studies, involving the induction of experimental models of MuSK-MG, have provided the strongest evidence concerning the pathogenic mechanisms underlying this disease. The data from MuSK-MG animal models induced by both passive and active immunization with MuSK demonstrate the role of the anti-MuSK Abs in the induction of both the weakness and the morphological and physiological NMJ changes observed in MuSK-MG (59, 60, 117–123).

## Passive Immunization Studies

A number of studies have assessed the effect of daily intraperitoneal injections into immunosuppressed mice of very large amounts of IgG (usually 35–50 mg per day) purified from (human) MuSK-MG serum. In one study, injections for 5 days produced reduced neuromuscular transmission but without clinical weakness (118). A second study made use of IgG purified from a severely affected patient injected for 14 days (total of 0.68 g), which resulted in weakness and weight loss. Histologic analysis of these animals found patchy reduction in NMJ AChR staining, reduced registration between nerve terminals and motor endplates (59) and reduced phosphorylation of the downstream components of the MuSK signaling pathway (60). The Abs also produce internalization of MuSK with more rapid degradation leading to reduced endplate MuSK concentrations (60, 117). The marked effectiveness of passive transfer of the IgG4 component of MuSK-MG serum is consistent with the role of these mechanisms in inducing the experimental disease (108). These observations, along with the absence of observed complement-mediated injury, support the hypothesis that the MuSK Abs induce the disease by blocking MuSK signaling *in vivo* with the resultant postsynaptic changes described above, as well as damage to the nerve terminals (see below).

## Active Immunization Studies

In rabbits (117) and, to a lesser extent in mice (118–123), repeatedly immunized with MuSK protein (of human or rat origin) over extended periods of time, induces mild weakness along with mild electrophysiologic evidence of disordered

neuromuscular transmission and varying degrees of reduction in motor endplate size. For mice there has been considerable variability among strains in the susceptibility to the active induction of experimental MuSK-MG (118–123). For C57BL6, the distribution of weakness and wasting follows the gradient of normal MuSK expression in individual muscles (120), which somewhat mimics the distribution of muscle involvement in MuSK-MG (see above).

In contrast to the variability in response and the requirement for repeated immunizations in mice, the experimental disease in inbred Lewis rats is highly stereotyped. A single immunization of mouse MuSK ectodomain in adjuvants results in reproducible severe weakness (death within 4 weeks), along with muscle wasting and electrodiagnostic abnormalities typical of MuSK-MG. Histologically, there are extensive postsynaptic and presynaptic changes. The NMJ morphologic findings include fragmented NMJs with varying degrees of postsynaptic muscle end plate destruction, along with abnormal nerve terminals. The presynaptic changes are characterized by reduced terminal size, ongoing terminal degeneration and lack of registration between endplate and nerve terminals. In addition, there is local axon sprouting, and extrajunctional dispersion of cholinesterase activity (61–63).

## PATHOGENESIS OF MuSK-MG

Data from analysis of both MuSK-MG and its animal models described above have contributed to the information concerning the pathogenesis of the human disease. With the identification of MuSK Abs in "seronegative MG" patients, the initial hypothesis was that the MuSK Abs indeed induce the disease. As noted above, at the time, the hypothesis was not universally accepted (103, 116, 117). The alternative hypothesis advanced was that these Abs are an epiphenomenon occurring in parallel with the disease or even occurring as a result of the disease (103, 116). The dual observations (see above) of (1) the ability of IgG isolated from patient serum, when injected into immunosuppressed mice, to induce a disease similar to human MuSK-MG, and (2) immunization of normal animals with purified MuSK protein, leading to the production of MuSK Abs, also results in a disease that is highly similar to human MuSK-MG, together strongly support the hypothesis that the MuSK Abs are the etiologic agents in this disease.

Multiple observations of neuromuscular junction histology from the various animal models, along with the few histologic observations available from MuSK-MG patients, all demonstrating the absence of inflammatory damage, suggests that the innate immune system, especially the complement cascade, does not play a role in this disease. This characteristic distinguishes MuSK-MG from AChR-MG. It should be noted, however, that the data from the mouse passive transfer models employing human anti-MuSK IgG are somewhat confounded by both the necessary pretreatment with immunosuppressive agents, the extremely high doses of human Ig required to induce disease and the necessity in this model for interaction between the injected human IgG and the recipient mouse's innate immune system. This heterologous interaction, theoretically, may not be strong enough to induce a vigorous inflammatory reaction. On the other hand, for the active immunization model, immunosuppressive agents are not employed and the Ab and the innate immune system components, e.g., complement proteins and inflammatory cells expressing Fc receptors, are autologous (syngeneic) and hence capable of inducing a strong inflammatory response. At least in humans, the absence of observed inflammation in the NMJ, may be the result of the high proportion of IgG4 anti-MuSK Abs in MuSK MG (see above).

Despite the lack of inflammatory damage to the neuromuscular junction in MuSK-MG and its animal models and the theoretical inability of the human anti-MuSK IgG4 to induce antigenic modulation (i.e., increased MuSK turnover), the concentration of AChR in the postsynaptic membrane is reduced, and, to a lesser extent, so is the MuSK concentration (60, 117). The current hypothesis is that the MuSK Abs act as antagonists to the MuSK function as a receptor kinase, with its natural ligand being the agrin/lrp4 complex. *In vitro*, MuSK Abs from MuSK-MG patients and from actively immunized animals block agrin-induced AChR clustering and downstream phosphorylation in muscle cells in tissue culture (14, 59, 106, 111, 124, 125). The latter observations and the development of the above abnormalities in the NMJs in adult animals (with fully developed neuromuscular junctions) in which experimental MuSK-MG is either actively or passively induced, suggests that MuSK plays a crucial role not only in the development of the NMJ, but also plays a role in the maintenance of this synapse in adult animals. Subsequent studies of adult animals in which MuSK has been inactivated or knocked down demonstrated similar NMJ changes (64, 65).

As noted above, presynaptic involvement is another characteristic of MuSK-MG that distinguishes it from AChR-MG. In the latter disease presynaptic dysfunction is absent except in the setting of severe NMJ inflammation (12). Otherwise, presynaptic activity, in fact, is increased (126). Similar to the human disease, the experimental models of MuSK-MG noted above exhibit non-inflammatory structural and functional abnormalities of both presynaptic and postsynaptic portions of the NMJ. The abnormal presynaptic function is most readily observed in the severe form of experimental MuSK-MG that occurs in the active-immunization rat model (63). In that disease model, both the cross-sectional area of individual nerve terminals is reduced as is the total nerve terminal cross-sectional area across the entire NMJ. In addition, the actively induced rat model and the passive transfer mouse model have both demonstrated abnormal registration between the nerve terminals and the muscle endplates. Whether the observed reduction in NMJ cholinesterase activity in MuSK-MG plays a role in the muscle weakness is yet to be determined.

## TREATMENT OF MuSK-MG

Early on, the treatment of seronegative MG simply followed the protocols developed for seropositive MG,

i.e., AChR-MG, including use of cholinesterase inhibitors, thymectomy, corticosteroids, plasma exchange and intravenous immunoglobulin and cytotoxic immunosuppressants. However, with the clear identification of the MuSK-MG subset of "seronegative" patients, data began to accumulate that a number of these treatments were ineffective, or even detrimental, in these patients. Thymectomy appears not to play a role in the treatment of this disease (78, 80, 96, 105, 127, 128). Some studies suggest other differences in response to treatment, including observations that MuSK-MG patients may respond poorly to intravenous immunoglobulin (78, 80, 96, 105, 129, 130). There is also a striking absence of improvement with cholinesterase inhibitors (101, 130–133). In fact, a number of MuSK-MG patients worsen in response these agents (101, 131). The latter observation may derive from diminished acetylcholinesterase concentrations at the NMJ, perhaps as a result of MuSK Ab-mediated blockade of ColQ binding to MuSK or Ab-mediated reduction in MuSK concentration (52, 53).

## Current Treatments

### Corticosteroids

Patients with MuSK-MG respond especially well to high dose corticosteroids (129, 134–136). This appears to be most often true for patients with rapidly progressive or aggressive disease. The effectiveness of steroid treatment, along with the absence of a role for thymectomy and the limited or absent role for cholinesterase inhibitors, make corticosteroids the foundation of the current treatment of this disease. However, about 15 percent of patients treated with high dose corticosteroids do not adequately respond, so called refractory disease. This figure is somewhat higher than the comparable data in AChR-MG (137, 138). Similar to the case in AChR-MG, the significant side effects of these agents often limit their effectiveness. In both diseases, the treatment protocols aim at inducing a remission with high doses, followed by slow tapering of the dose to the lowest effective dose (139). An additional issue in MuSK-MG is the muscle wasting that occurs uniquely in this form of autoimmune MG. In many patients, in spite of the early effectiveness of corticosteroids in inducing a clinical remission, the muscle wasting appears to continue to progress (135).

### Standard Cytotoxic Immunosuppressants

Cytotoxic agents effective in preventing and treating solid organ transplant rejection have been used as single immunosuppressive agents in the treatment of AChR-MG, including azathioprine, mycophenolate mofetil and cyclosporine, with some efficacy in inducing remission. Their major role in that disease, however, has been as steroid-sparing treatments, that is, effective in facilitating corticosteroid dose tapering (140). Anecdotal data for MuSK-MG suggest that these agents are even less effective in inducing remission than in AChR-MG but, similarly, may be useful as steroid-sparing medications (135, 140, 141).

### Immune-Directed Biologic Treatments

The B cell depleting agent rituximab, a chimeric anti-CD20 monoclonal Ab, has been especially effective in MuSK-MG. Because of its toxicity profile, including a 1:10,000 risk of the induction of progressive multifocal leukoencephalopathy, the drug has been used primarily in treatment of (steroid)-refractory patients. A significant number of reported studies, all limited by relatively small numbers of subjects, have shown efficacy in this disease (142–146). In a number of cases, treatment led to eventual elimination of the need for other immune-directed treatments, e.g., steroids, and without the necessity for repeated rituximab infusions (143–145). A recent set of clinical guidelines has supported earlier use of this agent when an initial standard treatment does not induce rapid remission (140). One successful protocol is to use two courses of rituximab at a dose of 375 mg per meter squared body surface area weekly for four doses, each course separated by 6 months. A 4-infusion course can then be repeated as needed (143). It appears that adding an infusion 1 month later and another 2 months later improves efficacy even further (147).

### Short-Term Immune-Directed Treatment

Plasma exchange has been a rapidly effective treatment for active AChR-MG. Intravenous immunoglobulin infusions have been equally effective and somewhat safer (148). Initial studies of plasma exchange in acute MuSK-MG demonstrated its efficacy in this disease (129, 134, 149). Unlike in AChR-MG, the efficacy of intravenous immunoglobulin in MuSK-MG appears to be less than that of plasma exchange (130, 134, 136, 150), but the data supporting the latter statement are much less robust (151, 152).

## Future Treatments: Antigen-Specific Agents

As noted above, the various immune-directed treatments currently in use have been reasonably effective in MuSK-MG. However, these treatments are all limited by their broad effect on the immune system: on both the pathogenic (autoimmune) components and the normally functioning components. As in all other autoimmune diseases, the treatment paradigm is to adjust drug dosages and timing to maximize the effect on the autoimmune portion of the immune response while reducing the "off-target" effect on the remainder of the immune system.

A theoretical means of focusing the treatment on the autoimmune portion is to employ an antigen-specific therapy, that is, one only targeting the attack on the auto-antigen. For an Ab-mediated disease such as MG, this would involve targeting the auto-Abs. One possibly means to accomplish this therapeutic effect involves physical removal of the auto-Abs, for example, by immunoadsorption plasmapheresis. For AChR-MG, such antigen-specific Ab removal employing an affinity column containing AChR antigen as the affinity agent has been examined. To date, this approach has been no more effective than gross removal of all Abs by plasma exchange (153, 154).

An alternative antigen-specific approach is to target the B cells that are secreting the auto-Abs. This approach is currently under study in both AChR-MG and in MuSK-MG, through the use of either genetically engineered Abs or genetically engineered T cells that target the pathogenic autoimmune B cells (155, 156).

## AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

LB and DR have received research funding from NINDS (1R21NS104516), Myasthenia Gravis Foundation of America, and Cabaletta Bio Inc.

## REFERENCES


1. Engel AG. Congenital myasthenic syndromes in 2018. *Curr Neurol Neurosci Rep*. (2018) 18:46. doi: 10.1007/s11910-018-0852-4
2. Rodriguez Cruz PM, Palace J, Beeson D. The neuromuscular junction and wide heterogeneity of congenital myasthenic syndromes. *Int J Mol Sci*. (2018) 19:1667. doi: 10.3390/ijms19061677
3. Lindstrom JM. Acetylcholine receptors and myasthenia. *Muscle Nerve*. (2000) 23:453–77.
4. Richman DP, Agius MA. Myasthenia gravis: pathogenesis and treatment. *Semin Neurol*. (1994) 14:106–10.
5. Toyka KV, Brachman DB, Pestronk A, Kao I. Myasthenia gravis: passive transfer from man to mouse. *Science*. (1975) 190:397–9.
6. Lennon VA, Lambert EH. Myasthenia gravis induced by monoclonal antibodies to acetylcholine receptors. *Nature*. (1980) 285:238–40.
7. Richman DP, Gomez CM, Berman PW, Burres SA, Fitch FW, Arnason BG. Monoclonal anti-acetylcholine receptor antibodies can cause experimental myasthenia. *Nature*. (1980) 286:738–9.
8. Tzartos SJ, Lindstrom JM. Monoclonal antibodies used to probe acetylcholine receptor structure: localization of the main immunogenic region and detection of similarities between subunits. *Proc Natl Acad Sci USA*. (1980) 77:755–9.
9. Patrick J, Lindstrom J. Autoimmune response to acetylcholine receptor. *Science*. (1973) 180:871–2.
10. Richman DP, Agius MA, Kirvan CA, Gomez CM, Fairclough RH, Dupont BL, et al. Antibody effector mechanisms in myasthenia gravis. The complement hypothesis. *Ann N Y Acad Sci*. (1998) 841:450–65.
11. Cetin H, Vincent A. Pathogenic mechanisms and clinical correlations in autoimmune myasthenic syndromes. *Semin Neurol*. (2018) 38:344–54. doi: 10.1055/s-0038-1660500
12. Maselli RA, Mass DP, Distad BJ, Richman DP. Anconeus muscle: a human muscle preparation suitable for in-vitro microelectrode studies. *Muscle Nerve*. (1991) 14:1189–92.
13. Engel AG, Tsujihata M, Lindstrom JM, Lennon VA. The motor end plate in myasthenia gravis and in experimental autoimmune myasthenia gravis. A quantitative ultrastructural study. *Ann N Y Acad Sci*. (1976) 274:60–79. doi: 10.1111/j.1749-6632.1976.tb47676.x
14. Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med*. (2001) 7:365–8.
15. Hopf C, Hoch W. Tyrosine phosphorylation of the muscle-specific kinase is exclusively induced by acetylcholine receptor-aggregating agrin fragments. *Eur J Biochem*. (1998) 253:382–9.
16. Jennings CG, Dyer SM, Burden SJ. Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. *Proc Natl Acad Sci USA*. (1993) 90:2895–9.
17. Stiegler AL, Burden SJ, Hubbard SR. Crystal structure of the frizzled-like cysteine-rich domain of the receptor tyrosine kinase MuSK. *J Mol Biol*. (2009) 393:1–9. doi: 10.1016/j.jmb.2009.07.091
18. Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, et al. Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron*. (1995) 15:573–84.
19. Vincent A, Newland C, Croxen R, Beeson D. Genes at the junction–candidates for congenital myasthenic syndromes. *Trends Neurosci*. (1997) 20:15–22. doi: 10.1016/s0166-2236(96)10066-7
20. Burden SJ, Huijbers MG, Remedio L. Fundamental molecules and mechanisms for forming and maintaining neuromuscular synapses. *Int J Mol Sci*. (2018) 19:490. doi: 10.3390/ijms19020490
21. Burden SJ, Yumoto N, Zhang W. The role of MuSK in synapse formation and neuromuscular disease. *Cold Spring Harb Perspect Biol*. (2013) 5:a009167. doi: 10.1101/cshperspect.a009167
22. Burden SJ. Building the vertebrate neuromuscular synapse. *J Neurobiol*. (2002) 53:501–11.
23. Burden SJ, Fuhrer C, Hubbard SR. Agrin/MuSK signaling: willing and Abl. *Nat Neurosci*. (2003) 6:653–4.
24. Hughes BW, Kusner LL, Kaminski HJ. Molecular architecture of the neuromuscular junction. *Muscle Nerve*. (2006) 33:445–61.
25. Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci*. (2001) 2:791–805. doi: 10.1038/35097557
26. Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature*. (2001) 410:1057–64.
27. Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, et al. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron*. (2001) 30:399–410. doi: 10.1016/s0896-6273(01)00287-2
28. Chen F, Liu Y, Sugiura Y, Allen PD, Gregg RG, Lin W. Neuromuscular synaptic patterning requires the function of skeletal muscle dihydropyridine receptors. *Nat Neurosci*. (2011) 14:570–7. doi: 10.1038/nn.2792
29. Ghazanfari N, Fernandez KJ, Murata Y, Morsch M, Ngo ST, Reddel SW, et al. Muscle specific kinase: organiser of synaptic membrane domains. *Int J Biochem Cell Biol*. (2011) 43:295–8. doi: 10.1016/j.biocel.2010.10.008
30. Hallock PT, Xu CF, Park TJ, Neubert TA, Curran T, Burden SJ. Dok-7 regulates neuromuscular synapse formation by recruiting Crk and Crk-L. *Genes Dev*. (2010) 24:2451–61. doi: 10.1101/gad.1977710
31. Henriquez JP, Salinas PC. Dual roles for Wnt signalling during the formation of the vertebrate neuromuscular junction. *Acta Physiol (Oxf)*. (2011) 204:128–36. doi: 10.1111/j.1748-1716.2011.02295.x
32. Hoch W. Molecular dissection of neuromuscular junction formation. *Trends Neurosci*. (2003) 26:335–7.
33. Kummer TT, Misgeld T, Sanes JR. Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. *Curr Opin Neurobiol*. (2006) 16:74–82. doi: 10.1016/j.conb.2005.12.003
34. Marques MJ, Conchello JA, Lichtman JW. From plaque to pretzel: fold formation and acetylcholine receptor loss at the developing neuromuscular junction. *J Neurosci*. (2000) 20:3663–75.
35. Flanagan-Steet H, Fox MA, Meyer D, Sanes JR. Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. *Development*. (2005) 132:4471–81.
36. Panzer JA, Gibbs SM, Dosch R, Wagner D, Mullins MC, Granato M, et al. Neuromuscular synaptogenesis in wild-type and mutant zebrafish. *Dev Biol*. (2005) 285:340–57.
37. Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, et al. Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell*. (2008) 135:334–42. doi: 10.1016/j.cell.2008.10.002
38. Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. *Development*. (2006) 133:4993–5000. doi: 10.1242/dev.02696
39. Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. LRP4 serves as a coreceptor of agrin. *Neuron*. (2008) 60:285–97. doi: 10.1016/j.neuron.2008.10.006
40. Borges LS, Yechikhov S, Lee YI, Rudell JB, Friese MB, Burden SJ, et al. Identification of a motif in the acetylcholine receptor beta subunit whose phosphorylation regulates rapsyn association and postsynaptic receptor localization. *J Neurosci*. (2008) 28:11468–76. doi: 10.1523/JNEUROSCI.2508-08.2008

41. Qu ZC, Moritz E, Huganir RL. Regulation of tyrosine phosphorylation of the nicotinic acetylcholine receptor at the rat neuromuscular junction. *Neuron*. (1990) 4:367–78.
42. Ramarao MK, Cohen JB. Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn. *Proc Natl Acad Sci USA*. (1998) 95:4007–12.
43. Okada K, Inoue A, Okada M, Murata Y, Kakuta S, Jigami T, et al. The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. *Science*. (2006) 312:1802–5.
44. Dai Z, Luo X, Xie H, Peng HB. The actin-driven movement and formation of acetylcholine receptor clusters. *J Cell Biol*. (2000) 150:1321–34.
45. Strochlic L, Cartaud A, Cartaud J. The synaptic muscle-specific kinase (MuSK) complex: new partners, new functions. *Bioessays*. (2005) 27:1129–35.
46. Lin W, Dominguez B, Yang J, Aryal P, Brandon EP, Gage FH, et al. Neurotransmitter acetylcholine negatively regulates neuromuscular synapse formation by a Cdk5-dependent mechanism. *Neuron*. (2005) 46:569–79.
47. Burden SJ. Synapse-specific gene expression. *Trends Genet*. (1993) 9:12–6.
48. DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, et al. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell*. (1996) 85:501–12.
49. Merlie JP, Sanes JR. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. *Nature*. (1985) 317:66–8.
50. Cartaud A, Stetzkowski-Marden F, Maoui A, Cartaud J. Agrin triggers the clustering of raft-associated acetylcholine receptors through actin cytoskeleton reorganization. *Biol Cell*. (2011) 103:287–301. doi: 10.1042/BC20110018
51. Madhavan R, Gong ZL, Ma JJ, Chan AW, Peng HB. The function of cortactin in the clustering of acetylcholine receptors at the vertebrate neuromuscular junction. *PLoS One*. (2009) 4:e8478. doi: 10.1371/journal.pone.0008478
52. Cartaud A, Strochlic L, Guerra M, Blanchard B, Lambergeon M, Krejci E, et al. MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. *J Cell Biol*. (2004) 165:505–15.
53. Sigoillot SM, Bourgeois F, Lambergeon M, Strochlic L, Legay C. ColQ controls postsynaptic differentiation at the neuromuscular junction. *J Neurosci*. (2010) 30:13–23. doi: 10.1523/jneurosci.4374-09.2010
54. Dimitropoulou A, Bixby JL. Motor neurite outgrowth is selectively inhibited by cell surface MuSK and agrin. *Mol Cell Neurosci*. (2005) 28:292–302. doi: 10.1016/j.mcn.2004.09.013
55. Yumoto N, Kim N, Burden SJ. Lrp4 is a retrograde signal for presynaptic differentiation at neuromuscular synapses. *Nature*. (2012) 489:438–42. doi: 10.1038/nature11348
56. Wu H, Lu Y, Shen C, Patel N, Gan L, Xiong WC, et al. Distinct roles of muscle and motoneuron LRP4 in neuromuscular junction formation. *Neuron*. (2012) 75:94–107. doi: 10.1016/j.neuron.2012.04.033
57. Kummer TT, Misgeld T, Lichtman JW, Sanes JR. Nerve-independent formation of a topologically complex postsynaptic apparatus. *J Cell Biol*. (2004) 164:1077–87.
58. Smith CL, Mittaud P, Prescott ED, Fuhrer C, Burden SJ. Src, Fyn, and Yes are not required for neuromuscular synapse formation but are necessary for stabilization of agrin-induced clusters of acetylcholine receptors. *J Neurosci*. (2001) 21:3151–60.
59. Cole RN, Reddel SW, Gervasio OL, Phillips WD. Anti-MuSK patient antibodies disrupt the mouse neuromuscular junction. *Ann Neurol*. (2008) 63:782–9.
60. Ghazanfari N, Morsch M, Reddel SW, Liang SX, Phillips WD. Muscle-specific kinase (MuSK) autoantibodies suppress the MuSK pathway and ACh receptor retention at the mouse neuromuscular junction. *J Physiol*. (2014) 592(Pt 13):2881–97. doi: 10.1113/jphysiol.2013.270207
61. Richman DP, Nishi K, Ferns MJ, Schnier J, Pytel P, Maselli RA, et al. Animal models of antimuscle-specific kinase myasthenia. *Ann N Y Acad Sci*. (2012) 1274:140–7. doi: 10.1111/j.1749-6632.2012.06782.x
62. Richman DP, Nishi K, Morell S, Maselli RA, Agius MA. Acute severe model of anti-muscle specific kinase (MuSK) myasthenia in lewis rats (Abstract). *Neurology*. (2008) 71:153.
63. Richman DP, Nishi K, Morell SW, Chang JM, Ferns MJ, Wollmann RL, et al. Acute severe animal model of anti-muscle-specific kinase myasthenia: combined postsynaptic and presynaptic changes. *Arch Neurol*. (2012) 69:453–60. doi: 10.1001/archneurol.2011.2200
64. Hesser BA, Henschel O, Witzemann V. Synapse disassembly and formation of new synapses in postnatal muscle upon conditional inactivation of MuSK. *Mol Cell Neurosci*. (2006) 31:470–80.
65. Kong XC, Barzaghi P, Ruegg MA. Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. *EMBO Rep*. (2004) 5:183–8.
66. Richman DP. The future of research in myasthenia. *JAMA Neurol*. (2015) 72:812–4. doi: 10.1001/jamaneurol.2014.4740
67. Stiegler AL, Burden SJ, Hubbard SR. Crystal structure of the agrin-responsive immunoglobulin-like domains 1 and 2 of the receptor tyrosine kinase MuSK. *J Mol Biol*. (2006) 364:424–33.
68. Zhang W, Coldefy AS, Hubbard SR, Burden SJ. Agrin binds to the N-terminal region of Lrp4 protein and stimulates association between Lrp4 and the first immunoglobulin-like domain in muscle-specific kinase (MuSK). *J Biol Chem*. (2011) 286:40624–30. doi: 10.1074/jbc.M111.279307
69. Hopf C, Hoch W. Dimerization of the muscle-specific kinase induces tyrosine phosphorylation of acetylcholine receptors and their aggregation on the surface of myotubes. *J Biol Chem*. (1998) 273:6467–73.
70. Xie MH, Yuan J, Adams C, Gurney A. Direct demonstration of MuSK involvement in acetylcholine receptor clustering through identification of agonist ScFv. *Nat Biotechnol*. (1997) 15:768–71.
71. Bergamin E, Hallock PT, Burden SJ, Hubbard SR. The cytoplasmic adaptor protein Dok7 activates the receptor tyrosine kinase MuSK via dimerization. *Mol Cell*. (2010) 39:100–9. doi: 10.1016/j.molcel.2010.06.007
72. Inoue A, Setoguchi K, Matsubara Y, Okada K, Sato N, Iwakura Y, et al. Dok-7 activates the muscle receptor kinase MuSK and shapes synapse formation. *Sci Signal*. (2009) 2:ra7. doi: 10.1126/scisignal.2000113
73. Jing L, Lefebvre JL, Gordon LR, Granato M. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. *Neuron*. (2009) 61:721–33. doi: 10.1016/j.neuron.2008.12.025
74. Messeant J, Dobbertin A, Girard E, Delers P, Manuel M, Mangione F, et al. MuSK frizzled-like domain is critical for mammalian neuromuscular junction formation and maintenance. *J Neurosci*. (2015) 35:4926–41. doi: 10.1523/JNEUROSCI.3381-14.2015
75. Remedio L, Gribble KD, Lee JK, Kim N, Hallock PT, Delestree N, et al. Diverging roles for Lrp4 and Wnt signaling in neuromuscular synapse development during evolution. *Genes Dev*. (2016) 30:1058–69. doi: 10.1101/gad.279745.116
76. Rodriguez Cruz PM, Cossins J, Cheung J, Maxwell S, Jayawant S, Herbst R, et al. Congenital myasthenic syndrome due to mutations in MUSK suggests that the level of MuSK phosphorylation is crucial for governing synaptic structure. *Hum Mutat*. (2020) 41:619–31. doi: 10.1002/humu.23949
77. McConville J, Farrugia ME, Beeson D, Kishore U, Metcalfe R, Newsom-Davis J, et al. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. *Ann Neurol*. (2004) 55:580–4.
78. Evoli A, Tonali PA, Padua L, Monaco ML, Scuderi F, Batocchi AP, et al. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. *Brain*. (2003) 126(Pt 10):2304–11.
79. Farrugia ME, Kennett RP, Hilton-Jones D, Newsom-Davis J, Vincent A. Quantitative EMG of facial muscles in myasthenia patients with MuSK antibodies. *Clin Neurophysiol*. (2007) 118:269–77.
80. Sanders DB, El Salem K, Massey JM, McConville J, Vincent A. Clinical aspects of MuSK antibody positive seronegative MG. *Neurology*. (2003) 60:1978–80.
81. Vincent A, Bowen J, Newsom-Davis J, McConville J. Seronegative generalised myasthenia gravis: clinical features, antibodies, and their targets. *Lancet Neurol*. (2003) 2:99–106. doi: 10.1016/s1474-4422(03)00306-5
82. Zhou L, McConville J, Chaudhry V, Adams RN, Skolasky RL, Vincent A, et al. Clinical comparison of muscle-specific tyrosine kinase (MuSK) antibody-positive and -negative myasthenic patients. *Muscle Nerve*. (2004) 30:55–60. doi: 10.1002/mus.20069
83. Wolfe GI, Oh SJ. Clinical phenotype of muscle-specific tyrosine kinase-antibody-positive myasthenia gravis. *Ann N Y Acad Sci*. (2008) 1132:71–5. doi: 10.1196/annals.1405.005
84. Farrugia ME, Bydder GM, Francis JM, Robson MD. Magnetic resonance imaging of facial muscles. *Clin Radiol*. (2007) 62:1078–86.
85. Farrugia ME, Robson MD, Clover L, Anslow P, Newsom-Davis J, Kennett R, et al. MRI and clinical studies of facial and bulbar muscle involvement

in MuSK antibody-associated myasthenia gravis. *Brain*. (2006) 129(Pt 6): 1481–92.
86. Ishii W, Matsuda M, Okamoto N, Shimojima Y, Yazaki M, Motomura M, et al. Myasthenia gravis with anti-MuSK antibody, showing progressive muscular atrophy without blepharoptosis. *Intern Med*. (2005) 44:671–2.
87. Benveniste O, Jacobson L, Farrugia ME, Clover L, Vincent A. MuSK antibody positive myasthenia gravis plasma modifies MURF-1 expression in C2C12 cultures and mouse muscle in vivo. *J Neuroimmunol*. (2005) 170:41–8.
88. Boneva N, Frenkian-Cuvelier M, Bidault J, Brenner T, Berrih-Aknin S. Major pathogenic effects of anti-MuSK antibodies in myasthenia gravis. *J Neuroimmunol*. (2006) 177:119–31.
89. Finsterer J. Turn/amplitude analysis to assess bulbar muscle wasting in MuSK positive myasthenia. *Clin Neurophysiol*. (2007) 118:1173–4.
90. Martignago S, Fanin M, Albertini E, Pegoraro E, Angelini C. Muscle histopathology in myasthenia gravis with antibodies against MuSK and AChR. *Neuropathol Appl Neurobiol*. (2009) 35:103–10.
91. Cenacchi G, Valentina P, Marina F, Elena P, Corrado A. Comparison of muscle ultrastructure in myasthenia gravis with anti-MuSK and anti-AChR antibodies. *J Neurol*. (2011) 258:746–52. doi: 10.1007/s00415-010-5823-x
92. Mantegazza R, Baggi F, Antozzi C, Confalonieri P, Morandi L, Bernasconi P, et al. Myasthenia gravis (MG): epidemiological data and prognostic factors. *Ann N Y Acad Sci*. (2003) 998:413–23.
93. Grob D, Brunner N, Namba T, Pagala M. Lifetime course of myasthenia gravis. *Muscle Nerve*. (2008) 37:141–9.
94. Osserman KE, Genkins G. Studies in myasthenia gravis: review of a twenty-year experience in over 1200 patients. *Mt Sinai J Med*. (1971) 38:497–537.
95. Niks EH, Kuks JB, Verschuuren JJ. Epidemiology of myasthenia gravis with anti-muscle specific kinase antibodies in the Netherlands. *J Neurol Neurosurg Psychiatry*. (2007) 78:417–8.
96. Niks EH, Kuks JB, Roep BO, Haasnoot GW, Verduijn W, Ballieux BE, et al. Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5. *Neurology*. (2006) 66:1772–4.
97. Lauriola L, Ranelletti F, Maggiano N, Guerriero M, Punzi C, Marsili F, et al. Thymus changes in anti-MuSK-positive and -negative myasthenia gravis. *Neurology*. (2005) 64:536–8.
98. Leite MI, Strobel P, Jones M, Micklem K, Moritz R, Gold R, et al. Fewer thymic changes in MuSK antibody-positive than in MuSK antibody-negative MG. *Ann Neurol*. (2005) 57:444–8.
99. Saka E, Topcuoglu MA, Akkaya B, Galati A, Onal MZ, Vincent A. Thymus changes in anti-MuSK-positive and -negative myasthenia gravis. *Neurology*. (2005) 65:782–3.
100. Marino M, Maiuri MT, Di Sante G, Scuderi F, La Carpia F, Trakas N, et al. T cell repertoire in DQ5-positive MuSK-positive myasthenia gravis patients. *J Autoimmun*. (2014) 52:113–21. doi: 10.1016/j.jaut.2013.12.007
101. Lavrnic D, Losen M, Vujic A, De Baets M, Hajdukovic LJ, Stojanovic V, et al. The features of myasthenia gravis with autoantibodies to MuSK. *J Neurol Neurosurg Psychiatry*. (2005) 76:1099–102.
102. Niks EH, Kuks JB, Wokke JH, Veldman H, Bakker E, Verschuuren JJ, et al. Pre- and postsynaptic neuromuscular junction abnormalities in musk myasthenia. *Muscle Nerve*. (2010) 42:283–8. doi: 10.1002/mus.21642
103. Selcen D, Fukuda T, Shen XM, Engel AG. Are MuSK antibodies the primary cause of myasthenic symptoms? *Neurology*. (2004) 62:1945–50.
104. Shiraishi H, Motomura M, Yoshimura T, Fukudome T, Fukuda T, Nakao Y, et al. Acetylcholine receptors loss and postsynaptic damage in MuSK antibody-positive myasthenia gravis. *Ann Neurol*. (2005) 57:289–93.
105. Evoli A, Batocchi AP, Lo MM, Servidei S, Padua L, Majolini L, et al. Clinical heterogeneity of seronegative myasthenia gravis. *Neuromuscul Disord*. (1996) 6:155–61.
106. Huijbers MG, Zhang W, Klooster R, Niks EH, Friese MB, Straasheijm KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc Natl Acad Sci USA*. (2013) 110:20783–8. doi: 10.1073/pnas.1313944110
107. Tao MH, Smith RI, Morrison SL. Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation. *J Exp Med*. (1993) 178:661–7.
108. Klooster R, Plomp JJ, Huijbers MG, Niks EH, Straasheijm KR, Detmers FJ, et al. Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. *Brain*. (2012) 135(Pt 4):1081–101. doi: 10.1093/brain/aws025
109. Trampert DC, Hubers LM, van de Graaf SFJ, Beuers U. On the role of IgG4 in inflammatory conditions: lessons for IgG4-related disease. *Biochim Biophys Acta Mol Basis Dis*. (2018) 1864(4 Pt B):1401–9.
110. Koneczny I, Stevens JA, De Rosa A, Huda S, Huijbers MG, Saxena A, et al. IgG4 autoantibodies against muscle-specific kinase undergo Fab-arm exchange in myasthenia gravis patients. *J Autoimmun*. (2017) 77:104–15. doi: 10.1016/j.jaut.2016.11.005
111. Koneczny I, Cossins J, Waters P, Beeson D, Vincent A. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1-3 can disperse preformed agrin-independent AChR clusters. *PLoS One*. (2013) 8:e80695. doi: 10.1371/journal.pone.0080695
112. Leite MI, Jacob S, Viegas S, Cossins J, Clover L, Morgan BP, et al. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain*. (2008) 131(Pt 7):1940–52. doi: 10.1093/brain/awn092
113. Huijbers MG, Vink AF, Niks EH, Westhuis RH, van Zwet EW, de Meel RH, et al. Longitudinal epitope mapping in MuSK myasthenia gravis: implications for disease severity. *J Neuroimmunol*. (2016) 291:82–8. doi: 10.1016/j.jneuroim.2015.12.016
114. Otsuka K, Ito M, Ohkawara B, Masuda A, Kawakami Y, Sahashi K, et al. Collagen Q and anti-MuSK autoantibody competitively suppress agrin/LRP4/MuSK signaling. *Sci Rep*. (2015) 5:13928. doi: 10.1038/srep13928
115. Takamori M, Nakamura T, Motomura M. Antibodies against Wnt receptor of muscle-specific tyrosine kinase in myasthenia gravis. *J Neuroimmunol*. (2013) 254:183–6. doi: 10.1016/j.jneuroim.2012.09.001
116. Lindstrom J. Is "seronegative" MG explained by autoantibodies to MuSK? *Neurology*. (2004) 62:1920–1.
117. Shigemoto K, Kubo S, Maruyama N, Hato N, Yamada H, Jie C, et al. Induction of myasthenia by immunization against muscle-specific kinase. *J Clin Invest*. (2006) 116:1016–24.
118. Viegas S, Jacobson L, Waters P, Cossins J, Jacob S, Leite MI, et al. Passive and active immunization models of MuSK-Ab positive myasthenia: electrophysiological evidence for pre and postsynaptic defects. *Exp Neurol*. (2012) 234:506–12. doi: 10.1016/j.expneurol.2012.01.025
119. Jha S, Xu K, Maruta T, Oshima M, Mosier DR, Atassi MZ, et al. Myasthenia gravis induced in mice by immunization with the recombinant extracellular domain of rat muscle-specific kinase (MuSK). *J Neuroimmunol*. (2006) 175:107–17.
120. Punga AR, Lin S, Oliveri F, Meinen S, Ruegg MA. Muscle-selective synaptic disassembly and reorganization in MuSK antibody positive MG mice. *Exp Neurol*. (2011) 230:207–17. doi: 10.1016/j.expneurol.2011.04.018
121. Mori S, Kubo S, Akiyoshi T, Yamada S, Miyazaki T, Hotta H, et al. Antibodies against muscle-specific kinase impair both presynaptic and postsynaptic functions in a murine model of myasthenia gravis. *Am J Pathol*. (2012) 180:798–810. doi: 10.1016/j.ajpath.2011.10.031
122. Ulusoy C, Kim E, Tuzun E, Huda R, Yilmaz V, Poulas K, et al. Preferential production of IgG1, IL-4 and IL-10 in MuSK-immunized mice. *Clin Immunol (Orlando, Fla)*. (2014) 151:155–63.
123. Patel V, Oh A, Voit A, Sultatos LG, Babu GJ, Wilson BA, et al. Altered active zones, vesicle pools, nerve terminal conductivity, and morphology during experimental MuSK myasthenia gravis. *PLoS One*. (2014) 9:e110571. doi: 10.1371/journal.pone.0110571
124. Cole RN, Ghazanfari N, Ngo ST, Gervasio OL, Reddel SW, Phillips WD. Patient autoantibodies deplete postsynaptic muscle specific kinase leading to disassembly of the ACh receptor scaffold and myasthenia gravis in mice. *J Physiol*. (2010) 588(Pt 17):3217–29. doi: 10.1113/jphysiol.2010.190298
125. Mori S, Yamada S, Kubo S, Chen J, Matsuda S, Shudou M, et al. Divalent and monovalent autoantibodies cause dysfunction of MuSK by distinct mechanisms in a rabbit model of myasthenia gravis. *J Neuroimmunol*. (2012) 244:1–7. doi: 10.1016/j.jneuroim.2011.12.005
126. Plomp JJ, Van Kempen GT, De Baets MB, Graus YM, Kuks JB, Molenaar PC. Acetylcholine release in myasthenia gravis: regulation at single end-plate level. *Ann Neurol*. (1995) 37:627–36.

127. Ponseti JM, Caritg N, Gamez J, Lopez-Cano M, Vilallonga R, Armengol M. A comparison of long-term post-thymectomy outcome of anti-AChR-positive, anti-AChR-negative and anti-MuSK-positive patients with non-thymomatous myasthenia gravis. *Expert Opin Biol Ther*. (2009) 9:1–8. doi: 10.1517/14712590802588831
128. Clifford KM, Hobson-Webb LD, Benatar M, Burns TM, Barnett C, Silvestri NJ, et al. Thymectomy may not be associated with clinical improvement in MuSK myasthenia gravis. *Muscle Nerve*. (2019) 59:404–10. doi: 10.1002/mus.26404
129. Guptill JT, Sanders DB. Update on muscle-specific tyrosine kinase antibody positive myasthenia gravis. *Curr Opin Neurol*. (2010) 23:530–5.
130. Pasnoor M, Wolfe GI, Nations S, Trivedi J, Barohn RJ, Herbelin L, et al. Clinical findings in MuSK-antibody positive myasthenia gravis: a U.S. experience. *Muscle Nerve*. (2010) 41:370–4. doi: 10.1002/mus.21533
131. Punga AR, Flink R, Askmark H, Stalberg EV. Cholinergic neuromuscular hyperactivity in patients with myasthenia gravis seropositive for MuSK antibody. *Muscle Nerve*. (2006) 34:111–5.
132. Evoli A, Lindstrom J. Myasthenia gravis with antibodies to MuSK: another step toward solving mystery? *Neurology*. (2011) 77:1783–4.
133. Morren J, Li Y. Myasthenia gravis with muscle-specific tyrosine kinase antibodies: a narrative review. *Muscle Nerve*. (2018) 58:344–58. doi: 10.1002/mus.26107
134. Reddel SW, Morsch M, Phillips WD. Clinical and scientific aspects of muscle-specific tyrosine kinase-related myasthenia gravis. *Curr Opin Neurol*. (2014) 27:558–65. doi: 10.1097/WCO.0000000000000136
135. Evoli A, Alboini PE, Damato V, Iorio R, Provenzano C, Bartoccioni E, et al. Myasthenia gravis with antibodies to MuSK: an update. *Ann N Y Acad Sci*. (2018) 1412:82–9. doi: 10.1111/nyas.13518
136. Guptill JT, Sanders DB, Evoli A. Anti-musk antibody myasthenia gravis: clinical findings and response to treatment in two large cohorts. *Muscle Nerve*. (2011) 44:36–40. doi: 10.1002/mus.22006
137. Suh J, Goldstein JM, Nowak RJ. Clinical characteristics of refractory myasthenia gravis patients. *Yale J Biol Med*. (2013) 86:255–60.
138. Silvestri NJ, Wolfe GI. Treatment-refractory myasthenia gravis. *J Clin Neuromuscul Dis*. (2014) 15:167–78. doi: 10.1097/CND.0000000000000034
139. Richman DP, Agius MA. Treatment of autoimmune myasthenia gravis. *Neurology*. (2003) 61:1652–61.
140. Sanders DB, Wolfe GI, Benatar M, Evoli A, Gilhus NE, Illa I, et al. International consensus guidance for management of myasthenia gravis: Executive summary. *Neurology*. (2016) 87:419–25. doi: 10.1212/WNL.0000000000002790
141. Sanders DB, Juel VC. MuSK-antibody positive myasthenia gravis: questions from the clinic. *J Neuroimmunol*. (2008) 20:85–9. doi: 10.1016/j.jneuroim.2008.05.032
142. Hehir MK, Hobson-Webb LD, Benatar M, Barnett C, Silvestri NJ, Howard JF Jr., et al. Rituximab as treatment for anti-MuSK myasthenia gravis: multicenter blinded prospective review. *Neurology*. (2017) 89:1069–77. doi: 10.1212/WNL.0000000000004341
143. Keung B, Robeson KR, DiCapua DB, Rosen JB, O'Connor KC, Goldstein JM, et al. Long-term benefit of rituximab in MuSK autoantibody myasthenia gravis patients. *J Neurol Neurosurg Psychiatry*. (2013) 84:1407–9.
144. Illa I, Diaz-Manera J, Rojas-Garcia R, Pradas J, Rey A, Blesa R, et al. Sustained response to Rituximab in anti-AChR and anti-MuSK positive Myasthenia Gravis patients. *J Neuroimmunol*. (2008) 20:90–4. doi: 10.1016/j.jneuroim.2008.04.039
145. Diaz-Manera J, Martinez-Hernandez E, Querol L, Klooster R, Rojas-Garcia R, Suarez-Calvet X, et al. Long-lasting treatment effect of rituximab in MuSK myasthenia. *Neurology*. (2012) 78:189–93. doi: 10.1212/WNL.0b013e3182407982
146. Nowak RJ, Dicapua DB, Zebardast N, Goldstein JM. Response of patients with refractory myasthenia gravis to rituximab: a retrospective study. *Ther Adv Neurol Disord*. (2011) 4:259–66. doi: 10.1177/1756285611411503
147. Cortes-Vicente E, Rojas-Garcia R, Diaz-Manera J, Querol L, Casasnovas C, Guerrero-Sola A, et al. The impact of rituximab infusion protocol on the long-term outcome in anti-MuSK myasthenia gravis. *Ann Clin Transl Neurol*. (2018) 5:710–6. doi: 10.1002/acn3.564
148. Gajdos P, Chevret S, Toyka K. Intravenous immunoglobulin for myasthenia gravis. *Cochrane Database Syst Rev*. (2008) 12:CD002277.
149. Deguchi K, Matsuzono K, Nakano Y, Kono S, Sato K, Deguchi S, et al. Anti-MuSK antibody-positive Myasthenia Gravis successfully treated with outpatient periodic weekly blood purification therapy. *Intern Med*. (2018) 57:1455–8. doi: 10.2169/internalmedicine.9466-17
150. Govindarajan R, Iyadurai SJ, Connolly A, Zaidman C. Selective response to rituximab in a young child with MuSK-associated myasthenia gravis. *Neuromuscul Disord*. (2015) 25:651–2. doi: 10.1016/j.nmd.2015.03.014
151. Takahashi H, Kawaguchi N, Nemoto Y, Hattori T. High-dose intravenous immunoglobulin for the treatment of MuSK antibody-positive seronegative myasthenia gravis. *J Neurol Sci*. (2006) 247:239–41.
152. Shibata-Hamaguchi A, Samuraki M, Furui E, Iwasa K, Yoshikawa H, Hayashi S, et al. Long-term effect of intravenous immunoglobulin on anti-MuSK antibody-positive myasthenia gravis. *Acta Neurol Scand*. (2007) 116:406–8.
153. Tzartos SJ, Bitzopoulou K, Gavra I, Kordas G, Jacobson L, Kostelidou K, et al. Antigen-specific apheresis of pathogenic autoantibodies from myasthenia gravis sera. *Ann N Y Acad Sci*. (2008) 1132:291–9. doi: 10.1196/annals.1405.017
154. Yeh JH, Chiu HC. Comparison between double-filtration plasmapheresis and immunoadsorption plasmapheresis in the treatment of patients with myasthenia gravis. *J Neurol*. (2000) 247:510–3.
155. Trinh VB, Foster AJ, Fairclough RH. Design, synthesis, and characterization of a 39 amino acid peptide mimic of the main immunogenic region of the Torpedo acetylcholine receptor. *Mol Immunol*. (2014) 59:79–90. doi: 10.1016/j.molimm.2014.01.002
156. Ellebrecht CT, Bhoj VG, Nace A, Choi EJ, Mao X, Cho MJ, et al. Reengineering chimeric antigen receptor T cells for targeted therapy of autoimmune disease. *Science*. (2016) 353:179–84. doi: 10.1126/science.aaf6756


**Conflict of Interest:** LB and DR have received research funding from NINDS (1R21NS104516), Myasthenia Gravis Foundation of America, and Cabaletta Bio Inc. DR is a member of the Advisory Board of Cabaletta Bio Inc.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 May 2020
doi: 10.3389/fimmu.2020.00809

# CD4$^+$ T Cells of Myasthenia Gravis Patients Are Characterized by Increased IL-21, IL-4, and IL-17A Productions and Higher Presence of PD-1 and ICOS

*Merve Çebi[1], Hacer Durmus[2], Fikret Aysal[3], Berker Özkan[4], Gizem Engin Gül[5], Arman Çakar[2], Mehmet Hocaoglu[1], Metin Mercan[6], Sibel P. Yentür[1], Melih Tütüncü[7], Vildan Yayla[6], Onur Akan[5], Öner Dogan[8], Yeşim Parman[2] and Güher Saruhan-Direskeneli[1]**

[1] Department of Physiology, Istanbul Medical Faculty, Istanbul University, Istanbul, Turkey, [2] Department of Neurology, Istanbul Medical Faculty, Istanbul University, Istanbul, Turkey, [3] Department of Neurology, Medipol University, Istanbul, Turkey, [4] Department of Thoracic Surgery, Istanbul Medical Faculty, Istanbul University, Istanbul, Turkey, [5] Okmeydani State Hospital, Istanbul, Turkey, [6] Bakirköy Sadi Konuk State Hospital, Istanbul, Turkey, [7] Department of Neurology, Cerrahpaşa Medical Faculty, Istanbul University Cerrahpaşa, Istanbul, Turkey, [8] Department of Pathology, Istanbul Medical Faculty, Istanbul University, Istanbul, Turkey

OPEN ACCESS

**Edited by:**
Rozen Le Panse,
Sorbonne Université, France

**Reviewed by:**
Paola Cavalcante,
Carlo Besta Neurological Institute (IRCCS), Italy
Howard A. Young,
National Cancer Institute at Frederick, United States

***Correspondence:**
Güher Saruhan-Direskeneli
gsaruhan@istanbul.edu.tr

**Specialty section:**
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

**Received:** 11 November 2019
**Accepted:** 08 April 2020
**Published:** 19 May 2020

**Citation:**
Çebi M, Durmus H, Aysal F, Özkan B, Gül GE, Çakar A, Hocaoglu M, Mercan M, Yentür SP, Tütüncü M, Yayla V, Akan O, Dogan Ö, Parman Y and Saruhan-Direskeneli G (2020) CD4$^+$ T Cells of Myasthenia Gravis Patients Are Characterized by Increased IL-21, IL-4, and IL-17A Productions and Higher Presence of PD-1 and ICOS. Front. Immunol. 11:809. doi: 10.3389/fimmu.2020.00809

Myasthenia gravis (MG) is an autoimmune disease mediated by autoantibodies predominantly against the acetylcholine receptor (AChR). Specific T cell subsets are required for long-term antibody responses, and cytokines secreted mainly from CD4$^+$ T cells regulate B cell antibody production. The aim of this study was to assess the differences in the cytokine expressions of CD4$^+$ T cells in MG patients with AChR antibodies (AChR-MG) and the effect of immunosuppressive (IS) therapy on cytokine activity and to test these findings also in MG patients without detectable antibodies (SN-MG). Clinically diagnosed AChR-MG and SN-MG patients were included. The AChR-MG patients were grouped as IS-positive and -negative and compared with age- and sex-matched healthy controls. Peripheral blood mononuclear cells were used for *ex vivo* intracellular cytokine production, and subsets of CD4$^+$ T cells and circulating follicular helper T (cTfh) cells were detected phenotypically by the expression of the chemokine and the costimulatory receptors. Thymocytes obtained from patients who had thymectomy were also analyzed. IL-21, IL-4, IL-10, and IL-17A productions in CD4$^+$ T cells were increased in AChR-MG compared to those in healthy controls. IS treatment enhanced IL-10 and reduced IFN-γ production in AChR-MG patients compared to those in IS-negative patients. Increased IL-21 and IL-4 productions were also demonstrated in SN-MG patients. Among CD4$^+$ T cells, Th17 cells were increased in both disease subgroups. Treatment induced higher proportions of Th2 cells in AChR-MG patients. Both CXCR5$^+$ and CXCR5$^-$ CD4$^+$ T cells expressed higher programmed cell death protein 1 (PD-1) and inducible costimulatory (ICOS) in AChR-MG and SN-MG groups, mostly irrespective of the treatment. Based on chemokine receptors on CXCR5$^+$PD-1$^+$ in CD4$^+$ T (cTfh) cells, in AChR-MG patients without treatment, the proportions of Tfh17 cells were higher

than those in the treated group, whereas the Tfh1 cells were decreased compared with those in the controls. The relevance of CXCR5 and PD-1 in the pathogenesis of AChR-MG was also suggested by the increased presence of these molecules on mature CD4 single-positive thymocytes from the thymic samples. The study provides further evidence for the importance of IL-21, IL-17A, IL-4, and IL-10 in AChR-MG. Disease-related $CD4^+$T cells are identified mainly as PD-$1^+$ or $ICOS^+$ with or without CXCR5, resembling cTfh cells in the circulation or probably in the thymus. AChR-MG and SN-MG seem to have some similar characteristics. IS treatment has distinctive effects on cytokine expression.

**Keywords: T follicular helper cells, PD-1, ICOS, IL-21, IL-4, IL-17, CXCR5, myasthenia gravis**

## INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease characterized by fatigable muscle weakness caused by pathologic autoantibodies. The majority of MG patients (80–85%) have autoantibodies against acetylcholine receptor (AChR). Autoantibodies against muscle-specific kinase (MuSK) are present in a smaller subgroup of patients (1, 2). A small proportion of MG patients [10–15%, classified as seronegative MG (SN-MG)] do not have detectable autoantibodies against these antigens. A clinical comparison between AChR-MG, MuSK-MG, and SN-MG has revealed that the SN-MG patients were closer to the AChR-MG patients rather than to the MuSK-MG patients (3). Several findings in SN-MG support the possible role of autoantibodies related to AChR which can be detected by more sensitive assays in patients considered to be seronegative (4–6).

Thymus, the organ for development of self-tolerance, reveals different abnormalities in MG subtypes. In early-onset patients, the thymus is typically enlarged and contains many follicular germinal centers with T and B cells similar to those seen in the lymph nodes (7). Thymic hyperplasia with follicular structures frequently accompanies AChR-MG and is also detected in some SN-MG patients (8). However, distinct gene signatures in thymic samples from AChR-MG and SN-MG have also been demonstrated, underlining the different mechanisms of these disease subtypes (9).

T follicular helper (Tfh) cells, as a specialized subset of $CD4^+$ T lymphocytes, are necessary for the generation of germinal centers (GC) in secondary lymphoid organs (10, 11). These cells are major producers of IL-21 which promotes B cell differentiation, antibody production, and Ig isotype switching, resulting in long-lasting antibody responses (12, 13). Tfh cells express transcription factor Bcl-6 and are characterized by their surface expression of C-X-C chemokine receptor type 5 (CXCR5), inducible costimulatory (ICOS), and programmed cell death protein 1 (PD-1) (14). Some studies have identified Tfh cells as total $CXCR5^+CD4^+$ T cells, while others have used subsets of $CD4^+$ T cells such as $CXCR5^+ICOS^+$, $CXCR5^+$PD-$1^+$, $CXCR5^+ICOS^+$PD-$1^+$, or $CXCR5^+$IL-$21^+$ (15). A circulating Tfh (cTfh) population has been described, which also expresses CXCR5, PD-1, and ICOS and can help B cell differentiation into plasma cells *via* IL-21 secretion (16).

An increase in the frequencies of cTfh populations is associated with several autoimmune diseases including rheumatoid arthritis (RA) (17), systemic lupus erythematosus (SLE) (18), and systemic sclerosis (SSc) (19). Recently, a pathologically expanded population of $CXCR5^-$PD-$1^{hi}CD4^+$ T cells called T peripheral helper (Tph) cells has been identified in the synovium of patients with RA, which could also promote plasma cell differentiation (20). $CXCR5^-$PD-$1^+CD4^+$T cell numbers and frequencies in blood positively correlated with plasma cells in patients with SSc (19). Both $CXCR5^-$PD-$1^+CD4^+$ and $CXCR5^+$PD-$1^+CD4^+$ T cells have been shown to produce high IL-21 (21). These findings implicate that the presence of the PD-1 molecule seems to be more effective than the presence of the CXCR5 molecule in antibody production.

Increased frequencies of $ICOS^{hi}$ or PD-$1^{hi}CXCR5^+CD4^+$ T cells with correlating serum AChR antibodies were reported in MG (22). A significant enrichment of activated ($ICOS^+$) cTfh ($CD4^+CXCR5^+$PD-$1^+$) cells has been assigned to Tfh subsets, namely, Tfh1 and Tfh17 cells, and these subsets were identified as the major source for IL-21 in generalized MG patients (22, 23). A demonstration of Tfh and B cells co-localized within the ectopic GC in MG thymus has also suggested the putative existence of intrathymic Tfh/B cell interaction playing a key role in this disease (24).

The pathogenesis of MG is generally characterized by various cytokines (25). Cytokine measurements in the sera revealed conflicting results: higher levels of IL-21 and IL-6 (23) or no significant increase in IL-21, IL-4, and IL-6 levels in AChR-MG patients (26) has been reported. Similarly, increased IL-17 in the sera of MG patients (27, 28) and similar levels among healthy controls (HC) in the sera or the culture supernatants of AChR-MG patients were demonstrated (26, 29).

A study measuring cytokine production from AChR-specific single-cell clones of MG patients demonstrated the co-expression of IFN-γ, IL-17, and GM-CSF, but not IL-10 (30). The heterogeneity of the disease and the effect of IS treatment may have caused these discrepancies between the studies which need clarification.

The anti-inflammatory properties of IS treatment result from the downregulation of pro-inflammatory or upregulation of anti-inflammatory genes. Several studies have demonstrated that glucocorticoids enhance the concentration of IL-10 in cultures

of peripheral blood mononuclear cells (PBMCs) from HC *in vitro* (31, 32). In addition, the IL-10 levels were increased in the sera of MG patients who received IS treatment, and the IFN-γ levels of these patients were also lower than those of the controls (26). On the other hand, some studies have shown the effects of glucocorticoids on the Tfh population. Glucocorticoids decreased the $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ Tfh cell population in SLE (33). In another study, the Tfh cells and the plasmablasts were decreased after steroid therapy in patients with IgG4-related disease (34).

IL-21, IL-4, IL-17A, IL-10, and IFN-γ were previously shown to be involved in the pathogenesis of MG (23, 26, 35). Based also on previous data, we aimed to further characterize the $CD4^{+}$ T cells producing these relevant cytokines in AChR-MG and SN-MG. Among the $CD4^{+}$ T cells, the role of cTfh cells or their functional molecules, PD-1 and ICOS, was investigated for differential contribution to disease development as well as to treatment responses in this study. The cytokines and their producer cells were compared *ex vivo* in the IS treatment groups. Moreover, changes in the thymic tissue cells of AChR-MG patients parallel with the peripheral T cells were also investigated.

# MATERIALS AND METHODS

## MG Patient Blood Samples

Blood samples from 95 MG patients and 64 HC were included in this study (**Table 1**). The diagnosis of MG was based on clinical presentation, electrophysiologic examination, and presence of AChR antibodies. Of the 73 AChR antibody-positive patients, 34 had early onset (<50 years, AChR-EO,) and 39 had late onset (≥50 years, AChR-LO). In the AChR-MG group, 31 patients were on IS drugs (steroid alone or steroid plus azathioprine, ISP group) at the time of blood sampling, whereas 42 patients were not receiving IS treatment (ISN). In this group, 24 patients were previously thymectomized and the pathological classifications are shown in **Table 1**. Additional 22 patients without AChR and MuSK antibodies were included in the SN-MG group, eight (36%) of whom were thymectomized and were receiving treatment at the time of blood sampling. Thymoma-associated MG cases were not included in this study. In the AChR-MG group, seven patients (women/men: 2/5, AChR-EO/AChR-LO: 2/5), who were initially not on IS treatment, were followed for 6 months after starting the treatment and tested again while under treatment. The age and gender distributions of the patients and HC were balanced to be not different from each other. AChR and MuSK antibodies were measured by radioimmunoassay (DLD Diagnostika GmBH, Germany) and ELISA (Euroimmune, Germany) with commercial kits.

This study was approved by the Ethical Review Board of the Istanbul Medical Faculty. Peripheral blood was obtained from the donors after acquiring informed consent.

## Thymic Tissue Cells

Thymic tissue samples were obtained separately from the blood samples during therapeutic thymectomy in MG patients (**Table 2**). From only five patients, peripheral blood was taken simultaneously at the time of thymectomy. According to the thymic pathology, 33 (89.2%) of the AChR-MG patients had thymic hyperplasia and only four patients (10.8%) had involution. The isolated tissue cells from non-myasthenic patients with different ages (0–51) and who were undergoing corrective cardiovascular surgery were evaluated as controls (Con). The samples were taken from the parenchymal parts of the specimens under the guidance of a pathologist and were processed within a few hours. Cell suspensions from the thymic samples were obtained by mechanical manipulation, filtration through a cell strainer (100 μm, Life Sciences), and separation using the density gradient method to obtain the mononuclear cells. The final thymocyte suspensions were washed twice in staining buffer (phosphate-buffered solution containing 1% bovine serum albumin, SB). The analysis of molecules on freshly isolated cells was performed using three-color immunofluorescence by flow cytometry as described below.

**TABLE 1 |** Characteristics of the patients included in the study for peripheral blood cell evaluations.

| Characteristics | AChR-MG patients ($n = 73$) | SN-MG patients ($n = 22$) | HC ($n = 64$) |
|---|---|---|---|
| Median age (years) | 53 (14–79) | 48 (18–73) | 47 (27–76) |
| Women (%) | 40 (55) | 15 (68) | 34 (53) |
| Disease onset (EO/LO) | 34/39 | 15/7 | – |
| Immunosuppressive treatment (%) | 31 (43) | 8 (36) | – |
| Thymectomy (%) | 24 (33) | 8 (36) | – |
| Hyperplasia | 18 | 2 | |
| Involution | 6 | 6 | |

*AChR-MG, myasthenia gravis patients with AChR antibodies; SN-MG, myasthenia gravis patients without detectable antibodies; EO, early onset; LO, late onset.*

**TABLE 2 |** Features of the donors included in the study for thymic samples.

| | M (%) | W (%) | Total | Median age | ISP (%) | ISN (%) |
|---|---|---|---|---|---|---|
| AChR-MG | 1 (3) | 36 (97) | 37 | 26 (9–51) | 16 (43.2) | 21 (56.8) |
| Hyperplasia | | | 33 | | | |
| Involution | | | 4 | | | |
| Con | 7 (36) | 13 (64) | 20 | 8 (0–51) | | |

*The myasthenia gravis patients were divided into immunosuppressive treatment positive (ISP) and negative (ISN) groups.*
*M, men; W, women; AChR-MG, myasthenia gravis patients with AChR antibodies; Con, thymic tissues from patients without myasthenia gravis.*

## Phenotypic Analyses of Peripheral Blood and Thymic Samples

PBMCs were separated by a gradient centrifugation procedure on a lymphocyte separation medium (Secoll Separation Media, Mannheim, Germany). After separation, freshly isolated cells were stained with the following fluorophore-conjugated antibodies: CD4 APC-Cy7 (clone: RFT-4g), ICOS PE-Cy7 (clone: ISA-3, eBioscience, ThermoFisher), CXCR5 FITC (CD185, clone: REA303), CXCR3 APC (CD183, clone: REA232), PD-1 PE-Vio 770 (clone: PD-1.3.1.3, Miltenyi Biotec), CD45RA PE-Cy5

(clone: HI100, BioLegend), and CCR6 PE (CD196, clone: 11A9, BD Bioscience).

The freshly isolated thymocytes and the PBMCs obtained at the time of thymectomy were stained with the following fluorochrome-conjugated antibodies: CD4 FITC (clone: RPA-T4, Beckman Coulter), CD8 APC (clone: RPA-T8), CXCR3 eFluor660 (CD183, clone: CEW33D, eBioscience), CCR6 PE (CD196, clone: 11A9), PD-1 PE (CD279, clone: MIH4, BD Bioscience), ICOS PE (clone: C398.4A, BioLegend), and CXCR5 PE (CD185, clone: 51505, R&D Systems). The samples were analyzed on an Attune Flow Cytometry (Thermofisher, USA). Fluorescence minus one control, which contains all flurochromes in a panel except for the target markers measured, was used to identify and to gate the cells.

## Intracellular Staining

Freshly isolated PBMCs were seeded in 48-well plates at a final concentration of $2 \times 10^6$ cells/ml in complete RPMI1640 medium supplemented with 2 mM L-glutamine, 100 IU/100 mg/ml penicillin/streptomycin (Sigma), and 10% fetal bovine serum (Gibco) and were stimulated by the cell stimulation cocktail (500X) containing phorbol 12-myristate 13-acetate and ionomycin (eBioscience, ThermoFisher) for 4 h at 37°C. After washing with SB, the cells were surface-stained with anti-CD4 antibody (clone: RPA-T4, BioLegend) for 20 min on ice. Then, the cells were permeabilized and fixed with a fixation/permeabilization solution (BD Bioscience) according to the manufacturer's instructions. The cells were stained with IL-21 PE (clone: 3A3-N2), IL-4 Alexa Fluor 488 (clone: 8D4-8), IL-17A PE (clone: eBio64), IFN-γ Alexa Fluor 488 (clone: 4S.B3), or IL-10 Alexa Fluor 488 (clone: JES3-9D7, eBiosciences Thermofisher) for 30 min on ice. The results were acquired by flow cytometry as discussed above.

## Statistical Analysis

In the statistical analysis measurements, Shapiro–Wilk test for normality was applied. As the distributions of variables were not normal in the sample populations, non-parametric tests (Kruskal–Wallis and Mann–Whitney U) were used with SPSS version 21. In the comparison of thymic samples, covariance analysis (ANCOVA) was applied for controlling the age differences between patients and controls. The data were presented as median values with interquartile ranges in the figures. A $p < 0.05$ was considered as statistically significant.

# RESULTS

## Elevated IL-21, IL-4, IL-17A, and IL-10 Production in MG Patients

To understand the differential role of cytokines in AChR-MG and SN-MG development, IL-21, IL-4, IL-17A, IFN-γ, and IL-10 productions of $CD4^+$ T cells were analyzed in the PBMCs *ex vivo*. The intracellular staining of $CD4^+$ T cells is presented in **Figure 1A**. IL-21, IL-4, IL-17A, and IL-10 productions were increased in AChR-MG patients ($n = 54$, $p < 0.001$, $p < 0.001$, $p = 0.001$, and $p = 0.014$, respectively), whereas IFN-γ was lower compared to that in HC ($n = 38$, $p = 0.023$) (**Figure 1B**). IL-21 and IL-4 were also increased in SN-MG patients ($n = 11$) ($p = 0.032$ and $p = 0.010$). IL-17A and IL-10 were also slightly higher than the controls but without significance. There were no significant differences in the cytokine profile between AChR-MG and SN-MG patients. Cytokine productions were also not different between AChR-EO and AChR-LO MG subgroups, patients with or without thymectomy, or according to the thymic pathologies (data not shown). However, both in AChR-MG and SN-MG groups, but not in HC, women produced higher IFN-γ than men (12.3 vs. 5.5%, $p = 0.008$ and 19.3 vs. 3.0%, $p = 0.034$; data not shown).

## Effects of IS Treatment on Cytokines

Cytokine production is influenced by IS treatment according to several studies (31, 32). This effect was evaluated only in AChR-MG patients separated as ISP ($n = 25$) or ISN ($n = 29$). IS treatment had an increasing effect on IL-10 production in ISP patients compared to that in HC as well as to the ISN patient group (both $p = 0.001$). IFN-γ production in ISN patients was lower than in HC ($p = 0.037$) (**Figure 2A**). The IFN-γ levels of ISP patients were similar to the ISN group and also relatively lower than those in the HC group. No significant effect of IS treatment on increased IL-21, IL-4, and IL-17A productions was observed (data not shown).

With another approach, seven AChR-MG patients who were initially not on IS treatment have been followed up to 6 months after starting the treatment. Measurements of the cytokines were performed in the same patients before and after the treatment. IL-10 increased in the $CD4^+$ T cells of all patients on IS treatment ($p = 0.028$), supporting the above finding of treatment. On the contrary, IFN-γ decreased with the effect of IS treatment ($p = 0.018$) (**Figure 2B**). No other effect of IS treatment on cytokines was shown (data not shown). In these seven patients, five had improved clinically with reduced symptoms. As only two out of 11 patients in the SN-MG group were on IS treatment, this effect was not evaluated in this group.

## $CD4^+$ T Cells and T Helper Subsets in MG Patients

The findings of increased cytokines in AChR-MG and SN-MG lead us to identify the $CD4^+$ T cells further and to characterize the cytokine producers in the blood. Among the lymphocytes, the $CD4^+$ T cells were significantly decreased in both AChR-MG ($n = 47$, $p < 0.001$) and SN-MG ($n = 16$, $p = 0.004$) groups compared to HC group ($n = 37$). These decreases were also observed in the $CD4^+$ T cells with the memory phenotype ($CD45RA^-$) in AChR-MG ($n = 30$, $p < 0.001$) and SN-MG ($n = 11$, $p = 0.003$) groups compared with HC group ($n = 25$) (**Figure 3A**). We further characterized these $CD4^+$ T cells in patients for their differentiation states into Th subgroups related to cytokine production. The Th subsets were identified by their differential expression of CXCR3 and CCR6 on $CD4^+$ T cells; the proportions of Th1: $CXCR3^+CCR6^-$, Th2: $CXCR3^-CCR6^-$, and Th17: $CXCR3^-CCR6^+$ were measured among the $CD4^+$ T cells (**Supplementary Figure 1**). When the distribution of the Th cell subsets was compared with HC, the

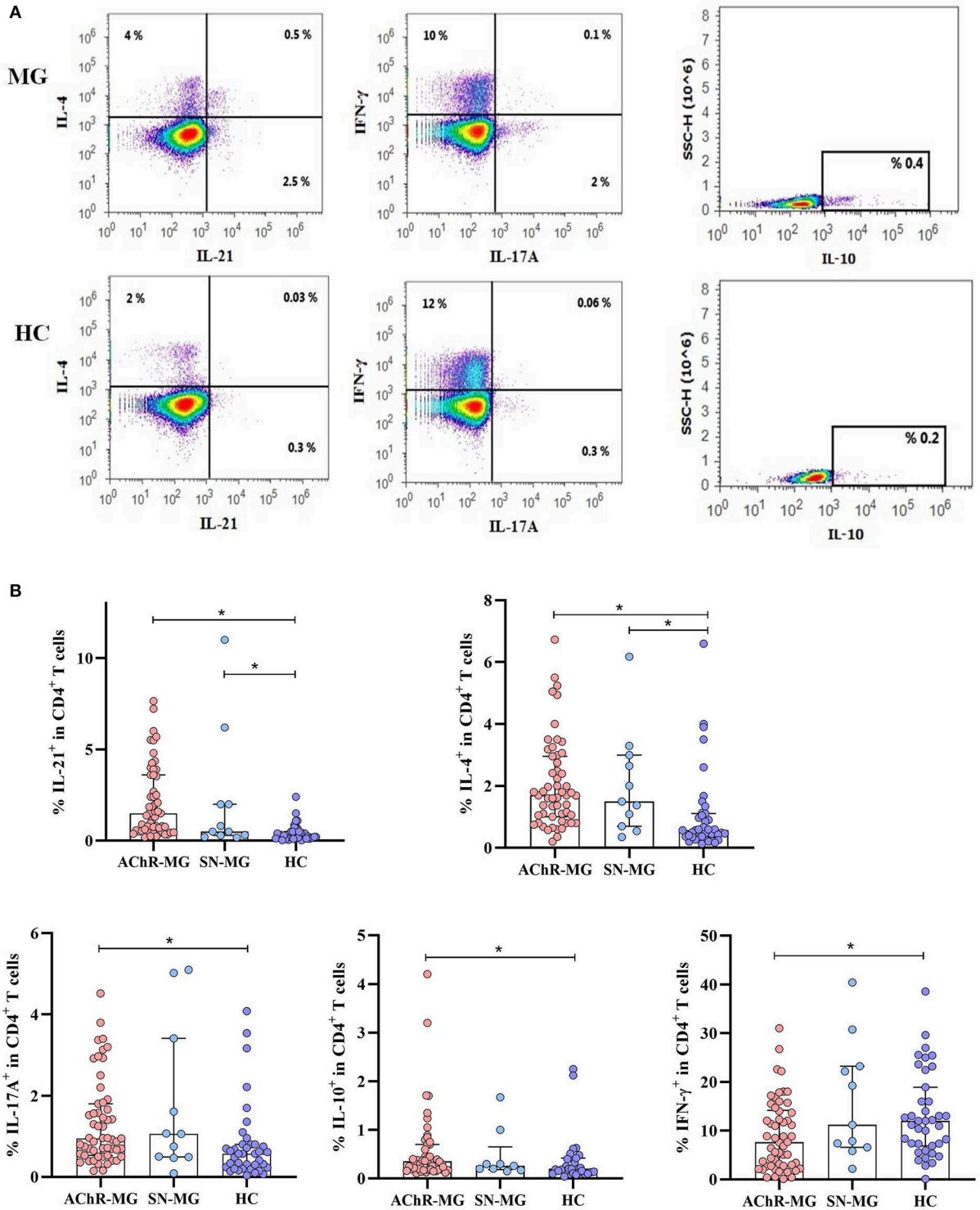



**FIGURE 1 |** Cytokine production of $CD4^+$ T cells in myasthenia gravis (MG) subgroups. **(A)** Measurement of intracellular IL-21, IL-4, IL-17A, IL-10, and IFN-γ in $CD4^+$ T cells of a patient and a healthy control (HC) by flow cytometry after 4 h of stimulation with phorbol 12-myristate 13-acetate and ionomycin in cell culture. **(B)** The AChR-MG ($n = 54$) patients had higher IL-21, IL-4, IL-17A, and IL-10 ($p < 0.001$, $p < 0.001$, $p = 0.001$, and $p = 0.014$) and lower IFN-γ ($p = 0.023$) in $CD4^+$ T cells compared to HC ($n = 38$). In the SN-MG group ($n = 11$), IL-21 ($p = 0.032$), and IL-4 ($p = 0.010$) were increased compared with those of HC. The results are compared with non-parametric tests (Kruskal–Wallis and Mann–Whitney *U*). * depicts a significant difference.

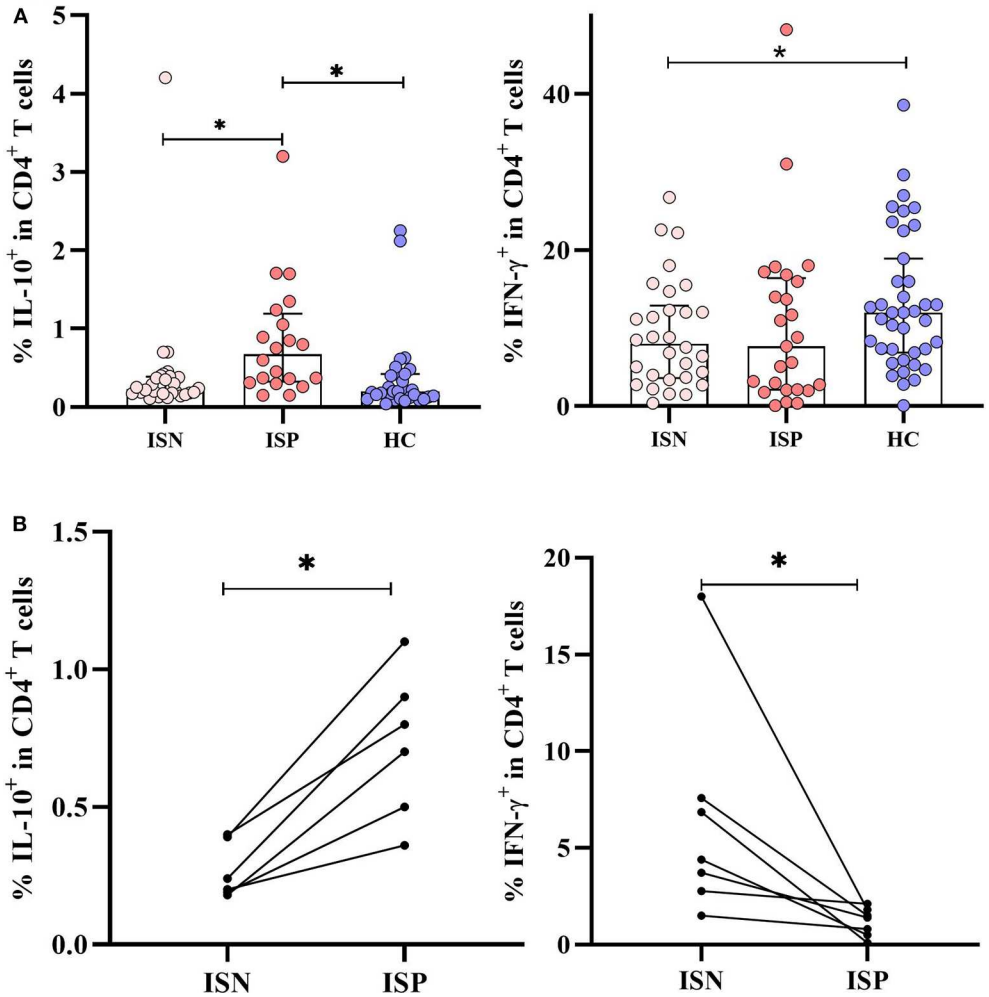



**FIGURE 2 |** Effect of immunosuppressive (IS) treatment in myasthenia gravis patients with AChR antibodies (AChR-MG) patients. **(A)** IL-10 production was increased in IS-positive patients ($n = 25$) compared to that of IS-negative patients ($n = 29$) and healthy control (HC) (both $p = 0.001$). The patients who were not receiving IS treatment had lower IFN-$\gamma^+$ CD4$^+$ T cells compared to HC ($p = 0.037$). **(B)** The effect of IS treatment in sequential measurements of seven patients: The initial IL-10 and IFN-γ values of AChR-MG patients and the values after IS treatment are shown ($p = 0.028$ and $p = 0.018$). The results are compared with non-parametric tests (Kruskal–Wallis and Mann–Whitney *U*). * depicts a significant difference.

Th17 cell population was increased both in AChR-MG and SN-MG patients ($p < 0.001$ and $p = 0.001$) (**Figure 3B**). Disease-onset age (EO vs. LO) as well as gender did not have any effect on the CD4$^+$ T cell subset distribution. In 32% of the AChR-MG group who had been thymectomized, cell distribution was also not different from that of the non-thymectomized patients (data not shown).

## Effect of IS Treatment on Th Cells

As the cytokine production was affected by the IS treatment, similar effects were investigated in the Th cell subtypes. IS treatment did not change the observed increase of Th17 subset in AChR-MG patients. Both ISP ($n = 26$) and ISN ($n = 21$) groups had higher Th17 cells compared to HC ($n = 37$, $p = 0.001$ and $p = 0.002$, respectively). However, IS treatment induced a relative increase of Th2 cells ($p = 0.021$) which were significantly lower in the untreated group than in HC ($p = 0.013$). Similar to AChR-MG, both ISP ($n = 7$) and ISN ($n = 9$) groups had higher Th17 cell populations ($p = 0.001$ and $p = 0.036$) in SN-MG (**Figure 3B**).

## PD-1 Expression Was Higher on CD4$^+$ T Cells in MG

In AChR-MG, higher productions of IL-21, IL-4, and also IL-17A were detected in total CD4$^+$ T cells. Among the CD4$^+$ T cells, the Tfh cells are considered as the major cell type for IL-21 and IL-4 productions (12, 13). The involvement of the cTfh cells in AChR-MG has also been reported (22, 23). To evaluate the functional state of the CD4$^+$ T cells and their relatedness with the cTfh cells, firstly PD-1 expression was examined on CD4$^+$ T cells with or without CXCR5 molecules (**Figure 4A**). We analyzed the blood cells of 35 AChR-MG and 12 SN-MG patients and 25 HC

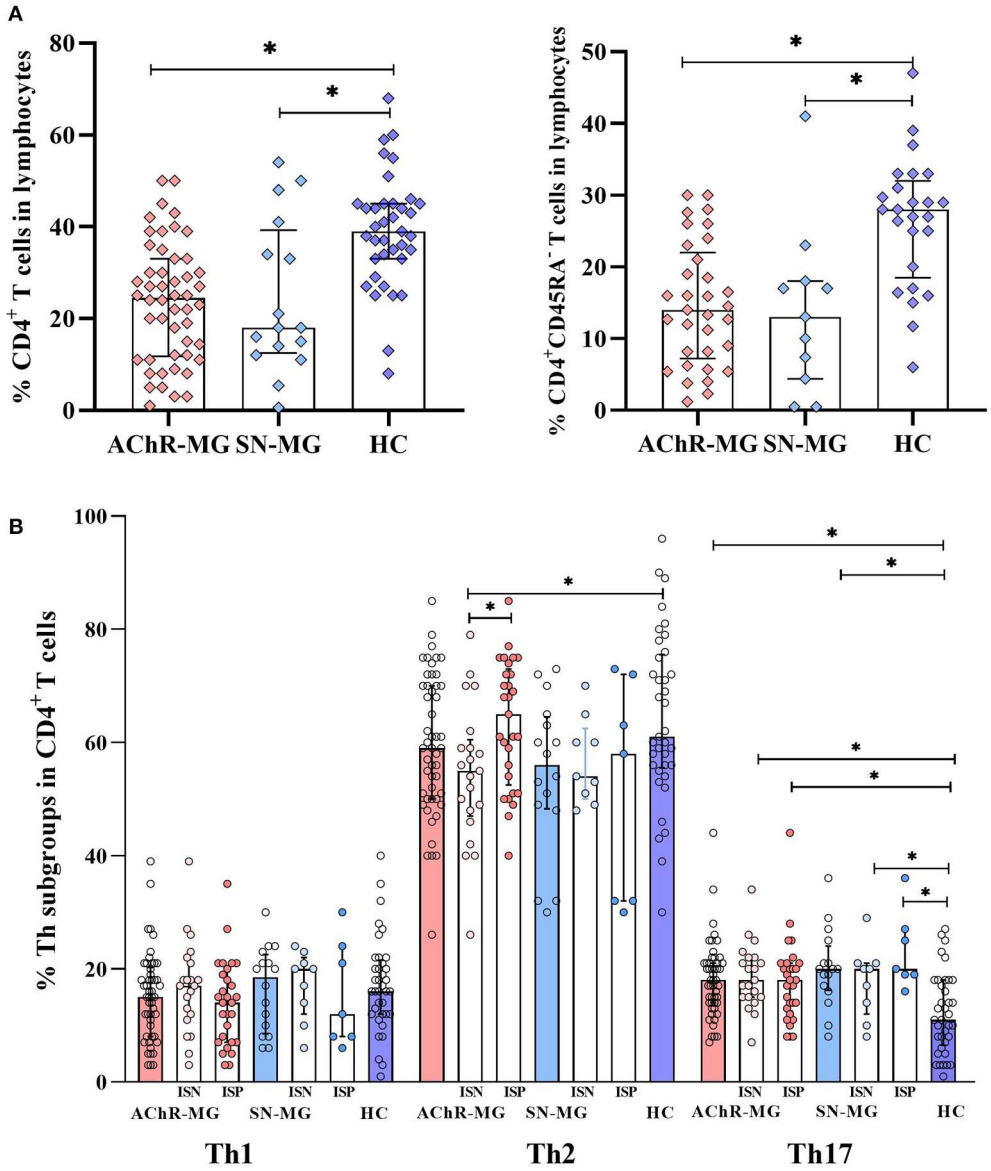



**FIGURE 3 |** Distribution of CD4+ T cells and T helper subsets in the study groups. **(A)** CD4+ T and CD4+CD45RA− T cells were lower in myasthenia gravis patients with AChR antibodies (AChR-MG) ($n = 47$, $n = 30$, both $p < 0.001$) and in myasthenia gravis patients without detectable antibodies (SN-MG) ($n = 16$, $p = 0.004$ and $n = 11$, $p = 0.003$) compared with those in HC ($n = 37$ and $n = 25$). **(B)** The Th17 ($CXCR3^{-}CCR6^{+}$) cell subset was increased in AChR-MG ($p < 0.001$) and SN-MG ($p = 0.001$) groups compared with that in HC. Both AChR-MG immunosuppressive (IS)-positive ($n = 26$) and IS-negative ($n = 21$) groups had higher Th17 cells compared to HC ($p = 0.002$ and $p = 0.001$). Immunosuppressive (IS) treatment induced a relative increase of Th2 cells ($p = 0.021$) which were significantly lower than those in HC ($p = 0.013$). Both IS-positive ($n = 7$) and IS-negative ($n = 9$) patients had higher Th17 cell populations ($p = 0.001$ and $p = 0.036$) in the SN-MG group. The results are compared with non-parametric tests (Mann–Whitney *U* test). * depicts a significant difference.

by flow cytometry. PD-1 expressing CD4+ T cells was higher in AChR-MG as well as in SN-MG patients compared to HC (both $p < 0.001$). Both $PD\text{-}1^{+}CXCR5^{+}$ (cTfh cells) and $PD\text{-}1^{+}CXCR5^{-}$ populations in CD4+ T cells were increased in AChR-MG patients ($p = 0.004$ and $p = 0.001$, respectively) compared with HC. Only the $PD\text{-}1^{+}CXCR5^{-}$ population in CD4+ T cells was significantly higher in SN-MG patients ($p = 0.006$, **Figure 4B**).

## ICOS Expression of CD4+ T Cell Was Increased in MG

ICOS and PD-1 are two molecules closely related to the function of Tfh cells. Previous studies have reported that ICOS and PD-1 are highly expressed on $CD4^{+}CXCR5^{+}$ T cells in the PBMCs of AChR-MG patients (22, 23, 36). As PD-1 was increased on total CD4+ T cells, covering $CD4^{+}CXCR5^{+}$ and $CD4^{+}CXCR5^{-}$ T cells, we analyzed ICOS expression also in

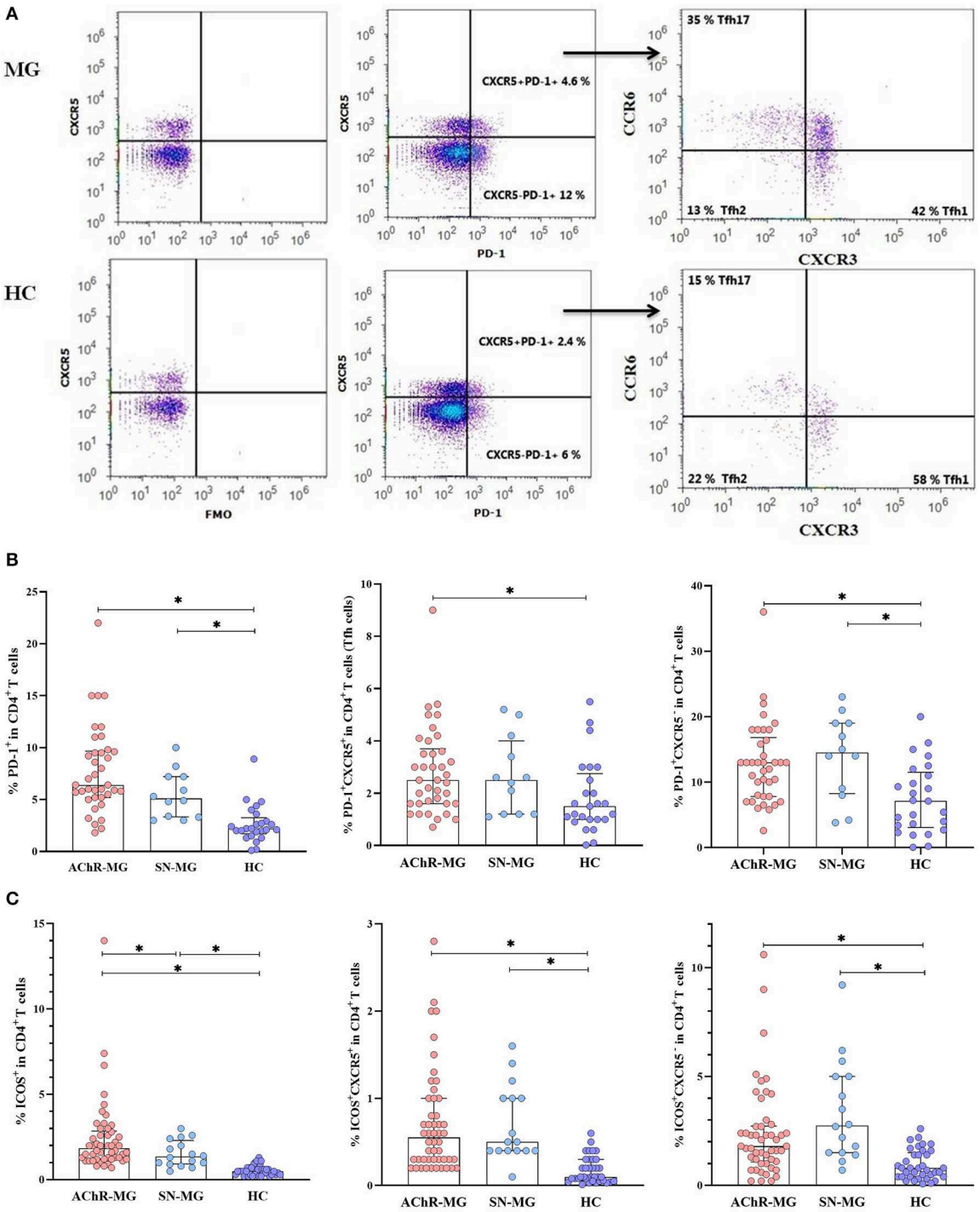



**FIGURE 4 |** Increase of PD-1 and ICOS expression on the CD4$^+$ T cells of myasthenia gravis (MG) patients. **(A)** The gating strategy to identify PD-1$^+$CXCR5$^+$ (cTfh) and PD-1$^+$CXCR5$^-$ populations in CD4$^+$ T cells and cTfh subsets by flow cytometry is shown. **(B)** The frequencies of PD-1$^+$ cells in CD4$^+$ T cells were higher in MG patients with AChR antibodies (AChR-MG) ($n = 35$) and in MG patients without detectable antibodies (SN-MG) ($n = 12$) compared with those of HC ($n = 25$) (both $p < 0.001$). Both PD-1$^+$CXCR5$^+$ (cTfh) and PD-1$^+$CXCR5$^-$ populations in CD4$^+$ T cells were higher in AChR-MG patients ($p = 0.004$ and $p = 0.001$), whereas PD-1$^+$CXCR5$^-$ in CD4$^+$ T cells were increased in SN-MG ($p = 0.006$) compared with those in HC. **(C)** The proportions of ICOS$^+$ cells in CD4$^+$ T cells were increased both in AChR-MG ($n = 47$) and in SN-MG ($n = 16$) patients compared with those in HC ($n = 37$, both $p < 0.001$), including CXCR5$^+$ (both $p < 0.001$) and CXCR5$^-$ (both $p < 0.001$) subgroups of CD4$^+$ T cells. The ICOS$^+$ CD4$^+$ T cells were lower in SN-MG than in AChR-MG ($p = 0.05$). The results are compared with non-parametric tests (Mann–Whitney *U* test). * depicts a significant difference.

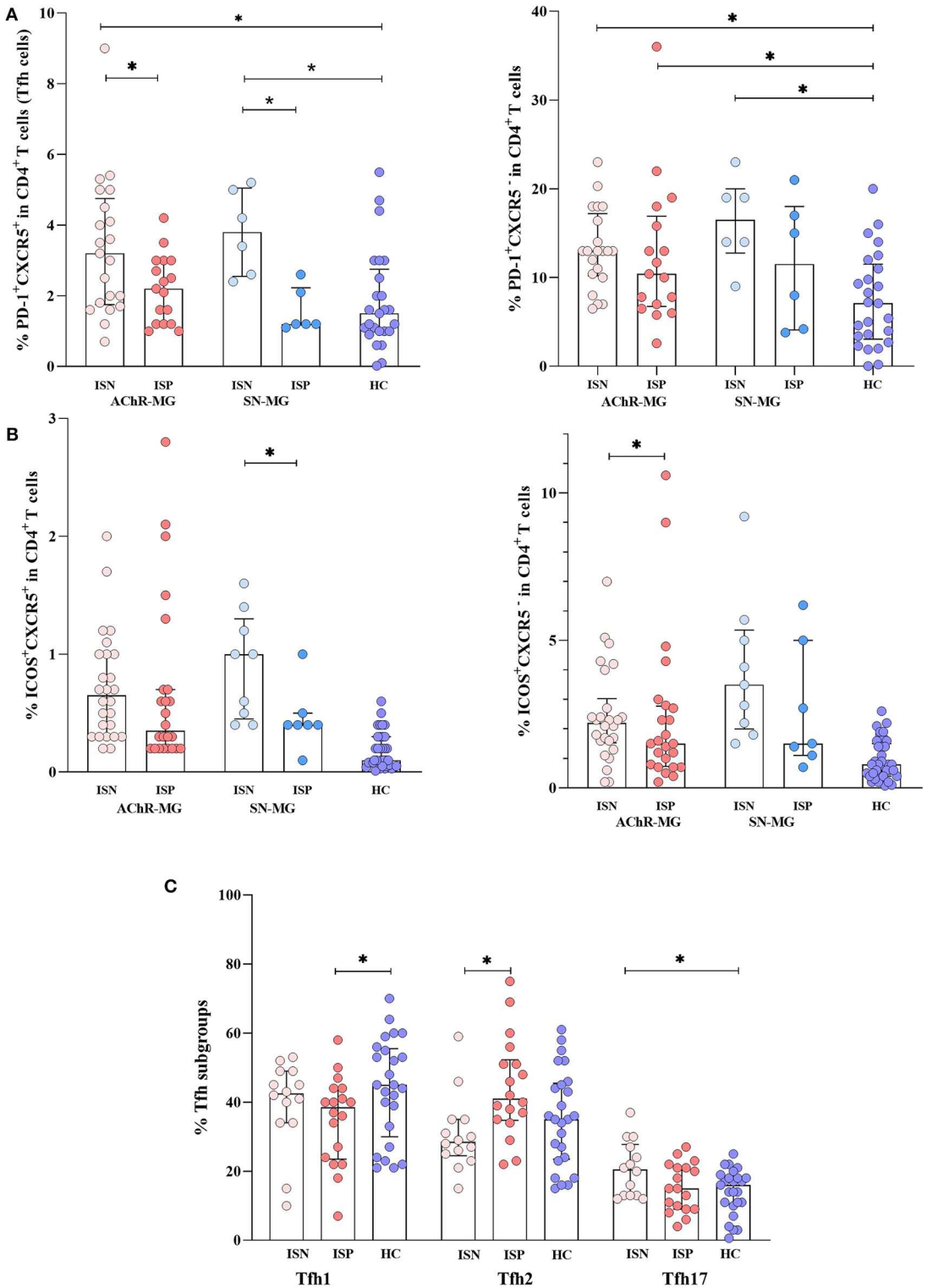



**FIGURE 5 |** The effect of immunosuppressive treatment on T cell subpopulations. **(A)** The PD-1$^+$CXCR5$^+$cells (cTfh cells) were higher in untreated patients of both myasthenia gravis (MG) patients with AChR antibodies (AChR-MG) and MG patients without detectable antibodies (SN-MG) groups compared with those in HC ($p = 0.001$ and $p = 0.007$). The immunosuppressive (IS) treatment decreased the cTfh populations in both AChR-MG and SN-MG patients ($p = 0.012$ and $p = 0.008$). The PD-1$^+$CXCR5$^-$ cell population was higher in the IS-negative (ISN) subgroups of both AChR-MG and SN-MG patients compared to that in HC ($p = 0.001$ and $p = 0.005$). The PD-1$^+$CXCR5$^-$ cell population remained significantly higher than that of HC in the IS-positive (ISP) group of AChR-MG ($p = 0.024$). **(B)** Independent of

*(Continued)*

**FIGURE 5 |** CXCR5, the ICOS$^+$ cells were higher in all subgroups. [These significant p values are not shown on the graph: ISN ($p < 0.001$) and ISP ($p = 0.007$) groups of AChR-MG and ISN ($p < 0.001$) and ISP ($p = 0.012$) groups of SN-MG for ICOS$^+$CXCR5$^-$ and ISN ($p < 0.001$) and ISP ($p < 0.001$) groups of AChR-MG and ISN ($p < 0.001$) and ISP ($p = 0.003$) groups of SN-MG for ICOS$^+$CXCR5$^+$)]. The ICOS$^+$ on CXCR5$^-$ cells in AChR-MG ($p = 0.037$) were higher, whereas on CXCR5$^+$ cells in SN-MG ($p = 0.029$) these were reduced with treatment. **(C)** An increase of Tfh2 ($p = 0.005$) was induced by treatment compared to that in untreated patients. Higher Tfh17 cells ($p = 0.036$) in untreated patients and lower Tfh1 cells in treated patients were detected ($p = 0.042$) compared to those in HC. The results are compared with non-parametric tests (Mann–Whitney *U* test). * depicts a significant difference.

these subpopulations separately in AChR-MG, SN-MG, and HC groups (**Supplementary Figure 2**). ICOS was increased on CD4$^+$ T cells in both disease subgroups (both $p < 0.001$) while being lower in SN-MG than in AChR-MG ($p = 0.05$, **Figure 4C**). The ICOS$^+$CXCR5$^+$ and ICOS$^+$CXCR5$^-$ subpopulations were both higher in AChR-MG (both $p < 0.001$) and SN-MG patients (both $p < 0.001$, **Figure 4C**). No differences were observed between disease onset and other subgroups, with ICOS being higher in all patients (data not shown).

## cTfh Cells

Defining the PD-1$^+$CXCR5$^+$ cells among CD4$^+$ T cells as cTfh cells (19, 33), we detected both PD-1 and CXCR5 molecules on CD4$^+$ T cells. The gating strategy of PD-1$^+$CXCR5$^-$ and PD-1$^+$CXCR5$^+$ (cTfh) cells in CD4$^+$ T cells and further cTfh subsets, such as Tfh1, Tfh2, and Tfh17, is shown in **Figure 4A**. To see a possible dominance of a cTfh subset in the increased population, we analyzed cTfh (PD-1$^+$CXCR5$^+$) cells by dividing these into subsets as cTfh1 (CXCR3$^+$CCR6$^-$), cTfh2 (CXCR3$^-$CCR6$^-$), and cTfh17 (CXCR3$^-$CCR6$^+$). The proportions of Tfh subsets did not differ in patients with AChR-MG and with SN-MG compared to HC (**Supplementary Figure 3**). The same comparisons performed in a smaller sample group on CD4$^+$CD45RA$^-$ memory T cell populations provided the same results (data not shown). No differences were observed between disease onset, thymectomy, or gender subgroups either.

## Effect of IS Treatment on PD-1$^+$, ICOS$^+$ CD4$^+$ T Cells, or Tfh Cells

A possible role of IS treatment also on the disease-relevant CD4$^+$ T cell populations has been evaluated by comparisons of PD-1, ICOS, and CXCR5 among CD4$^+$ T cells. Although PD-1 positivity on CD4$^+$ T cells was not affected significantly from IS treatment, PD-1$^+$CXCR5$^+$cells (cTfh cells) were higher in the untreated than in the treated patients ($p = 0.012$) and the HC ($p = 0.001$) in AChR-MG. A similar observation is also made in SN-MG: the untreated patients revealed an increase in PD-1$^+$CXCR5$^+$cells (cTfh cells) compared with the treated ($p = 0.008$) and HC ($p = 0.007$) groups.

The PD-1$^+$CXCR5$^-$ cell population was higher in the ISN subgroups of both AChR-MG and SN-MG patients compared to HC ($p = 0.001$ and $p = 0.005$, respectively; **Figure 5A**). The PD-1$^+$CXCR5$^-$ cell population remained significantly higher than HC in the ISP group of AChR-MG (p = 0.024), while the higher levels in six SN-MG patients on treatment were not different (**Figure 5A**).

Independent of CXCR5, the ICOS$^+$ cells were higher in the two disease groups with or without treatment compared with HC [ISN ($p < 0.001$) and ISP ($p = 0.007$) groups of AChR-MG, ISN ($p < 0.001$) and ISP ($p = 0.012$) groups of SN-MG for ICOS$^+$CXCR5$^-$, ISN ($p < 0.001$) and ISP ($p < 0.001$) groups of AChR-MG, and ISN ($p < 0.001$) and ISP ($p = 0.003$) groups of SN-MG for ICOS$^+$CXCR5$^+$)]. In AChR-MG ICOS$^+$ on CXCR5$^-$ cells ($p = 0.037$), while in SN-MG ICOS+ on CXCR5$^+$ cells ($p = 0.029$) were reduced with treatment (**Figure 5B**).

The proportions of Tfh1, Tfh2, and Tfh17 cells have also changed with IS treatment in AChR-MG. An increase of Tfh2 ($p = 0.005$) was induced by treatment compared to the untreated patients (**Figure 5C**). Higher Tfh17 cells ($p = 0.036$) in untreated patients and lower Tfh1 cells in treated patients were detected ($p = 0.042$) compared to HC. These findings supported the effect of IS treatment shown on IL-10 and IFN-γ productions by CD4$^+$ T cells in AChR-MG, whereas no differences were detected in SN-MG group (data not shown).

## CXCR5 and PD-1 Were Higher on Thymocytes in Thymus

Similar to the typical GC Tfh cells, cTfh with CD4$^+$CXCR5$^+$ phenotype are also considered as helpers of B cells in producing antibody *via* IL-21. In patients with thymic hyperplasia, the thymic tissue contains GC with similar organization to secondary or tertiary lymphoid tissues in MG (7). Moreover, similar changes of cytokines have been demonstrated in the thymus of patients (35). Along with the cTfh cells, we evaluated CXCR5, PD-1, or ICOS expression on thymocytes obtained from the thymic tissues to search for cells in the thymus resembling Tfh cells. However, as the maturational states of all thymocytes would not be the same, we firstly analyzed thymocytes for CD4 and CD8 expression patterns to characterize the cells. Thymocytes from 37 AChR-MG (33 hyperplasia and four involution cases) and 20 control thymi (Con) revealed changes in the maturational stages of the thymocytes in MG as reported before (37, 38) (**Figure 6**). Although the ages of the control tissue group varied considerably and revealed a significant difference from the ages of the patient group ($p = 0.023$), comparisons by controlling the effect of age also demonstrated significant differences between Con and AChR-MG tissue cells. In AChR-MG cases, higher proportions of CD4 single-positive (CD4$^+$CD8$^-$, SP, $p = 0.015$) and double-negative (DN, $p = 0.007$) thymocytes were observed compared to Con group. CD4 and CD8 double-positive (DP) thymocytes were decreased proportionally in AChR-MG group ($p = 0.012$). Comparisons revealed that the proportions of CD4$^+$CD8$^-$ SP ($p = 0.05$) and DN ($p = 0.001$) thymocytes were even higher and DP thymocytes ($p < 0.001$) were lower in patients on IS treatment at

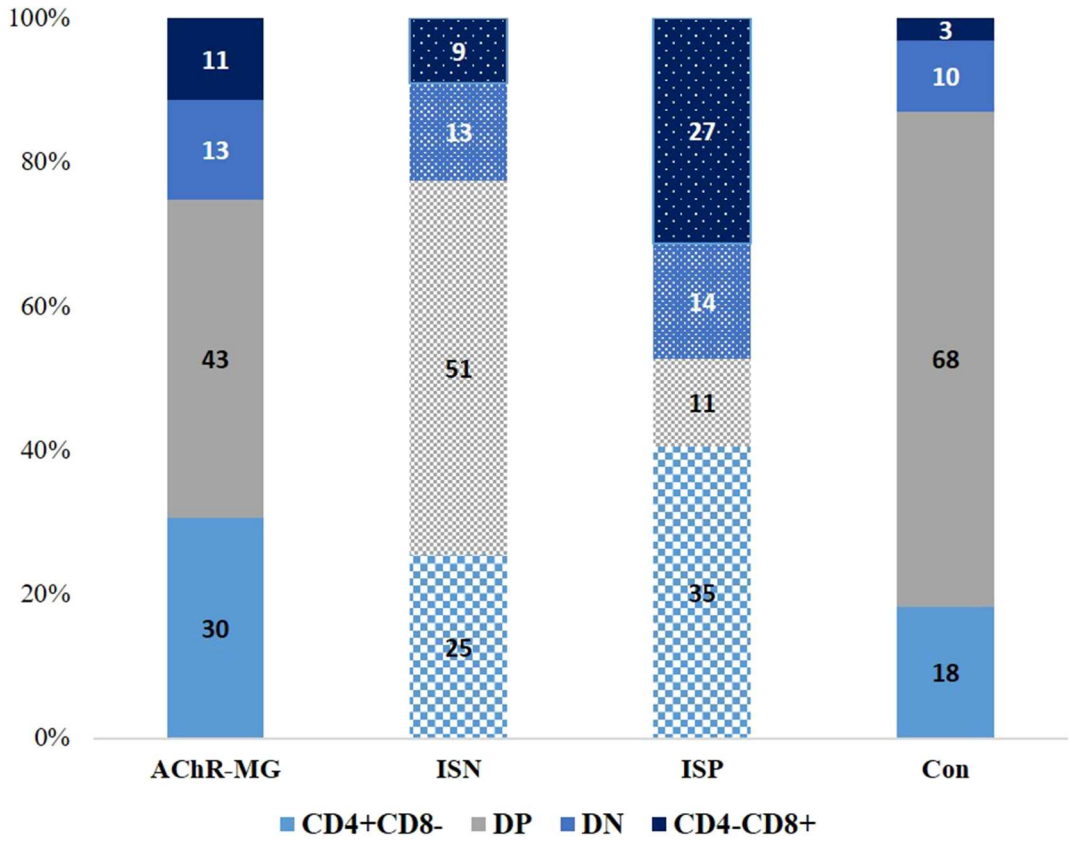



**FIGURE 6 |** Proportional distribution of the maturational stages of thymocytes in different groups. $CD4^+CD8^-$, $CD4^+$ $CD8^+$ (double-positive, DP), $CD4^-CD8^-$ (double-negative, DN), and $CD4^-CD8^+$ thymocytes in 37 myasthenia gravis patients with AChR antibodies (AChR-MG) [16 immunosuppressive (IS)-treated patients, ISP, and 22 not IS-treated patients, ISN] and 20 control (Con) patients. Higher proportions of CD4 single-positive ($CD4^+CD8^-$) and DN thymocytes were observed in AChR-MG cases compared to those in the Con group ($p = 0.015$ and $p = 0.007$). The CD4 and CD8 DP thymocytes were decreased in the AChR-MG group ($p = 0.002$) in comparison to those in the control group. The proportions of $CD4^+CD8^-$ ($p = 0.05$) and DN ($p = 0.001$) thymocytes were higher and DP thymocytes ($p < 0.001$) were decreased when the patients were on IS treatment compared with patients without treatment at the time of thymectomy. Parametric tests are used for comparisons of subpopulations by using age as covariate.

the time of thymectomy compared with samples from IS-naïve patients (**Figure 6**).

An increase of $CXCR5^+$ thymocytes in the thymi of AChR-MG patients was observed compared to Con tissues ($p = 0.002$) (**Figure 7**). The thymic samples from patients with or without IS treatment at the time of thymectomy (10 ISP and 14 ISN patients) revealed an increase in the treated patients compared to the untreated ones ($p = 0.033$) and Con group ($p = 0.007$), although the ISN patients also had higher CXCR5 on thymocytes ($p = 0.023$). The thymocyte subpopulation that expresses higher CXCR5 has been identified as the more mature $CD4^+CD8^-$SP thymocytes in AChR-MG compared to Con ($p = 0.004$, **Figure 7**). In IS treatment-receiving patients, $CXCR5^+$ cells in $CD4^+CD8^-$ SP population were higher than ISN group ($p = 0.016$) and Con ($p = 0.007$). However, these cells were also higher in ISN group compared with Con ($p = 0.042$).

When thymocytes were stained for PD-1 and ICOS expression, an increase of PD-1 was detected on the thymocytes and the $CD4^+CD8^-$ SP cells of AChR-MG patients (seven ISP and 11 ISN patients) compared to Con ($p = 0.039$ and $p = 0.028$, respectively) (**Figure 7**). The expression of ICOS did not show any significant differences in the groups.

In a small number of samples ($n = 5$), we also compared $CD4^+CXCR5^+$ cells between the thymus and the blood of MG patients. In this group, all patients had hyperplastic thymus and three of these patients were on IS treatment at the time of thymectomy when the PBMCs and thymic cells were obtained. The $CD4^+CXCR5^+$ population was higher in blood compared with thymus (11.8 vs. 0.5%). The distribution of chemokine receptors on $CD4^+CXCR5^+$ populations revealed lower Tfh2 (44 vs. 81%) and higher Tfh17 (36 vs. 10%) cell proportions in the blood compared to thymus in this small sample group (**Supplementary Figure 4**).

## DISCUSSION

Being a prototypic autoimmune disease mediated by autoantibodies, MG and its subtypes have been the subject of investigations on the interaction of T and B cells. In a previous study, we had shown increased IL-17A, IFN-γ,

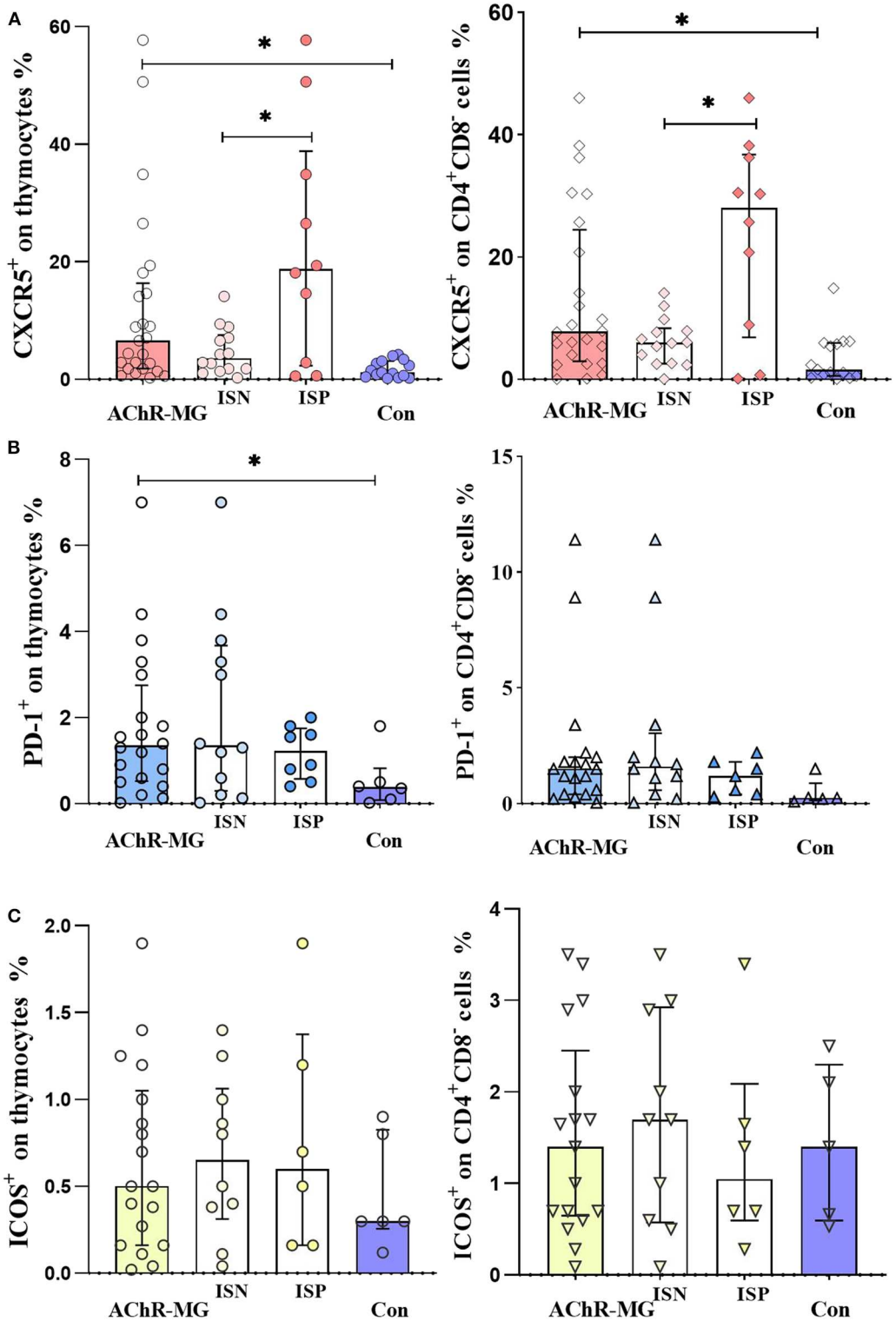



**FIGURE 7 |** CXCR5, PD-1, and ICOS expression on thymocytes. **(A)** CXCR5 increase on thymocytes of myasthenia gravis patients with AChR antibodies (AChR-MG) ($n = 24$) compared with control thymi (Con) ($n = 14$) ($p = 0.002$). Increase of CXCR5 on $CD4^+$ $CD8^-$ single-positive thymocyte population of AChR-MG patients compared with Con ($p = 0.004$). The proportions in IS-treated patients (ISP) were higher than in untreated patients (ISN) group ($p = 0.016$) and Con ($p = 0.007$), with the ISN group being higher than Con ($p = 0.042$) as well. **(B)** The PD-1 on thymocytes ($p = 0.039$) and on $CD4^+$ $CD8^-$ cells ($p = 0.028$) of AChR-MG patients ($n = 18$) were higher than those of Con ($n = 5$). **(C)** ICOS expression on thymocytes and on $CD4^+$ $CD8^-$ thymic cells. Non-parametric tests (Mann–Whitney *U* test) were used for comparisons between groups. * depicts a significant difference.

and IL-21 production in mainly MuSK-MG patients, whereas the AChR-MG patients, in comparison, did not reveal any differential cytokine production in response to non-specific T cell stimulation (26). In the present study, we demonstrate increased IL-4, in addition to IL-21 and IL-17A productions of $CD4^+$ T cells from IS treated as well as untreated AChR-MG patients. Although IL-4, IL-21, and IL-17A have pointed at the impact of cTfh cells, the cytokine-producing and *in vivo*-activated cells were identified not necessarily as cTfh cells but had surface markers such as PD-1 or ICOS with or without CXCR5. Findings in the thymic samples from AChR-MG patients revealed an IS treatment-related increase of CXCR5 as well. The results obtained from a smaller sample group of SN-MG patients provided support for their common immunological features with AChR-MG. The complex cytokine network of regulation for auto-antibody production has been elucidated further in this study.

## Cytokines in MG

The dysregulation of cytokines from Th1, Th17, and Tfh populations is associated with the pathogenesis of many autoimmune diseases (39). Imbalances of related cytokines are reported in MG with differences between studies. Higher levels of IL-21 and IL-6 (23) were not confirmed in the sera of AChR-MG patients in other studies (26, 29). Increased IL-17 (27, 28, 40) was not observed in other studies in the sera or the culture supernatants of AChR-MG patients. Variations in these measurements were reduced by cellular evaluation of the cytokines. The production of both IFN-γ and IL-17 and the absence of IL-10 expression in response to AChR of the $CCR6^+$ memory T cells in MG indicated a pro-inflammatory pathogenic phenotype with a recent approach (30). Thymic studies also supported the importance of IL-17, IFN-γ, IL-21, and TNF-α and related effects in MG (35, 41). The importance of IL-21 in MG has been shown to be associated with activated Tfh1 and Tfh17 cells being their major product (23). High frequencies of cTfh-Th17 cells with increased IL-21 mRNA expression confirmed this finding (36). The present study evaluated both Th- and Tfh-related cytokines. Th17-related activity and the relevance of IL-17 and IL-21 have also been demonstrated in MuSK-MG patients (26, 42). The findings of this study confirm the importance of IL-17 as a Th17-related cytokine and IL-21 as a Tfh cytokine *ex vivo* without stimulation in a relatively big sample. In addition to IL-17A and IL-21, a robust increase of IL-4 production in the $CD4^+$ T cells also provides evidence that the cytokine activity from AChR-MG patients exhibits a "Th17/Tfh" signature.

## Tfh or Other $CD4^+$ T Cell Types

Tolerance breakdown during B cell development (43) and aberrant selection in GC can increase the production of autoantibodies (44). Tfh cells are major immune regulators in B cell activation, differentiation into plasma cells, and antibody production in the lymphoid tissues (14). Isotype switching to IgG subclasses as in many autoimmune responses including MG is also subject to regulation by Tfh cells. With the observation of cTfh cells in the blood, several reports are published on the role of these cells in autoimmune diseases such as RA (17), SSc (19), and MG (23, 45). In MG, an increase of $PD\text{-}1^{hi}CXCR5^+CD4^+$ or $ICOS^{hi}CXCR5^+CD4^+$ T cell populations and a correlation with AChR antibodies were shown (22). Considering the ICOS expression of $CXCR5^+CD4^+$ cells as activation of cTfh, their higher IL-21 have also been demonstrated in AChR-MG (23). With a similar approach to the present study, when the cTfh cells are subtyped by receptors, the increased group has been determined as the cTfh–Th17 cells in the blood (36). The present results also confirmed the increase of cTfh cells defined as $PD\text{-}1^+CXCR5^+CD4^+$ T cells. $ICOS^+CXCR5^+CD4^+$ T cells were also increased in the present study group of two different disease subtypes. However, the observation of the parallel increase of the $CXCR5^-CD4^+$ T cells as $ICOS^+CD4^+$ and $PD\text{-}1^+CD4^+$ populations in both AChR-MG and SN-MG patients raised the possibility that another subtype of T cells can be involved in MG for this cytokine imbalance. The Tph cell group shown in the synovium of RA patients provided evidence for a new cell group with helper function to antibody production induced at the peripheral tissue (20). The peripheral or even thymic presence of PD-1- or ICOS-expressing cells may indicate similarly effective cell types in MG.

On the other hand, when the functional effects of cTfh cells in the blood of patients with SSc were compared, the positive correlation with plasma cell differentiation and the increase of IgG levels in co-cultures with B cells were mainly observed with $PD\text{-}1^+CXCR5^+$, but less with $PD\text{-}1^+CXCR5^-CD4^+$ T cells. Both $PD\text{-}1^+CXCR5^+$ and $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$T cells have been shown to produce high IL-21 (19). Although Tfh cells defined as $CD4^+CXCR5^+$ T cells appear to be responsible for antibody production in lymphoid tissues, the data suggest that PD-1 expression on $CD4^+$ T cells may be more important in the interaction of these cells with B cells. Both increases of ICOS and PD-1 expression on $CD4^+$ may contribute to the peripheral regulation of autoantibody production in AChR-MG. Further functional comparisons of these cell populations are needed to elucidate their effect in disease development.

The SN-MG patients revealed similarities to the AChR-MG patients. Although $CD4^+CXCR5^+PD\text{-}1^+$ (cTfh) cells were increased only in AChR-MG, both $PD\text{-}1^+CD4^+$ and $ICOS^+CD4^+$ T cell populations were higher in AChR-MG as well as in SN-MG. The Th17 population was increased in both disease subgroups compared to the HC. These findings suggest that AChR-MG and SN-MG patient groups, which are clinically similar to each other, may have a similar profile of cytokine regulations with some differences.

## IS Treatment Effect

In this study, changes induced by IS treatment in cytokine production and subsets of $CD4^+$ T cells were detected by measurements of separate groups as well as in sequential measurements in a group of AChR-MG patients. To rule out the variability of cytokine data in MG studies which may be IS treatment-related, relatively big groups and *ex vivo* cellular samples were studied.

IS treatment induced an increase of IL-10 and a decrease of IFN-γ production by $CD4^+$ T cells. In addition, Th2 and Tfh2 cell populations were increased in ISP patients, implicating an effect of IL-10 on the differentiation of Th2 and Tfh2 populations in AChR-MG. On the other hand, cTfh and cTfh1 populations were

decreased in ISP AChR-MG patients, which may be related to the lower IFN-γ levels of ISP patients. A decrease of Tfh population with IS treatment has been documented in SLE (33) and also in IgG4-related disease (34). In a recent report, $CD4^+PD\text{-}1^+$ and $CD4^+ICOS^+$ T cells were decreased after IS treatment in MG as well (45). Soluble forms of PD-1 and ICOSL were also increased in untreated patients and affected by IS treatment. However, we did not observe decreases of PD-1 or ICOS expression on $CD4^+$ T cells with IS treatment.

On the other hand, the treatment effects in SN-MG were not properly evaluated as the number of patients was not as high as those of the other groups and the distribution in the treatment groups was not balanced, which has been the main limitation of the present study.

### Thymic Changes Related to Peripheral Findings

In the thymic tissue of AChR-MG patients with hyperplasia, ectopic GC-like persistent structures are detected and evidences are presented that B cells are chemo-attracted and activated at these structures, resulting in antibody production (46). Co-localization of Tfh and B cell within the ectopic GC in MG thymus has also been reported (24). Evaluating the possible contribution of thymic pathology in autoantibody and cytokine production, we screened thymocytes for the relevant molecules. Preliminary observations provided support for the changes toward differentiation of Tfh cells in the thymic tissues of AChR-MG patients, mainly in more mature CD4 single-positive thymocytes and also related to IS treatment. However, functional evidence is lacking to prove this development. As previously reported, glucocorticoids induce atrophy in the thymus by acting on DP thymocytes which preferentially express the glucocorticoid receptors in the thymus (47). Locally produced, thymic endothelial cell-derived glucocorticoids in the thymus have also been shown to participate in antigen-specific thymocyte development (48, 49). In that sense, exogenous glucocorticoids may have an effect on antigen-specific T cell development and preferentially on the thymocyte development of a Tfh-like phenotype. As an alternative explanation, the active Tfh cells in the thymus may be insufficiently suppressed by glucocorticoids, leading to a persistent disease activity in MG, and may explain the favorable response to thymectomy.

## CONCLUSION

In conclusion, MG pathogenesis is associated with increased IL-21, IL-4, and IL-17A levels and higher frequencies of ICOS and PD-1 expressing $CD4^+$ T cells. AChR-MG and SN-MG patients have similar cytokine productions and subsets of $CD4^+$ T cell populations. Th2 or Tfh2 cells and IL-10 are mainly increased with the effect of IS treatment, which may have a compensating role. However, the production of IL-21, IL-17, and IL-4 are not affected by IS treatment. Blockade of T cell activation pathways of specific cell populations with distinctive surface markers which are not targeted by IS treatment will be a challenge for potential therapeutic approaches in MG.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Review Board of Istanbul Medical Faculty. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

MÇ, HD, YP, and GS-D contributed to the design of the study and to the planning of experiments. MÇ and GS-D wrote the manuscript. MC, HD, FA, BÖ, AÇ, GG, MM, MT, VY, OA, ÖD, YP, and GS-D were responsible for the ethical permission and for the clinical selection and diagnostic evaluation of patients and controls. MÇ, MH, and SY performed the experiments. MÇ and GS-D organized the figures and the tables.

## FUNDING

This study was supported by TÜBITAK (116S317) and Istanbul University Research Fund.

## ACKNOWLEDGMENTS

We are grateful to the patients and to Dr. Gözde Nezir, Dr. Mehmet Ali Akalin, and Dr. Özlem Güngör Tunçer.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00809/full#supplementary-material

**Supplementary Figure 1 |** Gating strategy to identify Th1 ($CXCR3^+CCR6^-$), Th2 ($CXCR3^-CCR6^-$) and Th17 ($CXCR3^-CCR6^+$) populations in $CD4^+$ T cells by flow cytometry is shown.

**Supplementary Figure 2 |** Gating strategy to identify $ICOS^+CXCR5^+$ (cTfh) and $ICOS^+CXCR5^-$ populations in $CD4^+$ T cells by flow cytometry is shown.

**Supplementary Figure 3 |** Tfh1($CXCR3^+CCR6^-$) Tfh2 ($CXCR3^-CCR6^-$) and Tfh17 ($CXCR3^-CCR6^+$) cell subsets among $CD4^+CXCR5^+$ T cells in AChR-MG and SN-MG groups compared with HC.

**Supplementary Figure 4 |** Thymic (t) and circulating (c) Tfh1($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$) and Tfh17 ($CXCR3^-CCR6^+$) cell subsets among $CD4^+CXCR5^+$ T cells detected in samples of five AChR-MG patients.

## REFERENCES


1. Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurol*. (2015) 14:1023–36. doi: 10.1016/S1474-4422(15)00145-3
2. Lindstrom J, Seybold M, Lennon, VA, Whittingham S, Duane DD. Antibody to acetylcholine receptor in myasthenia gravis. *Neurology*. (1976) 26:1054. doi: 10.1212/WNL.26.11.1054
3. Deymeer F, Gungor-Tuncer O, Ylmaz V, Parman Y, Serdaroglu P, Ozdemir C, et al. Clinical comparison of anti-MuSK- vs anti-AChR-positive and seronegative myasthenia gravis. *Neurology*. (2007) 68:609–11. doi: 10.1212/01.wnl.0000254620.45529.97
4. Leite MI, Jones M, Ströbel P, Marx A, Gold R, Niks E, et al. Myasthenia gravis thymus: complement vulnerability of epithelial and myoid cells, complement attack on them, and correlations with autoantibody status. *Am J Pathol*. (2007) 171:893–905. doi: 10.2353/ajpath.2007.070240
5. Plested CP, Tang T, Spreadbury I, Littleton ET, Kishore U, Vincent A. AChR phosphorylation and indirect inhibition of AChR function in seronegative MG. *Neurology*. (2002) 59:1682–8. doi: 10.1212/01.WNL.0000041625.41937.FF
6. Hong Y, Zisimopoulou P, Trakas N, Karagiorgou K, Stergiou C, Skeie GO, et al. Multiple antibody detection in 'seronegative' myasthenia gravis patients. *Eur J Neurol*. (2017) 24:844–50. doi: 10.1111/ene.13300
7. Truffault F, de Montpreville V, Eymard B, Sharshar T, Le Panse R, Berrih-Aknin S. Thymic germinal centers and corticosteroids in myasthenia gravis: an immunopathological study in 1035. cases and a critical review. *Clin Rev Allergy Immunol*. (2017) 52:108–24. doi: 10.1007/s12016-016-8558-3
8. Vincent A, Bowen J, Newsom-Davis J, McConville J. Seronegative generalised myasthenia gravis: clinical features, antibodies, and their targets. *Lancet Neurol*. (2003) 2:99–106. doi: 10.1016/S1474-4422(03)00306-5
9. Le Panse R, Cizeron-Clairac G, Bismuth J, Berrih-Aknin S. Microarrays reveal distinct gene signatures in the thymus of seropositive and seronegative myasthenia gravis patients and the role of CC chemokine ligand 21 in thymic hyperplasia. *J Immunol*. (2006) 177:7868–79. doi: 10.4049/jimmunol.177.11.7868
10. Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol*. (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
11. Arnold CN, Campbell DJ, Lipp M, Butcher EC. The germinal center response is impaired in the absence of T cell-expressed CXCR5. *Eur J Immunol*. (2007) 37:100–9. doi: 10.1002/eji.200636486
12. Kuchen S, Robbins R, Sims GP, Sheng C, Phillips TM, Lipsky PE, et al. Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. *J Immunol*. (2007) 179:5886–96. doi: 10.4049/jimmunol.179.9.5886
13. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol*. (2007) 179:8180–90. doi: 10.4049/jimmunol.179.12.8180
14. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*. (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
15. Gensous N, Charrier M, Duluc D, Contin-Bordes C, Truchetet M-E, Lazaro E, et al. T follicular helper cells in autoimmune disorders. *Front Immunol*. (2018) 9:1637. doi: 10.3389/fimmu.2018.01637
16. Morita R, Schmitt N, Bentebibel S-E, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood $CXCR5^{+}CD4^{+}$ T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. (2011) 34:108–21. doi: 10.1016/j.immuni.2010.12.012
17. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol*. (2012) 2012:827480. doi: 10.1155/2012/827480
18. Le Coz C, Joublin A, Pasquali J-L, Korganow A-S, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS ONE*. (2013) 8:e75319. doi: 10.1371/journal.pone.0075319
19. Ricard L, Jachiet V, Malard F, Ye Y, Stocker N, Rivière S, et al. Circulating follicular helper T cells are increased in systemic sclerosis and promote plasmablast differentiation through the IL-21 pathway which can be inhibited by ruxolitinib. *Ann Rheum Dis*. (2019) 78:539–50. doi: 10.1136/annrheumdis-2018-EWRR2019.31
20. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature*. (2017) 542:110–14. doi: 10.1038/nature20810
21. Kastirr I, Maglie S, Paroni M, Alfen JS, Nizzoli G, Sugliano E, et al. IL-21 Is a central memory T cell–associated cytokine that inhibits the generation of pathogenic Th1/17 effector cells. *J Immunol*. (2014) 193:3322–31. doi: 10.4049/jimmunol.1400775
22. Luo C, Li Y, Liu W, Feng H, Wang H, Huang X, et al. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *J Neuroimmunol*. (2013) 256:55–61. doi: 10.1016/j.jneuroim.2012.12.001
23. Zhang C-J, Gong Y, Zhu W, Qi Y, Yang C-S, Fu Y, et al. Augmentation of circulating follicular helper t cells and their impact on autoreactive B cells in myasthenia gravis. *J Immunol*. (2016) 197:2610–7. doi: 10.4049/jimmunol.1500725
24. Zhang X, Liu S, Chang T, Xu J, Zhang C, Tian F, et al. Intrathymic Tfh/B cells interaction leads to ectopic GCs formation and anti-AChR antibody production: central role in triggering MG occurrence. *Mol Neurobiol*. (2016) 53:120–31. doi: 10.1007/s12035-014-8985-1
25. Zhang G-X, Navikas V, Link H. Cytokines and the pathogenesis of myasthenia gravis. *Muscle Nerve*. (1997) 20:543–51. doi: 10.1002/(SICI)1097-4598(199705)20:5<543::AID-MUS2>3.0.CO;2-9
26. Yilmaz V, Oflazer P, Aysal F, Durmus H, Poulas K, Yentur SP, et al. Differential cytokine changes in patients with myasthenia gravis with antibodies against AChR and MuSK. *PLoS ONE*. (2015) 10:e0123546. doi: 10.1371/journal.pone.0123546
27. Roche JC, Capablo JL, Larrad L, Gervas-Arruga J, Ara JR, Sánchez A, et al. Increased serum interleukin-17 levels in patients with myasthenia gravis. *Muscle Nerve*. (2011) 44:278–80. doi: 10.1002/mus.22070
28. Wang Z, Wang W, Chen Y, Wei D. T helper type 17 cells expand in patients with myasthenia-associated thymoma. *Scand J Immunol*. (2012) 76:54–61. doi: 10.1111/j.1365-3083.2012.02703.x
29. Uzawa A, Kawaguchi N, Himuro K, Kanai T, Kuwabara S. Serum cytokine and chemokine profiles in patients with myasthenia gravis. *Clin Exp Immunol*. (2014) 176:232–7. doi: 10.1111/cei.12272
30. Cao Y, Amezquita RA, Kleinstein SH, Stathopoulos P, Nowak RJ, O'Connor KC. Autoreactive T cells from patients with myasthenia gravis are characterized by elevated IL-17, IFN-γ, and GM-CSF and diminished IL-10 production. *J Immunol*. (2016) 196:2075–84. doi: 10.4049/jimmunol.1501339
31. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. *In vitro* generation of interleukin 10–producing regulatory CD4+ T cells is induced by immunosuppressive drugs and inhibited by t helper type 1 (Th1)– and Th2-inducing cytokines. *J Exp Med*. (2002) 195:603–16. doi: 10.1084/jem.20011629
32. Mann EH, Gabryšová L, Pfeffer PE, O'Garra A, Hawrylowicz CM. High-dose IL-2 skews a glucocorticoid-driven IL-17+IL-10+ memory CD4+T cell response towards a single IL-10–producing phenotype. *J Immunol*. (2018) 202:684–93. doi: 10.4049/jimmunol.1800697
33. Feng X, Wang D, Chen J, Lu L, Hua B, Li X, et al. Inhibition of aberrant circulating tfh cell proportions by corticosteroids in patients with systemic lupus erythematosus. *PLoS ONE*. (2012) 7:e51982. doi: 10.1371/journal.pone.0051982
34. Kubo S, Nakayamada S, Zhao J, Yoshikawa M, Miyazaki Y, Nawata A, et al. Correlation of T follicular helper cells and plasmablasts with the development of organ involvement in patients with IgG4-related disease. *Rheumatology*. (2017) 57:514–24. doi: 10.1093/rheumatology/kex455
35. Gradolatto A, Nazzal D, Truffault F, Bismuth J, Fadel E, Foti M, et al. Both treg cells and tconv cells are defective in the myasthenia gravis thymus: roles of IL-17 and TNF-α. *J Autoimmun*. (2014) 52:53–63. doi: 10.1016/j.jaut.2013.12.015
36. Yang Y, Zhang M, Ye Y, Ma S, Fan L, Li Z. High frequencies of circulating Tfh-Th17 cells in myasthenia gravis patients. *Neurol Sci*. (2017) 38:1599–608. doi: 10.1007/s10072-017-3009-3
37. Li Q, Liu P, Xuan X, Zhang J, Zhang Y, Zhu Z, et al. CCR9 AND CCR7 are overexpressed in CD4–CD8– thymocytes of myasthenia gravis patients. *Muscle and Nerve*. (2017) 55:84–90. doi: 10.1002/mus.24999

38. Durelli L, Massazza U, Poccardi G, Ferrio MF, Cavallo R, Maggi G, et al. Increased thymocyte differentiation in myasthenia gravis: A dual-color immunofluorescence phenotypic analysis. *Ann Neurol*. (1990) 27:174–80. doi: 10.1002/ana.410270213
39. Kuchroo VK, Ohashi PS, Sartor RB, Vinuesa CG. Dysregulation of immune homeostasis in autoimmune diseases. *Nat Med*. (2012) 18:42–7. doi: 10.1038/nm.2621
40. Xie Y, Li HF, Jiang B, Li Y, Kaminski HJ, Kusner LL. Elevated plasma interleukin-17A in a subgroup of myasthenia gravis patients. *Cytokine*. (2016) 78:44–6. doi: 10.1016/j.cyto.2015.06.011
41. Villegas JA, Bayer AC, Ider K, Bismuth J, Truffault F, Roussin R, et al. IL-23/Th17 cell pathway: a promising target to alleviate thymic inflammation maintenance in myasthenia gravis. *J Autoimmun*. (2019) 98:59–73. doi: 10.1016/j.jaut.2018.11.005
42. Yi JSS, Guidon A, Sparks S, Osborne R, Juel VCC, Massey JMM, et al. Characterization of CD4 and CD8 T cell responses in MuSK myasthenia gravis. *J Autoimmun*. (2014) 52:130–8. doi: 10.1016/j.jaut.2013.12.005
43. Yurasov S, Nussenzweig MC. Regulation of autoreactive antibodies. *Curr Opin Rheumatol*. (2007) 19:421–6. doi: 10.1097/BOR.0b013e328277ef3b
44. Vinuesa CG, Sanz I, Cook MC. Dysregulation of germinal centres in autoimmune disease. *Nat Rev Immunol*. (2009) 9:845–57. doi: 10.1038/nri2637
45. Yan X, Gu Y, Wang C, Sun S, Wang X, Tian J, et al. Unbalanced expression of membrane-bound and soluble inducible costimulator and programmed cell death 1 in patients with myasthenia gravis. *Clin Immunol*. (2019) 207:68–78. doi: 10.1016/j.clim.2019.07.011
46. Berrih-Aknin S. Role of the thymus in autoimmune myasthenia gravis. *Clin Exp Neuroimmunol*. (2016) 7:226–37. doi: 10.1111/cen3.12319
47. Purton JF, Monk JA, Liddicoat DR, Kyparissoudis K, Sakkal S, Richardson SJ, et al. Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death. *J Immunol*. (2004) 173:3816–24. doi: 10.4049/jimmunol.173.6.3816
48. Ashwell JD, Lu FWM, Vacchio MS. Glucocorticoids in T cell development and function. *Annu Rev Immunol*. (2000) 18:309–45. doi: 10.1146/annurev.immunol.18.1.309
49. Taves MD, Mittelstadt PR, Presman DM, Hager GL, Ashwell JD. Single-cell resolution and quantitation of targeted glucocorticoid delivery in the thymus. *Cell Rep*. (2019) 26:3629–42. doi: 10.1016/j.celrep.2019.02.108


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 May 2020
doi: 10.3389/fimmu.2020.00776

# Autoimmune Pathology in Myasthenia Gravis Disease Subtypes Is Governed by Divergent Mechanisms of Immunopathology

*Miriam L. Fichtner[1,2†], Ruoyi Jiang[2†], Aoibh Bourke[3], Richard J. Nowak[1] and Kevin C. O'Connor[1,2*]*

[1] Department of Neurology, School of Medicine, Yale University, New Haven, CT, United States, [2] Department of Immunobiology, School of Medicine, Yale University, New Haven, CT, United States, [3] Trinity Hall, University of Cambridge, Cambridge, United Kingdom

Myasthenia gravis (MG) is a prototypical autoantibody mediated disease. The autoantibodies in MG target structures within the neuromuscular junction (NMJ), thus affecting neuromuscular transmission. The major disease subtypes of autoimmune MG are defined by their antigenic target. The most common target of pathogenic autoantibodies in MG is the nicotinic acetylcholine receptor (AChR), followed by muscle-specific kinase (MuSK) and lipoprotein receptor-related protein 4 (LRP4). MG patients present with similar symptoms independent of the underlying subtype of disease, while the immunopathology is remarkably distinct. Here we highlight these distinct immune mechanisms that describe both the B cell- and autoantibody-mediated pathogenesis by comparing AChR and MuSK MG subtypes. In our discussion of the AChR subtype, we focus on the role of long-lived plasma cells in the production of pathogenic autoantibodies, the IgG1 subclass mediated pathology, and contributions of complement. The similarities underlying the immunopathology of AChR MG and neuromyelitis optica (NMO) are highlighted. In contrast, MuSK MG is caused by autoantibody production by short-lived plasmablasts. MuSK MG autoantibodies are mainly of the IgG4 subclass which can undergo Fab-arm exchange (FAE), a process unique to this subclass. In FAE IgG4, molecules can dissociate into two halves and recombine with other half IgG4 molecules resulting in bispecific antibodies. Similarities between MuSK MG and other IgG4-mediated autoimmune diseases, including pemphigus vulgaris (PV) and chronic inflammatory demyelinating polyneuropathy (CIDP), are highlighted. Finally, the immunological distinctions are emphasized through presentation of biological therapeutics that provide clinical benefit depending on the MG disease subtype.

**Keywords: myasthenia gravis, B cells, B lymphocytes, autoimmunity, immunopathology, autoantibodies, AChR, MuSK**

OPEN ACCESS

***Edited by:***
*Sonia Berrih-Aknin, Institut National de la Santé et de la Recherche Médicale (INSERM), France*

***Reviewed by:***
*Fulvio Baggi, Carlo Besta Neurological Institute (IRCCS), Italy*
*Anne Cooke, University of Cambridge, United Kingdom*

***Correspondence:**
*Kevin C. O'Connor*
*kevin.oconnor@yale.edu*

*† These authors have contributed equally to this work*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *13 January 2020*
***Accepted:*** *06 April 2020*
***Published:*** *27 May 2020*

***Citation:***
*Fichtner ML, Jiang R, Bourke A, Nowak RJ and O'Connor KC (2020) Autoimmune Pathology in Myasthenia Gravis Disease Subtypes Is Governed by Divergent Mechanisms of Immunopathology. Front. Immunol. 11:776. doi: 10.3389/fimmu.2020.00776*

## INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disorder affecting neuromuscular transmission. MG patients suffer from muscle weakness and increased muscle fatigability due to diminished neuromuscular signaling (1, 2). The impairment in autoimmune MG is caused by autoantibodies that target components of the neuromuscular junction (NMJ) (1). The different subtypes of MG

are defined by the antigen specificity of the autoantibody (2, 3). The most common subtype of autoantibody-mediated MG (approximately 85% of patients) is characterized by autoantibodies against the nicotinic acetylcholine receptor (AChR) (2). In the remaining 15% of patients, autoantibodies targeting muscle-specific kinase (MuSK) (4) or lipoprotein receptor-related protein 4 (LRP4) (5, 6) can be found. Another small fraction of patients does not have detectable circulating autoantibodies to known targets. Accordingly, these patients are diagnosed as having seronegative MG (SNMG).

Numerous *in vitro* approaches have substantiated that autoantibodies against AChR and MuSK in MG are pathogenic (3, 7–11). Their pathogenic capacity has been further demonstrated through passive transfer of patient-derived serum or immunoglobulin (12), maternal-fetal autoantibody transmission (13, 14), and neonatal transfer (15, 16), all of which reproduce MG symptoms. The direct role of autoantibodies in the pathology of MG places it in a rare category of autoimmune diseases caused by autoantibodies with well-established pathogenic affects. Accordingly, MG serves as an archetype for B cell-mediated autoimmune disorders.

Although MG patients with different subtypes share similar disease presentations, the underlying immunopathology of several subtypes are remarkably distinct, contradicting the uniformity in the disease phenotype. MG subtypes share features broadly associated with MG, which can be elicited by clinical examination (17, 18). However, without the results of autoantibody testing in-hand, it is not possible to uniformly assess the subtype through clinical examination alone. Thus, autoantibody testing is necessary for establishing the MG subtype. AChR and MuSK MG, in particular, highlight the distinct immunopathology of the subtypes. The immunopathology of AChR MG is characterized by IgG subclasses (IgG1, IgG2, and IgG3) with effector functions that can mediate tissue damage at the NMJ. AChR-specific autoantibodies are thought to originate from long-lived plasma cells. Conversely, MuSK MG is largely caused by autoantibodies with an IgG subclass (IgG4) that mediates pathology through the direct disruption of AChR signaling by interfering with NMJ protein-protein interactions. Short-lived plasmablasts are thought to be the source of these autoantibodies (19). These stark differences in immunopathology have been elucidated through laboratory-based studies and reinforced through both successful and failed outcomes in the testing of biological therapeutics. A deeper understanding of the mechanisms underlying the differences in immunopathology is highly important for both the patient and clinician – the accurate determination of autoantibody-related subtype has important consequences for care. Treatments that are anticipated to work well in one subtype may not have a biological basis for use in the other subtype(s).

In this review, we focus on the most common subtypes of MG. Rare congenital, presynaptic autoimmune, and thymoma-associated subtypes of MG do exist, but they are not discussed here and are reviewed elsewhere (20–22). The LRP4 and SNMG subtypes are presented, but given the limited information about the underlying immunobiology, they are not emphasized throughout. Rather, the immunobiology underlying the AChR and MuSK subtypes of MG are highlighted. Particular attention is given to AChR and MuSK autoantibody characteristics, B cell subsets, mechanisms of immunopathology, and the effects of treatment with biological agents. Insight is drawn from laboratory-based research using human specimens, clinical trial outcomes, and parallels to other autoimmune diseases.

# IMMUNOPATHOLOGY OF AChR MYASTHENIA GRAVIS

## Characterization of B Cells in AChR Myasthenia Gravis

AChR MG can be divided into subtypes that are defined, in part, by age of onset and gender (23, 24). Patients who develop the disease before the age of 40–50 are often women. This subset is termed early-onset (EOMG), while those developing disease after the age of 40–50 fall into the late-onset LOMG category and are more often men. Patients in the EOMG category generally have conspicuous morphological changes of their thymus. This is primarily characterized by follicular hyperplasia and the presence of B cells and antibody secreting cells that organize into structures that share the characteristics of germinal centers (25–28). These structures are observed in approximately 70% of EOMG patients (29). There is a considerable amount of data that points to a major role in both the initiation and sustained production of AChR autoantibodies by B cells in the hyperplastic thymus. It was found that AChR-specific IgG (30) is present in the thymus along with activated B cells (31). A fraction of these activated B cells produces AChR-specific autoantibodies (32–34). The *in vitro* production of AChR autoantibodies by thymus-resident B cells can be spontaneous or driven by mitogens. Cells that spontaneously produce autoantibodies are most likely resident plasmablasts or plasma cells – both of which are known to occupy thymus tissue (32, 33, 35, 36). Thymic B cells requiring *in vitro* stimulation to produce autoantibodies are likely memory B cell populations, which require additional signals in order to differentiate to an antibody-secreting cell (ASC) phenotype (37, 38). Further confirmation that the thymus contributes to AChR autoantibody production was achieved through transplantation of thymus tissue from AChR MG patients into immunodeficient mice. AChR-specific autoantibodies were observed to deposit at the NMJ in these mice, demonstrating that AChR MG thymic tissue is sufficient for the production of AChR-specific antibodies and can cause muscle weakness in this rodent model (39). Sequencing of thymic B cell receptor repertoires identified clonal expansions of B cells in the thymus, although it has not been established if these expanded clones are specific for AChR (40, 41). The isolation of AChR-specific mAbs has provided additional details regarding the nature of the B cell repertoire producing them. Given that autoantibody-producing B cells are enriched in the thymus of many AChR MG patients (32, 36), several studies have used thymus tissue to isolate AChR-specific B cells. Sequencing of the antibody-variable regions afforded the characterization of these autoreactive B cells. It was demonstrated that B cells expressing AChR autoantibodies are

clonally heterogeneous, class switched, and have accumulated somatic hypermutations (41–43), all of which are properties of antigen-driven maturation. At this time, a limited set of human AChR-specific B cells have been isolated. Studies using newer single cell technologies are certain to provide larger sets, so that the B cell receptor repertoire characteristics of these autoantibody-producing cells can be better understood.

In both LOMG and EOMG patients, autoantibody-producing B cells can occupy other tissue compartments in addition to the thymus. Using *in vitro* cell culture approaches, it has been demonstrated that B cells expressing AChR autoantibodies exist within the circulation, lymph nodes, and in the bone marrow (44–48). Other studies have identified autoantibody-producing B cells in the circulation through production of recombinant human monoclonal AChR autoantibodies (mAb) from these cells (49).

## Properties of AChR-Specific Autoantibodies

The first recombinant AChR-specific autoantibodies were cloned from phage display libraries isolated from thymocyte-associated immunoglobulin sequences (31, 43, 50). Several additional AChR-specific human-derived mAbs have been produced using a number of different approaches, including single-cell technology (49). These recombinant human-derived mAbs emulate the properties of AChR autoantibodies found in the serum: They compete for binding to regions of the AChR recognized by serum-derived autoantibodies (50), and they possess pathogenic properties demonstrated through passive transfer of MG (49). These mAbs, coupled with investigations using human serum-derived AChR autoantibodies, have provided a clear illustration of the three AChR autoantibody pathogenic mechanisms. The first pathogenic mechanism is the inhibition of acetylcholine binding to the AChR. These autoantibodies can block this interaction by either binding to the same site on the AChR, or in proximity to the binding site, which results in inhibition of acetylcholine-dependent signaling at the NMJ (51–53). The second mechanism is termed antigen modulation, which results in internalization of the AChR following autoantibody-mediated crosslinking. Antibodies are structured as dimeric molecules that have two identical heavy and light chain pairs with two antigen binding sites and a constant region that determines the effector function. Monovalent antigen-binding fragments (Fabs), which are derived from whole antibodies, have one single antigen binding site. These Fabs have been shown to lack the ability to crosslink the AChR, while whole antibodies can crosslink the AChR through bivalent binding with two binding sites. Subsequent to the receptor crosslinking, there is internalization of the AChR, which diminishes the number of receptors at the NMJ (54, 55). Finally, the third pathogenic mechanism involves the immunoglobulin effector functions of the AChR autoantibodies. The effector functions of IgG1 and IgG3 are key properties of their pathogenic capacity. Among their principle effector functions is the ability to initiate the complement cascade. AChR autoantibodies are predominantly of the IgG1 or IgG3 subclass and effectively activate complement, leading to the formation of the membrane attack complex and consequent tissue damage at the NMJ (56–59). Early studies demonstrated that complement-mediated damage to the postsynaptic NMJ results in reduction of the postsynaptic junctional folds, elimination of AChR from the membrane, and an increase in synaptic distance (59, 60). It is unmistakable that complement plays a key role in AChR MG pathology, given the successful treatment of patients (61) with complement inhibitors (discussed below).

## Similarities Between Neuromyelitis Optica and AChR Myasthenia Gravis

Parallels between AChR MG and autoimmune neuromyelitis optica (NMO) suggest that additional studies on the role of complement in AChR MG are warranted. Like AChR MG, NMO is mediated by pathogenic autoantibodies, primarily of the IgG1 and IgG3 subclasses, that include complement activation among the mechanisms of autoimmune pathology (62). Studies of complement in NMO have led to a detailed understanding of pathogenic mechanisms, which may take place in AChR MG as well. In NMO, aquaporin-4 (AQP4)-IgGs targeting a distinct epitope in an extracellular loop, regardless of affinity, enhanced complement dependent cytotoxicity (CDC). Furthermore, particular AQP4 isoforms can form supramolecular orthogonal arrays that arrange in a manner that benefits autoantibody multimeric complexes supporting Fc-Fc interactions that are critical for CDC (63). Whether or not a similar phenomenon may occur in the context of AChR-specific MG has yet to be explored. However, at the NMJ, the AChR is tightly clustered by the intracellular scaffolding protein, rapsyn; thus, such organized formations of self-antigen may support Fc-Fc interactions facilitating efficient CDC, by AChR autoantibodies recognizing particular epitopes. Thus, parallels to pathogenic mechanisms that occur in NMO warrant investigation.

# IMMUNOPATHOLOGY OF MuSK MYASTHENIA GRAVIS

## Characterizations of B Cells in MuSK Myasthenia Gravis

Among the notable differences in the pathophysiology of MuSK and AChR MG is the role of the thymus in causing disease. As discussed previously, the thymus is a source for B cells specific for AChR in patients with AChR MG. While conspicuous in its pathogenic role in many AChR MG patients, abnormal thymus histopathology is not observed in patients with MuSK MG (64, 65). There are very few studies in which MuSK autoantibody-producing B cells have been identified and isolated. To date, these B cells have been found only in the circulation (66) and have a memory B cell or short-lived circulating plasmablast phenotype (19, 67). The variable region sequences of these autoantibodies revealed that they exhibit hallmarks of affinity maturation, including a high frequency of somatic hypermutation. They are oligoclonal, but the number of mAbs is currently too limited to draw firm conclusions about whether or not they share unique

repertoire properties with each other, such as restricted variable region gene usage.

## Properties of MuSK Myasthenia Gravis Autoantibodies

MuSK autoantibodies differ from their AChR-specific counterparts in terms of their subclass. MuSK autoantibodies are predominantly of the IgG4 subclass (68). Antibodies of the IgG4 subclass have an ambivalent role in immunity. In the context of allergy, especially studied in beekeepers, IgG4 antibodies dampen inflammatory reactions by competing with disease-mediating IgE antibodies for the same antigen, namely phospholipase A2 (69–71). In addition, beekeepers who were tolerant to bee venom had increased venom-specific IgG4 levels, while the venom-specific IgE levels were almost undetectable (69). This change toward protective IgG4 production seems to be modulated by sensitized regulatory T and B cells that secrete IL-10, leading to increased IgG4 and decreased IgE levels (72–74). The protective function of IgG observed in allergies has also been described in the autoimmune disease systemic lupus erythematosus (SLE) (75); the amount of complement deposition was negatively correlated with the amount of IgG4 antibodies in an *in vitro* deposition assay. The mechanism underlying this observation might be that IgG4 antibodies block and prevent autoantibodies of other subclasses and their effector functions – a similar approach using an antibody lacking effector functions called aquaporumab is currently under development in the treatment of NMO (76). In contrast to this regulatory and anti-inflammatory aspect of IgG4 immunology, several autoimmune disorders including MuSK MG, pemphigus vulgaris (PV), and chronic inflammatory demyelinating polyneuropathy (CIDP), include pathogenic IgG4 autoantibodies (77–79). IgG4 antibody effector function is clearly distinct from that of IgG1 antibodies. IgG4 subclass antibodies cannot activate the complement cascade via the classical pathway due to their poor affinity for C1q and Fc receptors (80–82). Thus, similar to the observed protective functions of IgG4 antibodies, MuSK autoantibodies exert their pathogenicity through blocking the interaction between MuSK and LRP4 that is required for the clustering of the AChR (83, 84).

An intriguing feature of human IgG4 antibodies is their exclusive ability to participate in "Fab-arm exchange" (**Figure 1**) (85). Fab-arm exchange (FAE) is a process whereby antibodies can dissociate to produce two identical half molecules each formed of a heavy chain and a light chain (**Figure 1**). These half molecules can then recombine, producing antibodies with two distinct variable regions which cannot crosslink identical antigens and are therefore functionally monovalent. FAE has been shown to play an important role in the immunopathology of MuSK MG (84). The mechanism of FAE is not fully understood, but key amino acid residues and conditions required for the exchange process have been elucidated. The Fc region plays a key role in the process of FAE. Two major interactions between the Fc regions are crucial for holding the two parts of the IgG molecule together: interchain disulfide bonds in the core hinge region and non-covalent interactions in the third constant heavy domain (CH3) of the respective chains (85, 86). Specific amino acids at these sites in the IgG4 molecule facilitate the dissociation of the two halves of the antibody and enable FAE. Although the sequences of the different IgG subclasses share similarities, some small changes have a large effect on the stability of the IgG molecule. The core hinge region in IgG1 contains the motif 226cys-pro-pro-cys-pro230 which confers stability. By contrast, IgG4 contains a serine at position 228 which enhances hinge flexibility and promotes dissociation of the molecule (87). Site-directed mutagenesis studies have shown that replacing the endogenous serine with a proline at position 228 in IgG4 reduces the formation of half molecules (88, 89) and prevents FAE from occurring *in vivo* and *in vitro* (89–91). Moreover, non-covalent interactions in the third constant (CH3) domain play a vital role in holding the two chains together (87). The lysine at position 409 in the CH3 region of IgG1 contributes to the stability of the molecule. Mutating this lysine to an arginine residue has been shown to destabilize the interchain links and lead to its dissociation into half molecules (92–95). Mutating lysine to arginine may not appear to be a particularly significant substitution but it should be noted that there are there are other examples of this mutation having rather profound effects *in vivo* (96, 97). Producing an arginine-to-lysine mutation in IgG4 CH3 was shown to cause a 10- to 100-fold change in the dissociation constant, which was enough to make the difference between enabling and inhibiting FAE (86, 98). FAE can occur under certain physiological conditions. Under non-reducing conditions IgG4 acts as a regular bivalent antibody (99). IgG4 can participate in half molecule exchange only under reducing conditions, which can be induced *in vitro* with low concentrations of the reducing agent glutathione (GSH) (85, 100). It is thought that the reduction of interchain disulfide bonds in the hinge region is a pre-requisite step for FAE (87, 101). Moreover, the reaction occurs more efficiently at physiological temperatures rather than at room temperature (85). Other factors contributing to FAE alongside amino-acid sequence, temperature, and reducing environment have been considered (e.g., time course for exchange, IgG ratio, and concentration of antibody), however, these factors have not been explored in depth (86, 102, 103). It was also found that plasma components have only a minor effect on the extent and duration of FAE *in vitro* (103).

## Comparison Between MuSK Myasthenia Gravis, Pemphigus Vulgaris, and Chronic Inflammatory Demyelinating Polyneuropathy

MuSK MG shares several features with other autoimmune diseases like PV and CIDP. PV is an autoimmune disorder characterized by autoantibodies that target integral parts within the skin structure that are important for cell adhesion (104–108), most often desmoglein 1 and desmoglein 3 (109, 110). Consequently, PV manifests with skin blisters, often involving the oral mucosal membrane (111). CIDP patients present similarly to MG with muscle weakness. CIDP is a heterogeneous autoimmune disease affecting peripheral nerves. Autoantibodies in CIDP interrupt the conduction along the nerves (112), in contrast to MG where the immunopathology is located at the

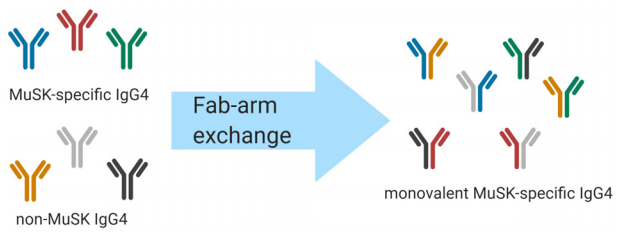



**FIGURE 1 |** Schematic of IgG4 Fab-arm exchange in MuSK MG. Human IgG4 antibodies can participate in a process termed Fab-arm exchange. In the MuSK autoantibody subtype of MG, MuSK-specific IgG4 autoantibodies can undergo Fab-arm exchange with other circulating IgG4 antibodies. The antibodies that were formerly divalent – with two identical antigen binding sites – become monovalent and bispecific after a heavy and light chain pair is switched with a heavy and light chain pair of another antibody. The process is thought to be critical for the development of pathogenic autoantibodies in MuSK MG.

NMJ. CIDP autoantibodies have been found to target contactin 1, neurofascin, and other self-antigens, which are associated with the node of Ranvier (79, 113). The autoantibodies in PV and CIDP are predominantly of the IgG4 subclass; thus sharing a key feature with MuSK MG (77–79). However, the only disease in which FAE has, to date, been shown to play a role in is MuSK MG (84). Yet, evidence is available showing that FAE may be a common occurrence in human biology. The biological therapeutic natalizumab was engineered using the IgG4 subclass; it has a wildtype core hinge region that does not contain the stabilizing serine to proline mutation at position 228 (114). It has been shown that natalizumab exchanges Fab-arms with endogenous human IgG4 in natalizumab-treated individuals (114). However, the role of FAE in healthy individuals is currently not known.

FAE plays a key role in the pathogenicity of MuSK autoantibodies. Several passive transfer models have shown that the IgG4 autoantibodies in MuSK MG are pathogenic *in vivo* (115, 116) and that serum-derived IgG4 autoantibodies are also pathogenic after Fab-arm exchange using an established *in vitro* assay (117). Using monoclonal patient isolated autoantibodies, we and others recently found that divalent MuSK autoantibodies could slightly induce agrin-independent AChR clustering by crosslinking MuSK at the NMJ leading to autophosphorylation of MuSK (66, 67). A stronger pathogenic effect of isolated MuSK autoantibodies was observed by testing these as monovalent Fabs (thus emulating FAE products); the monovalent Fabs could not crosslink MuSK on the cell surface and exerted their pathology through blocking the MuSK and LRP4 interaction, leading to a robust reduction in AChR clusters (66, 115–118).

The effect of valency on pathogenic autoantibodies in CIDP is not known at this time. The pathogenic effect of monovalent Fabs has been demonstrated for PV (119–122), but pathogenic autoantibodies in PV can be divalent as well, indicating that pathogenicity is not dependent on FAE as it is in MuSK MG. That FAE appears to be necessary for efficient pathology in MuSK MG, but not PV, may be explained by the very different functional contributions each of the antigen targets make to the cells in which they are expressed. MuSK is a transmembrane kinase, responsible for delivering a signal to induce AChR clustering. Desmogleins are adhesion-molecule superfamily members that mitigate cell-to-cell interactions. Therefore, when MuSK is crosslinked by a divalent autoantibody, which induces phosphorylation, the resulting affect is agonistic (*signal delivered*), and that is coupled with the blocking of the interaction between LPR4 and MuSK. When the autoantibody is monovalent (after FAE), the only effect is blocking LPR4 interaction and consequential pathology due to failed signaling for AChR clustering. On the other hand, in PV, autoantibody binding to the desmogleins interrupts their binding to partners; thus, monovalent or divalent autoantibody binding equally effect interference. Overall, not enough mechanistic detail is available regarding the role of FAE in CIDP, PV, and most intriguingly, normal immune responses in healthy individuals.

Finally, similarities in the immunopathology of CIDP, PV, and MuSK MG are also observed in responses to treatment. Patients with all three diseases respond very well to the treatment with the B cell depleting drug rituximab (123–125), the benefit of which can last for years (126). This effect seems to be common among autoimmune diseases that are mediated by IgG4 and is further discussed below.

## LRP4 AND SERONEGATIVE MYASTHENIA GRAVIS

Compared to what we know about AChR MG, there is a scarcity of information concerning the immunopathology of LRP4 MG and SNMG. However, that which is understood of the immune mechanisms contributing to LRP4 MG indicate that similarities to AChR MG can be found. Autoantibodies against LRP4 have been found in patients who were previously identified as seronegative (6, 127, 128). These autoantibodies were shown to disrupt the Agrin-LRP4 signaling and to be mainly of the complement activating IgG1 subclass (6). In contrast to findings, which show that that AChR and MuSK autoantibodies are specific for MG (129), LRP4 antibodies appear to cross disease boundaries. For example, LRP4 autoantibodies have been detected in some patients with amyotrophic lateral sclerosis (ALS) who presented with myasthenic symptoms (130, 131). The role of the thymus in LRP4 MG was recently investigated in a small pilot study (132). This study showed that there was a heterogeneity in thymus morphology among the four tested patients. Two out of these four patients seemed to benefit from thymectomy after a one-year follow up, while one of those two patients needed no additional treatment after thymectomy. Overall, there have been few investigations of the immunopathology that contributes to LPR4 MG, and thus caution should be taken such as not to generalize these early findings.

Some patients originally categorized as seronegative were later found to have detectable AChR, MuSK, or LRP4 autoantibodies due to either improved test sensitivity or increased titers above the lower limit reference on repeat measurement (133). Other SNMG patients remain defined by the absence of detectable

autoantibodies. This could be the consequence of sensitivity limitations within our current detection assays or due to the fact that other unidentified autoantigens are present within these patients. Indeed, when highly sensitive cell-based assays (CBAs) were introduced for the detection of AChR autoantibodies, a number of SNMG cases tested positive for circulating AChR autoantibodies. Similarly, both MuSK MG and LRP4 MG were identified through investigating novel targets in SNMG patients. Several new autoantibody targets within the NMJ, including agrin, collagen Q, cortactin, and the voltage-gated potassium channel, Kv1.4, have been proposed (134–138); however, these autoantibodies have not, as yet, been shown to have pathogenic capacity. Autoantibodies against the intracellular proteins, titin, and the ryanodine receptor were found to be potential candidate biomarkers for disease monitoring in MG (139–141). These autoantibodies together with other striational autoantibodies have been observed in patients with MG (142), however, their direct contribution to pathogenicity is unlikely, given the intracellular location of their targets.

Some SNMG patients respond to immunosuppression, IVIG, and plasmapheresis, which indicates that an autoantibody-mediated mechanism may contribute to the pathology (143, 144). The effect of thymectomy in SNMG is not clear. Some studies found positive therapeutic effects similar to seropositive MG (145–147), while other studies failed to show a beneficial effect (144, 148). Optimal treatment paradigms and outcomes for SNMG patients are uncertain. Further compounding the problem, SNMG patients are often not included in clinical trials in which autoantibody-positive patients participate. Consequently, these patients are not managed with standardized treatment approaches due to a lack of understanding of the disease mechanisms. It is reasonable to suspect that SNMG is, in many cases, a misnomer. Rather SNMG is likely a heterogeneous disease consisting of patients who have pathogenic autoantibodies directed against indeterminate NMJ targets, or known autoantibodies (AChR, MuSK, or LRP4) that are below the level of detection with current commercial diagnostic tests.

# THERAPEUTIC INTERVENTION FURTHER HIGHLIGHTS THE DIVERGENT IMMUNOPATHOLOGY OF AChR AND MuSK MYASTHENIA GRAVIS

The current standard of care for the treatment of MG largely targets MG symptoms rather than specific immune components underlying the disease subtypes. Immunosuppressive agents (i.e., corticosteroids) are used most often for this purpose (149). Cholinesterase inhibitors, such as pyridostigmine, prevent the degradation of acetylcholine and thus increase its availability at the NMJ, thus improving neuromuscular transmission (150–153). Some patients do not respond well to these therapies due to side effects or incomplete clinical benefit. Approaches that more directly target the immune system have recently shown promising effects. While such immune-modifying biologics in MG have proven to be therapeutically beneficial, they have also provided a unique opportunity to further understand MG immunopathology. Laboratory-based study of patient-derived material before and after such therapeutic interventions have been leveraged to provide immunomechanistic detail that would otherwise not be possible in laboratory-based translational investigations. These include B cell depletion, inhibition of complement, targeting the BAFF/APRIL system, resection of the thymus, and interruption of IgG recycling.

## Thymectomy

Thymectomy is a well-established treatment option in AChR MG. Even before experimental studies demonstrated the possibility that thymus resident B cells could produce AChR-specific autoantibodies, removal of the thymus had already been widely accepted as a treatment option for AChR MG (154, 155). Recently, thymectomy was formally confirmed to be beneficial in AChR MG patients compared to treatment with corticosteroids alone (156, 157). The thymus of around 70% of AChR MG patients is hyperplastic and populated by B and T cells, while the thymus of healthy subjects is involuted by adulthood (158, 159). Thymectomy was shown to generally lead to a reduction in overall AChR autoantibody titer, although AChR autoantibody titers almost never become undetectable (160, 161). In a subset of patients, only modest decreases of the AChR titer can be found (162). Whether patients with modest changes in AChR titer are less likely to go into remission is not known yet (160). About half (40–50%) of AChR patients experience long-term remission without relapse following thymectomy when followed for up to 20 years post-procedure (163, 164). While clinical improvement is observed in half the patients following thymectomy, complete remission is not achieved in many patients.

It is not clear why there is a heterogeneous response to thymectomy. Several retrospective studies explored factors associated with non-remission after thymectomy (162–164). Non-ocular MG (164), thymoma (162–164), specific surgical techniques (162, 163), duration of disease prior to resection (162), and age (163) have all been associated with a failure to respond to thymectomy. A series of studies have noted the failure of specific thymectomy approaches to remove the entire thymus, resulting in residual thymus tissue and symptoms (165, 166).

The thymus may be the site in which AChR B cells are initially activated and then mature. It is reasonable to speculate that autoantibody-producing B cell clones residing in the thymus can also populate compartments in the periphery. Consequently, surgical resection removes autoantibody-producing B cells but those which have emigrated from the thymus may continue to contribute to disease. The distribution of pathogenic AChR autoantibody-producing cells in anatomic compartments aside from the thymus, such as in the lymph nodes and bone marrow of patients with AChR MG (44, 45), must be considered. While thymectomy will remove a large fraction of thymus-resident autoreactive B cells and the cells that support their development, this treatment is performed only once the disease is established. This may be too late to halt disease progression, as those thymic B cells that have emigrated contribute to ongoing disease. Accordingly, combination therapies aimed at targeting residual thymus-related B cells may prove to be a valuable part of a

potential therapeutic strategy. The use of B cell depleting agents such as rituximab – currently used for the treatment of MG – may fit this approach well. While no controlled studies have specifically investigated the effect of B cell depletion following thymectomy, there is some evidence (167) showing that patients who underwent thymectomy respond to rituximab similarly to those who did not have the surgery. Firm conclusions cannot be drawn from restricted numbers of patients, but these data may again point toward the key role of rituximab-resistant plasma cells in AChR autoantibody production. Accordingly, consideration may also be given to anti-CD19 based treatments that would additionally target plasma cells thought to be spared by rituximab (168).

While it is clear that thymectomy depletes a population of B cells that secrete AChR-specific autoantibodies, it is unclear if therapeutic benefit arises from the removal of pathogenic B cells alone. That thymectomy includes the removal of pathogenic T cells, including Tregs that are defective in suppressing T cell proliferation and conventional T cells that resist Treg-mediated suppression (169), supports the idea that non-B cell related disease mechanisms may be interrupted by the procedure. Abnormal thymus histopathology is not observed in patients with MuSK MG (64, 65). Given the positive effect of thymectomy on AChR MG, including cases without measurable thymic abnormalities (170), thymectomy has been applied as a treatment option for MuSK MG. However, thymectomy has not been demonstrated to improve clinical outcomes for MuSK MG patients (170, 171). Consequently, thymectomy is a possible therapy for AChR MG only – regardless of the presence or absence of thymic abnormalities.

## B Cell Targeting Therapies

### Anti-CD20 and Anti-CD19 Antibodies

CD20 is a surface molecule that is expressed on B cells at almost every step of B cell differentiation. Only pro B, pre B I, and plasma cells do not express CD20 (172). The anti-CD20 mAb rituximab (RTX) is currently a treatment option for MuSK MG and has been trialed for the treatment of AChR MG. A case report on the successful treatment of AChR MG in a patient treated for non-Hodgkin's lymphoma offered the first evidence in support of the use of B cell depletion therapy in MG (173). Several groups have subsequently investigated the efficacy of RTX in the treatment of AChR and MuSK MG (125, 174, 175). These studies have demonstrated 100% complete stable remission for the use of RTX in MuSK MG (125, 174), while 56% of AChR patients experienced a relapse within an average of 36 months after treatment – a finding that was replicated in another similar independent study (125, 167). Although MuSK MG patients respond very well to treatment with RTX, relapses do occur, and the relapse rate is dependent on the applied RTX treatment protocol (176). Consistent reductions in AChR autoantibody titers and clinical improvement were demonstrated in a cohort study involving six patients (174). These results were similar to those from an independent study demonstrating symptomatic improvement for AChR patients undergoing therapy; however, no corresponding fall in AChR autoantibody titer was observed (125).

In general, there is a poor correlation between the titer of AChR-specific autoantibodies and overall clinical progress (125). The titer of MuSK autoantibodies associates with the clinical improvement observed after B cell depletion therapy, the same is not true in AChR MG. These contrasting results are well highlighted by a study (125) that demonstrated clear clinical improvement in both AChR and MuSK MG, but only MuSK autoantibody titers diminished, while intra-patient AChR autoantibody titers increased, decreased, or stayed the same. The poor correlation with clinical severity has been known since the earliest initial studies that established the use of assays measuring AChR autoantibody titers (160). This likely reflects the polyclonal nature of anti-AChR antibodies, their different specificities, subclasses, local concentrations and complement, modulating, and blocking activity. It is important to point out that the assays used to diagnose MG by measuring AChR binding, provide no information whatsoever on their pathogenic capacity. It is possible that a fraction of autoantibodies that bind in the laboratory assays have little pathogenic capacity *in vivo*. Furthermore, circulating AChR autoantibodies, by definition, are not present at the site where the disease pathology occurs (the NMJ). Combinations of these factors may contribute to a disassociation between circulating titer and disease severity. However, several studies have shown that intra-patient longitudinal AChR autoantibody titers may correlate with disease severity progress (160, 177). Establishing the use of relative – as opposed to absolute – AChR autoantibody as a trial endpoint may prove to be a useful biomarker in the future. Given that the RIA or CBA used to diagnose patients and provide AChR titer values are wholly unable to discriminate between the detection of these autoantibodies and their pathogenic properties, applying an assay suite that can quantitate the extent of AChR autoantibody-mediated complement activation, blocking, or modulating within an individual patient may associate with disease severity better than simple AChR binding titer measurements.

Recently, a phase-2 trial called BeatMG, designed to test the efficacy of RTX treatment in AChR patients with mild to severe disease, ended (ClinicalTrials.gov Identifier: NCT02110706), while a phase-3 trial called RINOMAX is currently in progress with patients that have moderate to severe disease (ClinicalTrials.gov Identifier: NCT02950155). The BeatMG study showed slightly favorable effects of treatment with rituximab especially in patients with more severe courses of disease, although there was no statistical difference between the rituximab and placebo groups (178). The data from the clinical trials aimed at investigating the efficacy of RTX in MuSK and AChR MG offer unique insight into the distinct immunopathology of these two MG subtypes (125, 167, 174). In general, RTX does not efficiently deplete tissue-localized B cells in lymph nodes, tonsils, and bone marrow (179–182). Moreover, RTX has been particularly efficacious in diseases mediated by pathogenic IgG4 antibodies such as PV, and CIDP in addition to MuSK MG (124, 183). Long-lived plasma cells populations residing in the thymus produce some of the circulating AChR-specific autoantibodies; plasma cells express low levels of CD20 (32, 34). In contrast, circulating plasmablasts, such as those that secrete MuSK-specific

autoantibodies, typically express higher levels of CD20 than their tissue resident plasma cell counterparts (19, 184), although some refractory B cell clones were found to emerge during relapse in MuSK MG after treatment with RTX (185). Thus, differences in the efficacy of RTX in AChR and MuSK MG may reflect differences in the tissue localization of disease-causing B cell subsets and/or the susceptibility of different autoantibody-producing B cell subsets (*plasmablasts in MuSK MG and plasma cells in AChR MG, each expressing different levels of CD20*) to anti-CD20 depletion.

Although it is well-understood that MuSK MG patients responds remarkably well to B cell depletion therapy, there are patients who respond less well to this treatment and a small fraction who do not improve (186). This highlights the heterogeneity that is invariably observed among MG patients. While it is not understood why non-responders have emerged, one can speculate that some MuSK patients may produce autoantibodies from a subset of B cells that do not express CD20, such as plasma cells as in AChR MG, or they utilize plasmablasts with low surface CD20 expression levels. Other possible mechanisms include diminished complement activity, the mechanism by which anti-CD20 mediates cell death.

Alternative strategies of B cell depletion therapy have recently emerged, including mAbs targeting CD19. CD19 is a gene surface marker that is expressed over a wider range of B cell subsets than CD20. CD19 is expressed before the expression of CD20 in pro-B cells and declines after the expression of CD20 in plasma cells. A larger proportion of plasma cells expresses CD19 in comparison to CD20 (187). Thus, anti-CD19 agents could potentially enhance the depletion of disease-causing plasma cell populations. MEDI-155 or inebilizumab is an IgG monoclonal antibody that was initially demonstrated to be more effective than anti-CD20 depletion in EAE, a mouse model for multiple sclerosis (188, 189). This study showed that the improved efficacy of anti-CD19 depletion could be explained by the depletion of plasma cells in the bone marrow. Consequently, two phase 1 trials were initiated for its use in relapsing-remitting multiple sclerosis (190) and systemic sclerosis (191) with promising results. Moreover, a phase-2/3 clinical trial was initiated for its use in the treatment of NMO called NM-omentum (192). The NM-omentum trial showed a clear efficacy of the treatment with anti-CD19 over placebo (168). The treatment with anti-CD19 therapy is a possible option for both MuSK and AChR MG. In comparison to anti-CD20 based therapy, targeting CD19 could have an increased effect on the AChR autoantibody-producing B cell subsets.

**TABLE 1 |** Autoimmune characteristics differentiating AChR and MuSK MG subsets and consequent response to immunomodulating therapies.

| | AChR MG subtype | MuSK MG subtype |
|---|---|---|
| **Immunomechanisms** | | |
| Thymus | Hyperplasia (in a subset) | Normal |
| Autoantibody IgG subclass | IgG1 and IgG3 | IgG4 |
| Role for complement | Major | Not significant |
| B cell subtype responsible for autoantibody production | $CD20^{neg}$ Plasma cells | Plasmablasts |
| **Treatment response** | | |
| Thymectomy | Clinical benefit | Clinical benefit not observed |
| Complement inhibitors | Clinical benefit | Clinical benefit not expected |
| Anti-CD20 (rituximab) | Some clinical benefit | Clinical benefit observed |
| FcRn inhibition | Clinical benefit expected | Clinical benefit expected |
| Anti-CD19 | Clinical benefit expected | Clinical benefit expected |
| Proteasome inhibitors | Clinical benefit expected | Clinical benefit not expected |

## Proteasome Inhibitors

Given that plasma cells are suspected of playing an important role in the production of disease-causing autoantibodies in AChR MG, direct plasma cell depletion with proteasome inhibitors has been proposed for the treatment of this MG disease subtype. Bortezomib is such a proteasome inhibitor, and it was shown that it could directly deplete plasma cells (193–195). The proteasome is connected to cell homeostasis and promotes protein clearance of cell apoptosis associated and misfolded proteins (196). Bortezomib was demonstrated to be efficacious for the treatment of hematologic autoimmune diseases such as autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), and thrombotic thrombocytopenic purpura (TTP) in a phase-2 trial (197). Bortezomib (198) and other proteasome inhibitors (199) were first shown to be beneficial in EAMG, a mouse model for MG. Moreover, *in vitro* studies of AChR MG patients showed that bortezomib can eliminate thymus-derived plasma cell populations, reducing pathogenic IgG as well as total IgG levels (194). Consequently, a phase-2 trial called TAVAB that investigated the use of bortezomib in generalized AChR MG, rheumatoid arthritis (RA), and SLE was initiated in 2014, although results are still not yet available (ClinicalTrials.gov Identifier: NCT02102594) (200). The plasmablasts that are thought to produce autoantibodies in MuSK MG are not targeted by proteasome inhibitors. Accordingly, proteasome inhibitors may be a possible treatment option for AChR MG only.

## Targeting the BAFF/APRIL System

The survival of B-cells is regulated in part by the BAFF/APRIL system. This system consists of the two ligands B-cell activating factor (BAFF/BLyS/TALL-1) and a proliferation-inducing ligand (APRIL), and the three receptors, B-cell activating factor receptor (BAFF-R), B-cell maturation Ag (BCMA), and transmembrane activator and CAML interactor (TACI) (201). BAFF and APRIL are B cell stimulatory molecules that promote B cell proliferation, autoimmunity, somatic hypermutation and mediate B cell survival (202). The BAFF/APRIL system is a highly balanced system controlling B cell survival and proliferation. High levels of BAFF lead to an imbalance towards B cell proliferation. The occurrence of high levels of ligands and soluble receptors of the

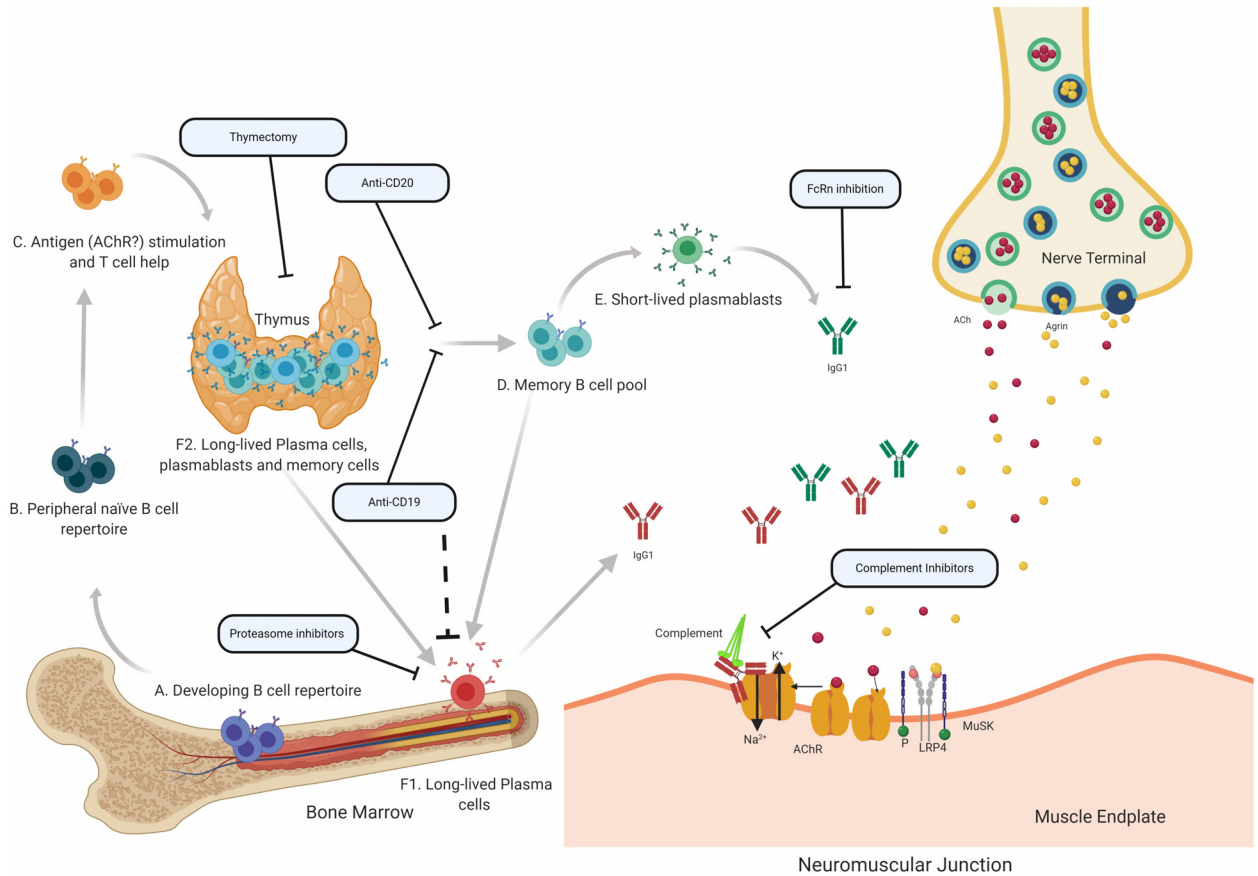



**FIGURE 2 |** Speculative mechanisms of AChR MG immunopathology. The proposed mechanistic path to autoantibody production in AChR MG begins with naïve B cells (Steps A and B), which likely encounter self-antigen(s) and receive T cell help in the thymus (C). They can then differentiate into autoantibody specific memory B cells (D), which can be activated into antibody-secreting short-lived plasmablasts (E) or antibody-secreting long-lived plasma cells (F), which reside in the bone marrow (F1) and may also be present in the thymus (F2) of some patients with AChR MG. It is thought that long-lived plasma cells in the bone marrow and thymus make major contributions to AChR autoantibody production. Autoantibodies migrate to the NMJ where they bind to the AChR hindering the neuromuscular transmission by directly interrupting acetylcholine signaling at the AChR. Most of the antibodies are of the IgG1 subclass which can induce the complement cascade. Several therapeutic strategies target different parts of this process in AChR MG. Thymectomy is thought to directly remove autoantibody-producing B cell and other pathogenic cell subsets. B cell depletion, mediated by anti-CD20 antibodies is thought to remove autoreactive B cells, which includes memory cells and a subset of plasmablasts. Anti-CD19 antibodies can additionally target further subsets of plasmablasts and subsets of plasma cells. Proteasome inhibitors target plasma cells and may target the disease-causing long-lived plasma cells more efficiently. FcRn inhibitors increase the elimination of circulating IgGs, which is expected to also reduce the levels of pathogenic autoantibodies. Inhibiting complement activity, with complement inhibitors, results in the disruption of the pathogenic effector functions mediated by AChR autoantibodies.

BAFF/APRIL system is associated with B cell pathologies (203–206) and high levels of BAFF are linked to autoimmunity (203, 207, 208). The anti-BAFF antibody belimumab was shown to be efficacious in the treatment of SLE in a phase-3 randomized controlled trial and it was approved by the FDA for the treatment of SLE (209). Elevated serum BAFF levels have been observed in both AChR MG and MuSK MG patients, and BAFF levels were shown to correlate with autoantibody titer (210–212). The results from a recent randomized controlled trial on the use of belimumab in AChR MG, however, failed to meet the primary endpoint of a change in quantitative myasthenia gravis (QMG) score (213). High serum BAFF levels have been shown to correlate with poor responses to rituximab in RA and Sjogren's disease, raising the possibility that combination therapy with B cell depleting agents may hold promise (81, 214, 215). Several other approaches targeting the BAFF/APRIL system have been investigated, including atacicept (a soluble decoy receptor for BAFF and APRIL) which showed beneficial effects in SLE and RA (216–218). However, adverse effects in its use for the treatment of multiple sclerosis indicate that it may have a more complex role within the immune system (219). Additionally, γ-secretase inhibitors were recently described as an add-on therapy for multiple myeloma, as the inhibition of the shedding of BCMA was shown to work synergistically with CAR-T cell therapy (220). BAFF-R, BCMA, and TACI are expressed differently during all steps of B cell development (172). Consequently, targeting the BAFF/APRIL system is a potential therapeutic avenue for both MuSK and AChR MG.

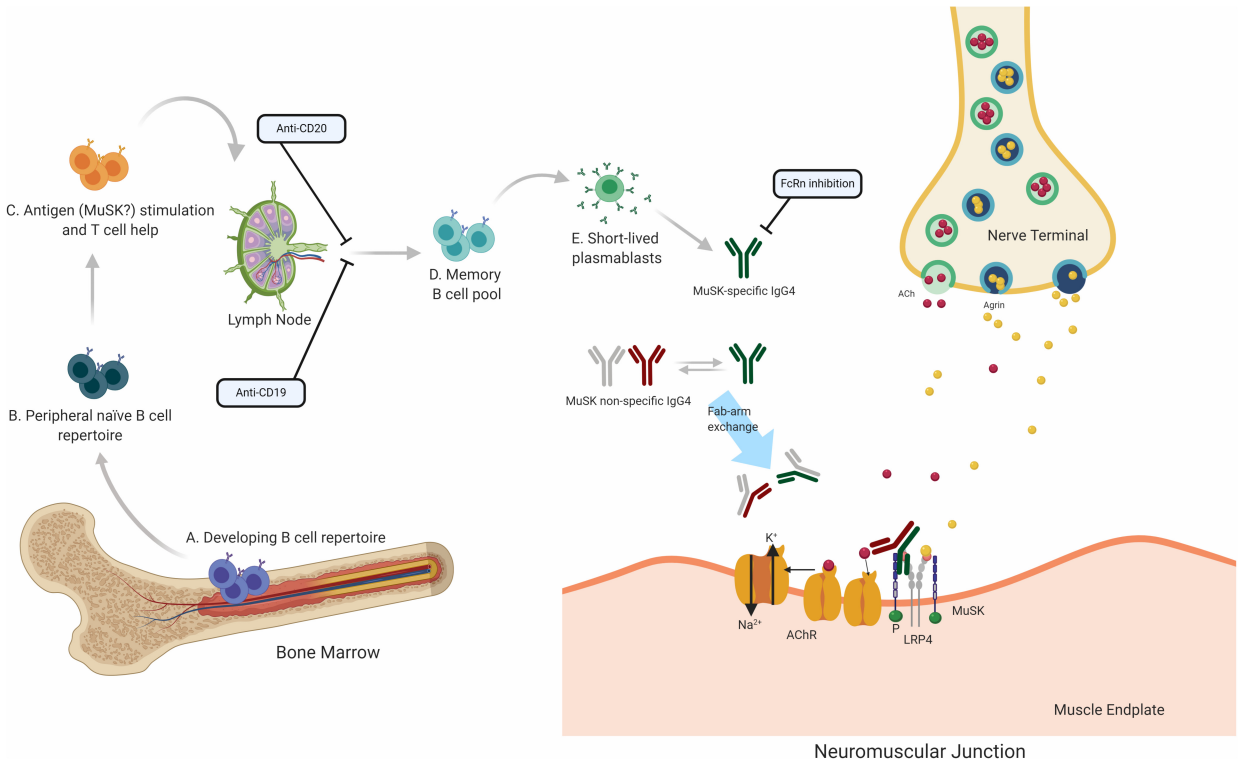



**FIGURE 3 |** Speculative mechanisms of MuSK MG immunopathology. The proposed mechanistic path to autoantibody production in MuSK MG begins with naïve B cells (Steps A and B), which likely encounter self-antigen(s) and receive T cell help in the lymphoid tissue (C). They then differentiate into memory B cells (D) and antibody-secreting plasmablasts (E). Most autoantibodies in MuSK MG are of the IgG4 subclass. Antibodies of the IgG4 subclass can undergo the process of Fab-arm exchange with other antibodies of the IgG4 subclass. Consequently, the divalent mono-specific MuSK autoantibodies become monovalent bispecific autoantibodies. These autoantibodies migrate to the neuromuscular junction where they bind to MuSK hindering the neuromuscular transmission by blocking the LRP4 and MuSK pathway which is important for the clustering of the AChR. Several therapeutic strategies target different parts of this process in MuSK MG. B cell depletion by anti-CD20 antibodies is thought to remove B cells expressing CD20 which includes memory cells and a subset of plasmablasts. Anti CD19 antibodies can additionally target further subsets of plasmablasts. FcRn inhibitors increase the elimination of circulating IgGs, which is expected to also reduce the levels of pathogenic autoantibodies.

## Complement Inhibitors

AChR MG autoantibodies are mainly of the complement-inducing IgG1 subclass. Accordingly, the complement system has been shown to be an effective target for the treatment of AChR MG. Two different therapies are available and have been tested in refractory AChR positive generalized MG. The first, eculizumab, is a humanized mAb that binds to C5 and thus inhibits the terminal complement pathway (221). Eculizumab showed positive effects in paroxysmal nocturnal hemoglobinuria (PNH) (222, 223) and was shown to be beneficial in atypical hemolytic uremic syndrome (aHUS) (224–226). Additionally, eculizumab was successfully tested in a clinical trial for the treatment of NMO with a primary endpoint of total relapse frequency (PREVENT Study; ClinicalTrials.gov Identifier: NCT01997229) (227, 228). After a promising pilot phase-2 trial of eculizumab in AChR positive generalized MG (229, 230), a phase-3 clinical trial of eculizumab was initiated (REGAIN; ClinicalTrials.gov Identifier: NCT01997229) (231, 232). Although the study did not achieve its primary endpoint of a statistical difference in the Myasthenia Gravis-specific Activities of Daily Living scale (MG-ADL) score for patients, additional sensitivity analyses of different MG-related scores including the MG-ADL showed improvements in the eculizumab group in comparison to the placebo group. Therefore, eculizumab was approved for the treatment of generalized AChR MG. Pointing again to heterogeneity within MG patient subtypes, it was interesting to observe that in the phase-3 clinical trial, 40% of AChR autoantibody-positive patients did not meet the trial endpoint. Furthermore, it is now appreciated that some patients have a conspicuous and rapid response to eculizumab, while others do not respond or have a more protracted improvement. These results may reflect heterogeneity among patients in terms of the relative fractions of AChR autoantibody-mediated complement activation, blocking or modulating functions (discussed above).

Zilucoplan – a small molecule (synthetic macrocyclic peptide) – binds to C5 and inhibits the terminal complement pathway (233). A phase-2 trial showed significant improvement in generalized MG patients, leading to the approval of a phase-3 clinical trial which is currently in progress (RAISE; ClinicalTrials.gov Identifier: NCT04115293). In contrast to AChR MG, MuSK MG autoantibodies are mainly of the IgG4 subclass, which does not activate complement (discussed above).

Thus, treatment with complement inhibitors, at this time, is likely to be mostly beneficial for AChR MG patients. Treatment of patients with SNMG or LRP4 MG with complement inhibitors could provide highly valuable information regarding mechanisms of immunopathology. In SNMG, beneficial outcomes would point toward autoantibody-mediated pathology, thus providing key insight toward understanding this disease subset. In LRP4 MG such outcomes would further support the role of complement activating autoantibodies in disease pathology.

### FcRn Inhibitors

Human IgG is present at high concentration in serum (approximately 7–17 mg/mL). The half-life of circulating human IgG is between 3 and 4 weeks. This high-circulating level and long half-life are not exclusively dependent on synthesis, but rather due to continuous salvage and recycling. The IgG recycling pathway is mediated by the neonatal Fc receptor (FcRn) (234). FcRn inhibitors, which block the interaction of FcRn with IgGs, effect degradation and fast clearance of IgGs and are leveraged as such as therapeutics for IgG-mediated diseases (235). An early stage trial of one FcRn inhibitor called efgartigimod in patients with AChR MG showed a reduction in the titer of pathogenic autoantibodies that was associated with an improvement in disease severity (ClinicalTrials.gov NCT02965573, EudraCT 2016-002938-73) (236). These findings suggest that FcRn inhibitors may be a valuable treatment approach for MG. Although FcRn inhibitors have not yet been formally tested in MuSK MG, they reduce the circulating levels of all IgG subclasses (including IgG4) (237). Consequently, this treatment modality has the potential to be effective in treating MuSK MG as well as AChR MG. Again, this treatment paradigm could be leveraged to provide highly valuable information regarding autoantibody-mediated mechanisms of immunopathology of LRP4 MG and especially SNMG as discussed above.

## CONCLUSION

Translational laboratory-based research and clinical trials have both provided considerable evidence supporting the idea that the immunopathology of AChR and MuSK MG is distinct (summary in **Table 1**). In general terms, AChR MG is characterized by a key role for the thymus in its immunopathology and by autoantibodies of the complement activating IgG1 subclass, which are produced by plasma cells residing in the bone marrow, thymus, and other tissues (**Figure 2**). By comparison, MuSK MG autoantibodies are mainly of the IgG4 subclass, which undergo Fab-arm exchange as a prerequisite for pathogenic capacity. MuSK MG autoantibodies are thought to be produced by circulating short-lived plasmablasts (**Figure 3**). An understanding of these differences is valuable for defining different mechanisms that underlie human autoimmune disease. They are also highly important in considering treatment options, since an understanding of the immunopathology can inform such decisions. Within both the AChR and MuSK subtypes, further heterogeneity in disease course and wide-ranging response to treatment have both been observed. Furthermore, the immunomechanisms underlying SNMG and LRP4 MG still need to be more thoroughly understood. Accordingly, additional studies directed toward understanding the immunopathology, which associates with MG subtypes and the heterogeneity within each subtype, are needed. In such efforts, it is critically important that clinical trial leadership and laboratory-based translational research groups form partnerships so that highly valuable specimens, which provide deep insight into mechanisms, are properly curated and investigated.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

KO'C was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIH) through awards R01-AI114780 and R21-AI142198, NIH through the Rare Diseases Clinical Research Consortia of the NIH (Award Number U54-NS115054), and a Neuromuscular Disease Research Program award from the Muscular Dystrophy Association (MDA) under award number MDA575198. MF is a recipient of the James Hudson Brown – Alexander Brown Coxe Postdoctoral Fellowship in the Medical Sciences. AB was funded by a Taylor Travel Award from Trinity Hall, University of Cambridge.

## ACKNOWLEDGMENTS

The authors thank Dr. Steven H. Kleinstein from the Yale University School of Medicine for critically reading the manuscript critically and Karen Boss for expert editing and proofreading. The figures were created with biorender.com by MF.

## REFERENCES

1. Gilhus NE, Skeie GO, Romi F, Lazaridis K, Zisimopoulou P, Tzartos S. Myasthenia gravis-autoantibody characteristics and their implications for therapy. *Nat Rev Neurol.* (2016) 12:259–68. doi: 10.1038/nrneurol.2016.44
2. Vincent A. Unravelling the pathogenesis of myasthenia gravis. *Nat Rev Immunol.* (2002) 2:797–804. doi: 10.1038/nri916
3. Vincent A, Beeson D, Lang B. Molecular targets for autoimmune and genetic disorders of neuromuscular transmission. *Eur J Biochem.* (2000) 267:6717–28. doi: 10.1046/j.1432-1033.2000.01785.x
4. Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med.* (2001) 7:365–8. doi: 10.1038/85520
5. Zisimopoulou P, Evangelakou P, Tzartos J, Lazaridis K, Zouvelou V, Mantegazza R, et al. A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis. *J Autoimmun.* (2014) 52:139–45. doi: 10.1016/j.jaut.2013.12.004

6. Higuchi O, Hamuro J, Motomura M, Yamanashi Y. Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. *Ann Neurol.* (2011) 69:418–22. doi: 10.1002/ana.22312
7. Koneczny I, Cossins J, Vincent A. The role of muscle-specific tyrosine kinase (MuSK) and mystery of MuSK myasthenia gravis. *J Anat.* (2013) 224:29–35. doi: 10.1111/joa.12034
8. Jacob S, Viegas S, Leite MI, Webster R, Cossins J, Kennett R, et al. Presence and pathogenic relevance of antibodies to clustered acetylcholine receptor in ocular and generalized myasthenia gravis. *Arch Neurol.* (2012) 69:994–1001. doi: 10.1001/archneurol.2012.437
9. Lindstrom JM, Engel AG, Seybold ME, Lennon VA, Lambert EH. Pathological mechanisms in experimental autoimmune myasthenia gravis. II. Passive transfer of experimental autoimmune myasthenia gravis in rats with anti-acetylcholine recepotr antibodies. *J Exp Med.* (1976) 144:739–53.
10. Oda K, Korenaga S, Ito Y. Myasthenia gravis: passive transfer to mice of antibody to human and mouse acetylcholine receptor. *Neurology.* (1981) 31:282–7.
11. Sterz R, Hohlfeld R, Rajki K, Kaul M, Heininger K, Peper K, et al. Effector mechanisms in myasthenia gravis: end-plate function after passive transfer of IgG. Fab, and F(ab')2 hybrid molecules. *Muscle Nerve.* (1986) 9:306–12. doi: 10.1002/mus.880090404
12. Toyka KV, Brachman DB, Pestronk A, Kao I. Myasthenia gravis: passive transfer from man to mouse. *Science* (New York, NY). (1975) 190:397–9.
13. Melber D. Maternal-fetal transmission of myasthenia gravis with acetylcholine-receptor antibody. *N Engl J Med.* (1988) 318:996.
14. Vernet-der Garabedian B, Lacokova M, Eymard B, Morel E, Faltin M, Zajac J, et al. Association of neonatal myasthenia gravis with antibodies against the fetal acetylcholine receptor. *J Clin Invest.* (1994) 94:555–9. doi: 10.1172/JCI117369
15. Donaldson JO, Penn AS, Lisak RP, Abramsky O, Brenner T, Schotland DL. Antiacetylcholine receptor antibody in neonatal myasthenia gravis. *Am J Dis Child.* (1981) 135:222–6.
16. Keesey J, Lindstrom J, Cokely H. Anti-acetylcholine receptor antibody in neonatal myasthenia gravis. *N Engl J Med.* (1977) 296:55. doi: 10.1056/NEJM197701062960125
17. Benatar M. A systematic review of diagnostic studies in myasthenia gravis. *Neuromus Dis.* (2006) 16:459–67. doi: 10.1016/j.nmd.2006.05.006
18. Golnik KC, Pena R, Lee AG, Eggenberger ER. An ice test for the diagnosis of myasthenia gravis. *Ophthalmology.* (1999) 106:1282–6. doi: 10.1016/s0161-6420(99)00709-5
19. Stathopoulos P, Kumar A, Nowak RJ, O'Connor KC. Autoantibody-producing plasmablasts after B cell depletion identified in muscle-specific kinase myasthenia gravis. *JCI Insight.* (2017) 2:e94263–75. doi: 10.1172/jci.insight.94263
20. Newsom-Davis J, Murray N, Wray D, Lang B, Prior C, Gwilt M, et al. Lambert-Eaton myasthenic syndrome: electrophysiological evidence for a humoral factor. *Muscle Nerve.* (1982) 5:S17–20.
21. Engel AG, Shen XM, Selcen D, Sine SM. Congenital myasthenic syndromes: pathogenesis, diagnosis, and treatment. *Lancet Neurol.* (2015) 14:420–34. doi: 10.1016/s1474-4422(14)70201-7
22. Filosso PL, Galassi C, Ruffini E, Margaritora S, Bertolaccini L, Casadio C, et al. Thymoma and the increased risk of developing extrathymic malignancies: a multicentre study. *Eur J Cardio Thorac Surgery.* (2013) 44:219–24; discussion 224. doi: 10.1093/ejcts/ezs663
23. Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurol.* (2015) 14:1023–36. doi: 10.1016/S1474-4422(15)00145-3
24. Berrih-Aknin S, Frenkian-Cuvelier M, Eymard B. Diagnostic and clinical classification of autoimmune myasthenia gravis. *J Autoimmun.* (2014) 48-49:143–8. doi: 10.1016/j.jaut.2014.01.003
25. Safar D, Berrih-Aknin S, Morel E. In vitro anti-acetylcholine receptor antibody synthesis by myasthenia gravis patient lymphocytes: correlations with thymic histology and thymic epithelial-cell interactions. *J Clin Immunol.* (1987) 7:225–34.
26. Staber FG, Fink U, Sack W. Letter: B lymphocytes in the thymus of patients with myasthenia gravis. *N Engl J Med.* (1975) 292:1032–3.
27. Roxanis I, Micklem K, McConville J, Newsom-Davis J, Willcox N. Thymic myoid cells and germinal center formation in myasthenia gravis; possible roles in pathogenesis. *J Neuroimmunol.* (2002) 125:185–97. doi: 10.1016/s0165-5728(02)00038-3
28. Marx A, Pfister F, Schalke B, Saruhan-Direskeneli G, Melms A, Strobel P. The different roles of the thymus in the pathogenesis of the various myasthenia gravis subtypes. *Autoimmun Rev.* (2013) 12:875–84. doi: 10.1016/j.autrev.2013.03.007
29. Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J Autoimmun.* (2014) 52:90–100. doi: 10.1016/j.jaut.2013.12.011
30. Mittag T, Kornfeld P, Tormay A, Woo C. Detection of anti-acetylcholine receptor factors in serum and thymus from patients with myasthenia gravis. *N Engl J Med.* (1976) 294:691–4. doi: 10.1056/NEJM197603252941303
31. Leprince C, Cohen-Kaminsky S, Berrih-Aknin S, Vernet-Der Garabedian B, Treton D, Galanaud P, et al. Thymic B cells from myasthenia gravis patients are activated B cells. Phenotypic and functional analysis. *J Immunol.* (1990) 145:2115–22.
32. Hill ME, Shiono H, Newsom-Davis J, Willcox N. The myasthenia gravis thymus: a rare source of human autoantibody-secreting plasma cells for testing potential therapeutics. *J Neuroimmunol.* (2008) 201–202:50–6. doi: 10.1016/j.jneuroim.2008.06.027
33. Scadding GK, Vincent A, Newsom-Davis J, Henry K. Acetylcholine receptor antibody synthesis by thymic lymphocytes: correlation with thymic histology. *Neurology.* (1981) 31:935–43.
34. Vincent A, Scadding GK, Thomas HC, Newsom-Davis J. In-vitro synthesis of anti-acetylcholine-receptor antibody by thymic lymphocytes in myasthenia gravis. *Lancet (London, England).* (1978) 1:305–7.
35. Willcox HN, Newsom-Davis J, Calder LR. Cell types required for anti-acetylcholine receptor antibody synthesis by cultured thymocytes and blood lymphocytes in myasthenia gravis. *Clin Exp Immunol.* (1984) 58: 97–106.
36. Newsom-Davis J, Willcox N, Calder L. Thymus cells in myasthenia gravis selectively enhance production of anti-acetylcholine-receptor antibody by autologous blood lymphocytes. *N Engl J Med.* (1981) 305:1313–8. doi: 10.1056/NEJM198111263052203
37. Lisak RP, Levinson AI, Zweiman B, Kornstein MJ. Antibodies to acetylcholine receptor and tetanus toxoid: in vitro synthesis by thymic lymphocytes. *J Immunol.* (1986) 137:1221–5.
38. Levinson AI, Zweiman B, Lisak RP, Dziarski A, Moskovitz AR. Thymic B-cell activation in myasthenia gravis. *Neurology.* (1984) 34: 462–8.
39. Schonbeck S, Padberg F, Hohlfeld R, Wekerle H. Transplantation of thymic autoimmune microenvironment to severe combined immunodeficiency mice. A new model of myasthenia gravis. *J Clin Invest.* (1992) 90:245–50. doi: 10.1172/JCI115843
40. Vrolix K, Fraussen J, Losen M, Stevens J, Lazaridis K, Molenaar PC, et al. Clonal heterogeneity of thymic B cells from early-onset myasthenia gravis patients with antibodies against the acetylcholine receptor. *J Autoimmun.* (2014) 52:101–12. doi: 10.1016/j.jaut.2013.12.008
41. Sims GP, Shiono H, Willcox N, Stott DI. Somatic hypermutation and selection of B cells in thymic germinal centers responding to acetylcholine receptor in myasthenia gravis. *J Immunol.* (2001) 167:1935–44. doi: 10.4049/jimmunol.167.4.1935
42. Cardona A, Pritsch O, Dumas G, Bach JF, Dighiero G. Evidence for an antigen-driven selection process in human autoantibodies against acetylcholine receptor. *Mol Immunol.* (1995) 32: 1215–23.
43. Graus YF, de Baets MH, Parren PW, Berrih-Aknin S, Wokke J, van Breda Vriesman PJ, et al. Human anti-nicotinic acetylcholine receptor recombinant Fab fragments isolated from thymus-derived phage display libraries from myasthenia gravis patients reflect predominant specificities in serum and block the action of pathogenic serum antibodies. *J Immunol.* (1997) 158:1919–29.
44. Fujii Y, Monden Y, Hashimoto J, Nakahara K, Kawashima Y. Acetylcholine receptor antibody-producing cells in thymus and lymph nodes in myasthenia gravis. *Clin Immunol Immunopathol.* (1985) 34: 141–6.

45. Fujii Y, Monden Y, Hashimoto J, Nakahara K, Kawashima Y. Acetylcholine receptor antibody production by bone marrow cells in a patient with myasthenia gravis. *Neurology.* (1985) 35:577–79.
46. Fujii Y, Hashimoto J, Monden Y, Ito T, Nakahara K, Kawashima Y. Specific activation of lymphocytes against acetylcholine receptor in the thymus in myasthenia gravis. *J Immunol.* (1986) 136:887–91.
47. Newsom-Davis J, Willcox N, Scadding G, Calder L, Vincent A. Anti-acetylcholine receptor antibody synthesis by cultured lymphocytes in myasthenia gravis: thymic and peripheral blood cell interactions. *Ann N Y Acad Sci.* (1981) 377:393–402.
48. Lisak RP, Laramore C, Zweiman B, Moskovitz A. In vitro synthesis of antibodies to acetylcholine receptor by peripheral blood mononuclear cells of patients with myasthenia gravis. *Neurology.* (1983) 33:604–8. doi: 10.1212/wnl.33.5.604
49. Makino T, Nakamura R, Terakawa M, Muneoka S, Nagahira K, Nagane Y, et al. Analysis of peripheral B cells and autoantibodies against the anti-nicotinic acetylcholine receptor derived from patients with myasthenia gravis using single-cell manipulation tools. *PLoS One.* (2017) 12:e0185976. doi: 10.1371/journal.pone.0185976
50. Saxena A, Stevens J, Cetin H, Koneczny I, Webster R, Lazaridis K, et al. Characterization of an anti-fetal AChR monoclonal antibody isolated from a myasthenia gravis patient. *Sci Rep.* (2017) 7:14426. doi: 10.1038/s41598-017-14350-8
51. Hara H, Hayashi K, Ohta K, Itoh N, Nishitani H, Ohta M. Detection and characterization of blocking-type anti-acetylcholine receptor antibodies in sera from patients with myasthenia gravis. *Clin Chem.* (1993) 39:2053–7.
52. Whiting P, Vincent A, Newsom-Davis J. Monoclonal antibodies to Torpedo acetylcholine receptor. Characterisation of antigenic determinants within the cholinergic binding site. *Eur J Biochem.* (1985) 150:533–9.
53. Almon RR, Andrew CG, Appel SH. Serum globulin in myasthenia gravis: inhibition of alpha-bungarotoxin binding to acetylcholine receptors. *Science* (New York, NY). (1974) 186:55–7. doi: 10.1126/science.186.4158.55
54. Drachman DB, Angus CW, Adams RN, Michelson JD, Hoffman GJ. Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. *N Engl J Med.* (1978) 298:1116–22. doi: 10.1056/NEJM197805182982004
55. Loutrari H, Kokla A, Tzartos SJ. Passive transfer of experimental myasthenia gravis via antigenic modulation of acetylcholine receptor. *Eur J Immunol.* (1992) 22:2449–52. doi: 10.1002/eji.1830220939
56. Rodgaard A, Nielsen FC, Djurup R, Somnier F, Gammeltoft S. Acetylcholine receptor antibody in myasthenia gravis: predominance of IgG subclasses 1 and 3. *Clin Exp Immunol.* (1987) 67:82–8.
57. Nakano S, Engel AG. Myasthenia gravis: quantitative immunocytochemical analysis of inflammatory cells and detection of complement membrane attack complex at the end-plate in 30 patients. *Neurology.* (1993) 43:1167–72.
58. Lefvert AK, Cuenoud S, Fulpius BW. Binding properties and subclass distribution of anti-acetylcholine receptor antibodies in myasthenia gravis. *J Neuroimmunol.* (1981) 1:125–35.
59. Engel AG, Lambert EH, Howard FM. Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations. *Mayo Clin Proc.* (1977) 52:267–80.
60. Engel AG, Sakakibara H, Sahashi K, Lindstrom JM, Lambert EH, Lennon VA. Passively transferred experimental autoimmune myasthenia gravis. Sequential and quantitative study of the motor end-plate fine structure and ultrastructural localization of immune complexes (IgG and C3), and of the acetylcholine receptor. *Neurology.* (1979) 29:179–88. doi: 10.1212/wnl.29.2.179
61. Muppidi S, Utsugisawa K, Benatar M, Murai H, Barohn RJ, Illa I, et al. Long-term safety and efficacy of eculizumab in generalized myasthenia gravis. *Muscle Nerve.* (2019) 60:14–24. doi: 10.1002/mus.26447
62. Bennett JL, O'Connor KC, Bar-Or A, Zamvil SS, Hemmer B, Tedder TF, et al. B lymphocytes in neuromyelitis optica. *Neurol Neuroimmunol Neuroinflamm.* (2015) 2:e104. doi: 10.1212/nxi.0000000000000104
63. Soltys J, Liu Y, Ritchie A, Wemlinger S, Schaller K, Schumann H, et al. Membrane assembly of aquaporin-4 autoantibodies regulates classical complement activation in neuromyelitis optica. *J Clin Invest.* (2019) 130:2000–13. doi: 10.1172/JCI122942
64. Leite MI, Strobel P, Jones M, Micklem K, Moritz R, Gold R, et al. Fewer thymic changes in MuSK antibody-positive than in MuSK antibody-negative MG. *Ann Neurol.* (2005) 57:444–8. doi: 10.1002/ana.20386
65. Lauriola L, Ranelletti F, Maggiano N, Guerriero M, Punzi C, Marsili F, et al. Thymus changes in anti-MuSK-positive and -negative myasthenia gravis. *Neurology.* (2005) 64:536–8. doi: 10.1212/01.WNL.0000150587.71497.B6
66. Huijbers MG, Vergoossen DL, Fillie-Grijpma YE, van Es IE, Koning MT, Slot LM, et al. MuSK myasthenia gravis monoclonal antibodies: Valency dictates pathogenicity. *Neurol Neuroimmunol Neuroinflamm.* (2019) 6:e547. doi: 10.1212/NXI.0000000000000547
67. Takata K, Stathopoulos P, Cao M, Mane-Damas M, Fichtner ML, Benotti ES, et al. Characterization of pathogenic monoclonal autoantibodies derived from muscle-specific kinase myasthenia gravis patients. *JCI Insight* (2019) 4:e127167. doi: 10.1172/jci.insight.127167
68. Niks EH, van Leeuwen Y, Leite MI, Dekker FW, Wintzen AR, Wirtz PW, et al. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. *J Neuroimmunol.* (2008) 195:151–6. doi: 10.1016/j.jneuroim.2008.01.013
69. Varga EM, Kausar F, Aberer W, Zach M, Eber E, Durham SR, et al. Tolerant beekeepers display venom-specific functional IgG4 antibodies in the absence of specific IgE. *J Allergy Clin Immunol.* (2013) 131:1419–21. doi: 10.1016/j.jaci.2012.08.037
70. Garcia-Robaina JC, de la Torre-Morin F, Vazquez-Moncholi C, Fierro J, Bonnet-Moreno C. The natural history of Apis-specific IgG and IgG4 in beekeepers. *Clin Exp Allergy.* (1997) 27:418–23. doi: 10.1111/j.1365-2222.1997.tb00727.x
71. Trampert DC, Hubers LM, van de Graaf SFJ, Beuers U. On the role of IgG4 in inflammatory conditions: lessons for IgG4-related disease. *Biochim Biophys Acta Mol Basis Dis.* (2018) 1864:1401–9. doi: 10.1016/j.bbadis.2017.07.038
72. Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol.* (1998) 160:3555–61.
73. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol.* (2013) 131:1204–12. doi: 10.1016/j.jaci.2013.01.014
74. Meiler F, Klunker S, Zimmermann M, Akdis CA, Akdis M. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. *Allergy.* (2008) 63:1455–63. doi: 10.1111/j.1398-9995.2008.01774.x
75. Pan Q, Guo L, Wu J, Cai J, Liao H, Lan Q, et al. Association between IgG4 autoantibody and complement abnormalities in systemic lupus erythematosus. *Mediators of inflammation.* (2016) 2016:2196986. doi: 10.1155/2016/2196986
76. Duan T, Tradtrantip L, Phuan PW, Bennett JL, Verkman AS. Affinity-matured 'aquaporumab' anti-aquaporin-4 antibody for therapy of seropositive neuromyelitis optica spectrum disorders. *Neuropharmacology.* (2020) 162:107827. doi: 10.1016/j.neuropharm.2019.107827
77. Huijbers MG, Zhang W, Klooster R, Niks EH, Friese MB, Straasheijm KR, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc Natl Acad Sci USA.* (2013) 110:20783–8. doi: 10.1073/pnas.1313944110
78. Futei Y, Amagai M, Ishii K, Kuroda-Kinoshita K, Ohya K, Nishikawa T. Predominant IgG4 subclass in autoantibodies of pemphigus vulgaris and foliaceus. *J Dermatol Sci.* (2001) 26:55–61. doi: 10.1016/s0923-1811(00)00158-4
79. Ng JK, Malotka J, Kawakami N, Derfuss T, Khademi M, Olsson T, et al. Neurofascin as a target for autoantibodies in peripheral neuropathies. *Neurology.* (2012) 79:2241–8. doi: 10.1212/WNL.0b013e31827689ad
80. van der Zee JS, van Swieten P, Aalberse RC. Inhibition of complement activation by IgG4 antibodies. *Clin Exp Immunol.* (1986) 64:415–22.
81. Becerra E, De La Torre I, Leandro MJ, Cambridge G. B cell phenotypes in patients with rheumatoid arthritis relapsing after rituximab: expression of B cell-activating factor-binding receptors on B cell subsets. *Clin Exp Immunol.* (2017) 190:372–83. doi: 10.1111/cei.13024
82. Jefferis R. *Molecular Structure of Human IgG Subclasses. The Human IgG Subclasses*. Oxford: Pergamon Press (1990). p. 15–31. doi: 10.1016/C2009-0-00373-0

83. Ohno K, Otsuka K, Ito M. Roles of collagen Q in MuSK antibody-positive myasthenia gravis. *Chem Biol Interact.* (2016) 259:266–70. doi: 10.1016/j.cbi.2016.04.019
84. Koneczny I, Cossins J, Waters P, Beeson D, Vincent A. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1-3 can disperse preformed agrin-independent AChR clusters. *PLoS One.* (2013) 8:e80695. doi: 10.1371/journal.pone.0080695
85. van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK, Martinez-Martinez P, Vermeulen E, et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science (New York, NY).* (2007) 317:1554–7. doi: 10.1126/science.1144603
86. Labrijn AF, Rispens T, Meesters J, Rose RJ, den Bleker TH, Loverix S, et al. Species-specific determinants in the IgG CH3 domain enable Fab-arm exchange by affecting the noncovalent CH3-CH3 interaction strength. *J Immunol (Baltimore, Md* (1950) 187:3238–46. doi: 10.4049/jimmunol.1003336
87. Yang X, Wang F, Zhang Y, Wang L, Antonenko S, Zhang S, et al. Comprehensive analysis of the therapeutic IgG4 antibody pembrolizumab: hinge modification blocks half molecule exchange in vitro and in vivo. *J Pharmac Sci.* (2015) 104:4002–14. doi: 10.1002/jps.24620
88. Aalberse RC, Schuurman J. IgG4 breaking the rules. *Immunology.* (2002) 105:9–19. doi: 10.1046/j.0019-2805.2001.01341.x
89. Angal S, King DJ, Bodmer MW, Turner A, Lawson AD, Roberts G, et al. A single amino acid substitution abolishes the heterogeneity of chimeric mouse/human (IgG4) antibody. *Mol Immunol.* (1993) 30:105–8. doi: 10.1016/0161-5890(93)90432-b
90. Bloom JW, Madanat MS, Marriott D, Wong T, Chan SY. Intrachain disulfide bond in the core hinge region of human IgG4. *Protein Sci.* (1997) 6:407–15. doi: 10.1002/pro.5560060217
91. Lewis KB, Meengs B, Bondensgaard K, Chin L, Hughes SD, Kjaer B, et al. Comparison of the ability of wild type and stabilized human IgG(4) to undergo Fab arm exchange with endogenous IgG(4)in vitro and in vivo. *Mol Immunol.* (2009) 46:3488–94. doi: 10.1016/j.molimm.2009.07.009
92. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol.* (2014) 5:520. doi: 10.3389/fimmu.2014.00520
93. Rispens T, Ooievaar-De Heer P, Vermeulen E, Schuurman J, van der Neut Kolfschoten M, Aalberse RC. Human IgG4 binds to IgG4 and conformationally altered IgG1 via Fc-Fc interactions. *J Immunol.* (1950) 2009:4275–81. doi: 10.4049/jimmunol.0804338
94. Saphire EO, Stanfield RL, Crispin MD, Parren PW, Rudd PM, Dwek RA, et al. Contrasting IgG structures reveal extreme asymmetry and flexibility. *J Mol Biol.* (2002) 319:9–18. doi: 10.1016/s0022-2836(02)00244-9
95. Rispens T, Leeuwen A, Vennegoor A, Killestein J, Aalberse RC, Wolbink GJ, et al. Measurement of serum levels of natalizumab, an immunoglobulin G4 therapeutic monoclonal antibody. *Anal Biochem.* (2011) 411:271–6. doi: 10.1016/j.ab.2011.01.001
96. Chacko S, Silverton E, Kam-Morgan L, Smith-Gill S, Cohen G, Davies D. Structure of an antibody-lysozyme complex unexpected effect of conservative mutation. *J Mol Biol.* (1995) 245:261–74. doi: 10.1006/jmbi.1994.0022
97. Teillet F, Lacroix M, Thiel S, Weilguny D, Agger T, Arlaud GJ, et al. Identification of the site of human mannan-binding lectin involved in the interaction with its partner serine proteases: the essential role of Lys55. *J Immunol.* (1950) 2007:5710–6. doi: 10.4049/jimmunol.178.9.5710
98. Rose RJ, Labrijn AF, van den Bremer ET, Loverix S, Lasters I, van Berkel PH, et al. Quantitative analysis of the interaction strength and dynamics of human IgG4 half molecules by native mass spectrometry. *Structure (London, England).* (1993) 2011:1274–82. doi: 10.1016/j.str.2011.06.016
99. Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy.* (2009) 39:469–77. doi: 10.1111/j.1365-2222.2009.03207.x
100. Schuurman J, Van Ree R, Perdok GJ, Van Doorn HR, Tan KY, Aalberse RC. Normal human immunoglobulin G4 is bispecific: it has two different antigen-combining sites. *Immunology.* (1999) 97:693–8. doi: 10.1046/j.1365-2567.1999.00845.x
101. King DJ, Adair JR, Angal S, Low DC, Proudfoot KA, Lloyd JC, et al. Expression, purification and characterization of a mouse-human chimeric antibody and chimeric Fab' fragment. *Biochem J.* (1992) 281(Pt 2):317–23. doi: 10.1042/bj2810317
102. Rispens T, Ooijevaar-de Heer P, Bende O, Aalberse RC. Mechanism of immunoglobulin G4 Fab-arm exchange. *J Am Chem Soc.* (2011) 133:10302–11. doi: 10.1021/ja203638y
103. Silva JP, Vetterlein O, Jose J, Peters S, Kirby H. The S228P mutation prevents in vivo and in vitro IgG4 Fab-arm exchange as demonstrated using a combination of novel quantitative immunoassays and physiological matrix preparation. *J Biol Chem.* (2015) 290:5462–9. doi: 10.1074/jbc.M114.600973
104. Eyre RW, Stanley JR. Human autoantibodies against a desmosomal protein complex with a calcium-sensitive epitope are characteristic of pemphigus foliaceus patients. *J Exp Med.* (1987) 165:1719–24. doi: 10.1084/jem.165.6.1719
105. Jones JC, Yokoo KM, Goldman RD. Further analysis of pemphigus autoantibodies and their use in studies on the heterogeneity, structure, and function of desmosomes. *J Cell Biol.* (1986) 102:1109–17. doi: 10.1083/jcb.102.3.1109
106. Stanley JR. Pemphigus. Skin failure mediated by autoantibodies. *JAMA.* (1990) 264:1714–7. doi: 10.1001/jama.264.13.1714
107. Stanley JR. Pemphigus and pemphigoid as paradigms of organ-specific, autoantibody-mediated diseases. *J Clin Investigat.* (1989) 83:1443–8. doi: 10.1172/jci114036
108. Stanley JR, Koulu L, Klaus-Kovtun V, Steinberg MS. A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol.* (1950) 1986:1227–30.
109. Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell.* (1991) 67:869–77. doi: 10.1016/0092-8674(91)90360-b
110. Rubinstein N, Stanley JR. Pemphigus foliaceus antibodies and a monoclonal antibody to desmoglein I demonstrate stratified squamous epithelial-specific epitopes of desmosomes. *Am J Dermatopathol.* (1987) 9:510–4. doi: 10.1097/00000372-198712000-00007
111. Sauvages FBD. *Nosologia Methodica Sistens Morborum Classes*. Amsterdam: Fratres de Tournes (1768).
112. Rizzuto N, Simonati A. Chronic inflammatory demyelinating polyneuropathy. *Int J Tissue React.* (1985) 7:521–6.
113. Querol L, Nogales-Gadea G, Rojas-Garcia R, Martinez-Hernandez E, Diaz-Manera J, Suarez-Calvet X, et al. Antibodies to contactin-1 in chronic inflammatory demyelinating polyneuropathy. *Ann Neurol.* (2013) 73:370–80. doi: 10.1002/ana.23794
114. Labrijn AF, Buijsse AO, van den Bremer ET, Verwilligen AY, Bleeker WK, Thorpe SJ, et al. Therapeutic IgG4 antibodies engage in Fab-arm exchange with endogenous human IgG4 in vivo. *Nat Biotechnol.* (2009) 27:767–71. doi: 10.1038/nbt.1553
115. Cole RN, Reddel SW, Gervasio OL, Phillips WD. Anti-MuSK patient antibodies disrupt the mouse neuromuscular junction. *Ann Neurol.* (2008) 63:782–9. doi: 10.1002/ana.21371
116. Ter Beek WP, Martinez-Martinez P, Losen M, de Baets MH, Wintzen AR, Verschuuren JJ, et al. The effect of plasma from muscle-specific tyrosine kinase myasthenia patients on regenerating endplates. *Am J Pathol.* (2009) 175:1536–44. doi: 10.2353/ajpath.2009.090040
117. Koneczny I, Stevens JA, De Rosa A, Huda S, Huijbers MG, Saxena A, et al. IgG4 autoantibodies against muscle-specific kinase undergo Fab-arm exchange in myasthenia gravis patients. *J Autoimmun.* (2017) 77:104–15. doi: 10.1016/j.jaut.2016.11.005
118. Fichtner ML, Vieni C, Redler RL, Kolich L, Jiang R, Takata K, et al. Self-antigen driven affinity maturation is required for pathogenic monovalent IgG4 autoantibody development. *bioRxiv.* (2020) [Preprint]. doi: 10.1101/2020.03.14.988758
119. Rock B, Labib RS, Diaz LA. Monovalent Fab' immunoglobulin fragments from endemic pemphigus foliaceus autoantibodies reproduce the human disease in neonatal Balb/c mice. *J Clin Invest.* (1990) 85:296–9. doi: 10.1172/jci114426
120. Payne AS, Ishii K, Kacir S, Lin C, Li H, Hanakawa Y, et al. Genetic and functional characterization of human pemphigus vulgaris monoclonal autoantibodies isolated by phage display. *J Clin Invest.* (2005) 115:888–99. doi: 10.1172/JCI24185

121. Ishii K, Lin C, Siegel DL, Stanley JR. Isolation of pathogenic monoclonal anti-desmoglein 1 human antibodies by phage display of pemphigus foliaceus autoantibodies. *J Invest Dermatol.* (2008) 128:939–48. doi: 10.1038/sj.jid.5701132
122. Yamagami J, Kacir S, Ishii K, Payne AS, Siegel DL, Stanley JR. Antibodies to the desmoglein 1 precursor proprotein but not to the mature cell surface protein cloned from individuals without pemphigus. *J Immunol.* (2009) 183:5615–21. doi: 10.4049/jimmunol.0901691
123. Ran NA, Payne AS. Rituximab therapy in pemphigus and other autoantibody-mediated diseases. *F1000Res.* (2017) 6:83. doi: 10.12688/f1000research.9476.1
124. Querol L, Rojas-Garcia R, Diaz-Manera J, Barcena J, Pardo J, Ortega-Moreno A, et al. Rituximab in treatment-resistant CIDP with antibodies against paranodal proteins. *Neurol Neuroimmunol Neuroinflam.* (2015) 2:e149. doi: 10.1212/nxi.0000000000000149
125. Diaz-Manera J, Martinez-Hernandez E, Querol L, Klooster R, Rojas-Garcia R, Suarez-Calvet X, et al. Long-lasting treatment effect of rituximab in MuSK myasthenia. *Neurology.* (2012) 78:189–93. doi: 10.1212/WNL.0b013e3182407982
126. Colliou N, Picard D, Caillot F, Calbo S, Le Corre S, Lim A, et al. Long-term remissions of severe pemphigus after rituximab therapy are associated with prolonged failure of desmoglein B cell response. *Sci Transl Med.* (2013) 5:175ra130. doi: 10.1126/scitranslmed.3005166
127. Pevzner A, Schoser B, Peters K, Cosma NC, Karakatsani A, Schalke B, et al. Anti-LRP4 autoantibodies in AChR- and MuSK-antibody-negative myasthenia gravis. *J Neurol.* (2012) 259:427–35. doi: 10.1007/s00415-011-6194-7
128. Zhang B, Tzartos JS, Belimezi M, Ragheb S, Bealmear B, Lewis RA, et al. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. *Arch Neurol.* (2012) 69:445–51. doi: 10.1001/archneurol.2011.2393
129. Stathopoulos P, Chastre A, Waters P, Irani S, Fichtner ML, Benotti ES, et al. Autoantibodies against neurologic antigens in nonneurologic autoimmunity. *J Immunol.* (2019) 202:2210–19. doi: 10.4049/jimmunol.1801295
130. Takahashi H, Noto YI, Makita N, Kushimura-Okada Y, Ishii R, Tanaka A, et al. Myasthenic symptoms in anti-low-density lipoprotein receptor-related protein 4 antibody-seropositive amyotrophic lateral sclerosis: two case reports. *BMC Neurol.* (2016) 16:229. doi: 10.1186/s12883-016-0758-1
131. Tzartos JS, Zisimopoulou P, Rentzos M, Karandreas N, Zouvelou V, Evangelakou P, et al. LRP4 antibodies in serum and CSF from amyotrophic lateral sclerosis patients. *Ann Clin Transl Neurol.* (2014) 1:80–7. doi: 10.1002/acn3.26
132. Koneczny I, Rennspiess D, Marcuse F, Dankerlui N, Abdul Hamid M, Mane-Damas M, et al. Characterization of the thymus in Lrp4 myasthenia gravis: Four cases. *Autoimmun Rev.* (2019) 18:50–5. doi: 10.1016/j.autrev.2018.07.011
133. Lazaridis K, Tzartos SJ. Autoantibody specificities in myasthenia gravis; implications for improved diagnostics and therapeutics. *Front Immunol.* (2020) 11:212–212. doi: 10.3389/fimmu.2020.00212
134. Romi F, Suzuki S, Suzuki N, Petzold A, Plant GT, Gilhus NE. Anti-voltage-gated potassium channel Kv1.4 antibodies in myasthenia gravis. *J Neurol.* (2012) 259:1312–6. doi: 10.1007/s00415-011-6344-y
135. Gasperi C, Melms A, Schoser B, Zhang Y, Meltoranta J, Risson V, et al. Anti-agrin autoantibodies in myasthenia gravis. *Neurology.* (2014) 82:1976–83. doi: 10.1212/WNL.0000000000000478
136. Gallardo E, Martinez-Hernandez E, Titulaer MJ, Huijbers MG, Martinez MA, Ramos A, et al. Cortactin autoantibodies in myasthenia gravis. *Autoimmun Rev.* (2014) 13:1003–7. doi: 10.1016/j.autrev.2014.08.039
137. Zoltowska Katarzyna M, Belaya K, Leite M, Patrick W, Vincent A, Beeson D. Collagen Q–a potential target for autoantibodies in myasthenia gravis. *J Neurol Sci.* (2015) 348:241–4. doi: 10.1016/j.jns.2014.12.015
138. Zhang B, Shen C, Bealmear B, Ragheb S, Xiong WC, Lewis RA, et al. Autoantibodies to agrin in myasthenia gravis patients. *PLoS One.* (2014) 9:e91816. doi: 10.1371/journal.pone.0091816
139. Skeie GO, Aarli JA, Gilhus NE. Titin and ryanodine receptor antibodies in myasthenia gravis. *Acta Neurol Scand Suppl.* (2006) 183:19–23. doi: 10.1111/j.1600-0404.2006.00608.x
140. Aarli JA, Stefansson K, Marton LS, Wollmann RL. Patients with myasthenia gravis and thymoma have in their sera IgG autoantibodies against titin. *Clin Exp Immunol.* (1990) 82:284–8. doi: 10.1111/j.1365-2249.1990.tb05440.x
141. Mygland A, Tysnes OB, Matre R, Volpe P, Aarli JA, Gilhus NE. Ryanodine receptor autoantibodies in myasthenia gravis patients with a thymoma. *Ann Neurol.* (1992) 32:589–91. doi: 10.1002/ana.410320419
142. Williams CL, Lennon VA. Thymic B lymphocyte clones from patients with myasthenia gravis secrete monoclonal striational autoantibodies reacting with myosin, alpha actinin, or actin. *J Exp Med.* (1986) 164:1043–59. doi: 10.1084/jem.164.4.1043
143. Deymeer F, Gungor-Tuncer O, Yilmaz V, Parman Y, Serdaroglu P, Ozdemir C, et al. Clinical comparison of anti-MuSK- vs anti-AChR-positive and seronegative myasthenia gravis. *Neurology.* (2007) 68:609–11. doi: 10.1212/01.wnl.0000254620.45529.97
144. Evoli A, Tonali PA, Padua L, Monaco ML, Scuderi F, Batocchi AP, et al. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. *Brain.* (2003) 126:2304–11. doi: 10.1093/brain/awg223
145. Yuan HK, Huang BS, Kung SY, Kao KP. The effectiveness of thymectomy on seronegative generalized myasthenia gravis: comparing with seropositive cases. *Acta Neurol Scand.* (2007) 115:181–4. doi: 10.1111/j.1600-0404.2006.00733.x
146. Guillermo GR, Téllez-Zenteno JF, Weder-Cisneros N, Mimenza A, Estañol B, Remes-Troche JM, et al. Response of thymectomy: clinical and pathological characteristics among seronegative and seropositive myasthenia gravis patients. *Acta Neurol Scand.* (2004) 109:217–21. doi: 10.1034/j.1600-0404.2003.00209.x
147. Soliven BC, Lange DJ, Penn AS, Younger D, Jaretzki A III, Lovelace RE, et al. Seronegative myasthenia gravis. *Neurology.* (1988) 38:514–7. doi: 10.1212/wnl.38.4.514
148. Romi F, Aarli JA, Gilhus NE. Seronegative myasthenia gravis: disease severity and prognosis. *Eur J Neurol.* (2005) 12:413–8. doi: 10.1111/j.1468-1331.2005.01137.x
149. Sanders DB, Wolfe GI, Narayanaswami P. Developing treatment guidelines for myasthenia gravis. *Ann N Y Acad Sci.* (2018) 1412:95–101. doi: 10.1111/nyas.13537
150. Mehndiratta MM, Pandey S, Kuntzer T. Acetylcholinesterase inhibitor treatment for myasthenia gravis. *Cochr Datab Syst Rev.* (2014) Cd006986. doi: 10.1002/14651858.CD006986.pub3
151. Walker MB. Treatment of myasthenia gravis with physostigmine. *Lancet* (London, England). (1934) 2:1198–9.
152. Engel AG, Lambert EH, Santa T. Study of long-term anticholinesterase therapy. Effects on neuromuscular transmission and on motor end-plate fine structure. *Neurology.* (1973) 23:1273–81. doi: 10.1212/wnl.23.12.1273
153. Gold R, Hohlfeld R, Toyka KV. Progress in the treatment of myasthenia gravis. *Ther Adv Neurol Dis.* (2008) 1:36–51. doi: 10.1177/1756285608093888
154. Blalock A, Mason MF, Morgan HJ, Riven SS. Myasthenia gravis and tumors of the thymic region: report of a case in which the tumor was removed. *Ann Surg.* (1939) 110:544–61. doi: 10.1097/00000658-193910000-00005
155. Eaton LM, Clagett OT, et al. Thymectomy in treatment of myasthenia gravis; report based on 32 cases. *Arch Neurol Psychiatry.* (1949) 61:467–98. doi: 10.1001/archneurpsyc.1949.02310110002001
156. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo HC, Marx A, et al. Randomized trial of thymectomy in myasthenia gravis. *N Engl J Med.* (2016) 375:511–22. doi: 10.1056/NEJMoa1602489
157. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo HC, Marx A, et al. Long-term effect of thymectomy plus prednisone versus prednisone alone in patients with non-thymomatous myasthenia gravis: 2-year extension of the MGTX randomised trial. *Lancet Neurol.* (2019) 18:259–68. doi: 10.1016/S1474-4422(18)30392-2
158. Cron MA, Maillard S, Villegas J, Truffault F, Sudres M, Dragin N, et al. Thymus involvement in early-onset myasthenia gravis. *Ann N Y Acad Sci.* (2018) 1412:137–45. doi: 10.1111/nyas.13519
159. Berrih-Aknin S, Morel E, Raimond F, Safar D, Gaud C, Binet JP, et al. The role of the thymus in myasthenia gravis: immunohistological and immunological studies in 115 cases. *Ann N Y Acad Sci.* (1987) 505:50–70. doi: 10.1111/j.1749-6632.1987.tb51282.x

160. Vincent A, Newsom-Davis J, Newton P, Beck N. Acetylcholine receptor antibody and clinical response to thymectomy in myasthenia gravis. *Neurology.* (1983) 33:1276–82. doi: 10.1212/WNL.33.10.1276
161. Okumura M, Ohta M, Takeuchi Y, Shiono H, Inoue M, Fukuhara K, et al. The immunologic role of thymectomy in the treatment of myasthenia gravis: implication of thymus-associated B-lymphocyte subset in reduction of the anti-acetylcholine receptor antibody titer. *J Thorac Cardiovasc Surg.* (2003) 126:1922–8. doi: 10.1016/S0022
162. Kim H, Lim YM, Lee EJ, Oh YJ, Kim KK. Factors predicting remission in thymectomized patients with acetylcholine receptor antibody-positive myasthenia gravis. *Muscle Nerve.* (2018) 58:796–800. doi: 10.1002/mus.26300
163. Kaufman AJ, Palatt J, Sivak M, Raimondi P, Lee D-S, Wolf A, et al. Thymectomy for myasthenia gravis : complete stable remission and associated prognostic factors in over 1000 Cases. *Sem Thor Cardiovas Surgery.* (2016) 28:561–8. doi: 10.1053/j.semtcvs.2016.04.002
164. Yu S, Li F, Chen B, Lin J, Yang M, Fu X, et al. Eight-year follow-up of patients with myasthenia gravis after thymectomy. *Acta Neurol Scand.* (2015) 131:94–101. doi: 10.1111/ane.12289
165. Masaoka A, Monden Y, Seike Y, Tanioka T, Kagotani K. Reoperation after transcervical thymectomy for myasthenia gravis. *Neurology.* (1982) 32:83–5. doi: 10.1212/wnl.32.1.83
166. Miller RG, Filler-Katz A, Kiprov D, Roan R. Repeat thymectomy in chronic refractory myasthenia gravis. *Neurology.* (1991) 41:923–4. doi: 10.1212/wnl.41.6.923
167. Robeson KR, Kumar A, Keung B, DiCapua DB, Grodinsky E, Patwa HS, et al. Durability of the rituximab response in acetylcholine receptor autoantibody-positive myasthenia gravis. *JAMA Neurol.* (2016) 74:60–6. doi: 10.1001/jamaneurol.2016.4190
168. Cree BAC, Bennett JL, Kim HJ, Weinshenker BG, Pittock SJ, Wingerchuk DM, et al. Inebilizumab for the treatment of neuromyelitis optica spectrum disorder (N-MOmentum): a double-blind, randomised placebo-controlled phase 2/3 trial. *Lancet (London, England).* (2019) 394:1352–63. doi: 10.1016/S0140-6736(19)31817-3
169. Gradolatto A, Nazzal D, Truffault F, Bismuth J, Fadel E, Foti M, et al. Both Treg cells and Tconv cells are defective in the Myasthenia gravis thymus: roles of IL-17 and TNF-alpha. *J Autoimmun.* (2014) 52:53–63. doi: 10.1016/j.jaut.2013.12.015
170. Pompeo E, Tacconi F, Massa R, Mineo D, Nahmias S, Mineo TC. Long-term outcome of thoracoscopic extended thymectomy for nonthymomatous myasthenia gravis. *Eur J Cardio Thor surgery.* (2009) 36:164–9. doi: 10.1016/j.ejcts.2009.02.021
171. Clifford KM, Hobson-Webb LD, Benatar M, Burns TM, Barnett C, Silvestri NJ, et al. Thymectomy may not be associated with clinical improvement in MuSK myasthenia gravis. *Muscle Nerve.* (2019) 59:404–10. doi: 10.1002/mus.26404
172. Krumbholz M, Derfuss T, Hohlfeld R, Meinl E. B cells and antibodies in multiple sclerosis pathogenesis and therapy. *Nat Rev Neurol.* (2012) 8:613–23. doi: 10.1038/nrneurol.2012.203
173. Gajra A, Vajpayee N, Grethlein SJ. Response of myasthenia gravis to rituximab in a patient with non-Hodgkin lymphoma. *Am J Hematol.* (2004) 77:196–7. doi: 10.1002/ajh.20169
174. Nowak RJ, DiCapua DB, Zebardast N, Goldstein JM. Response of patients with refractory myasthenia gravis to rituximab: a retrospective study. *Ther Adv Neurol Dis.* (2011) 4:259–66. doi: 10.1177/1756285611411503
175. Keung B, Robeson KR, DiCapua DB, Rosen JB, O'Connor KC, Goldstein JM, et al. Long-term benefit of rituximab in MuSK autoantibody myasthenia gravis patients. *J Neurol Neurosurg Psychiatry.* (2013) 84:1407–9. doi: 10.1136/jnnp-2012-303664
176. Cortes-Vicente E, Rojas-Garcia R, Diaz-Manera J, Querol L, Casasnovas C, Guerrero-Sola A, et al. The impact of rituximab infusion protocol on the long-term outcome in anti-MuSK myasthenia gravis. *Ann Clin Transl Neurol.* (2018) 5:710–6. doi: 10.1002/acn3.564
177. Besinger UA, Toyka KV, Homberg M, Heininger K, Hohlfeld R, Fateh-Moghadam A. Myasthenia gravis: long-term correlation of binding and bungarotoxin blocking antibodies against acetylcholine receptors with changes in disease severity. *Neurology.* (1983) 33:1316–21. doi: 10.1212/wnl.33.10.1316
178. Nowak RJ, Coffey C, Goldstein JM, Dimachkie MM, Benatar M, Huq SN, et al. B cell targeted treatment in myasthenia gravis A phase 2 trial of rituximab in MG: topline results. *Papper Presended at the 70th American Academy of Neurology Annual Meeting.* Los Angeles, CA (2018).
179. Mamani-Matsuda M, Cosma A, Weller S, Faili A, Staib C, Garcon L, et al. The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells. *Blood.* (2008) 111:4653–9. doi: 10.1182/blood-2007-11-123844
180. Leandro MJ, Cambridge G, Ehrenstein MR, Edwards JC. Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis. *Arthr Rheumatism.* (2006) 54:613–20. doi: 10.1002/art.21617
181. Anolik JH, Friedberg JW, Zheng B, Barnard J, Owen T, Cushing E, et al. B cell reconstitution after rituximab treatment of lymphoma recapitulates B cell ontogeny. *Clin Immunol (Orlando, Fla).* (2007) 122:139–45. doi: 10.1016/j.clim.2006.08.009
182. Ramwadhdoebe TH, van Baarsen LGM, Boumans MJH, Bruijnen STG, Safy M, Berger FH, et al. Effect of rituximab treatment on T and B cell subsets in lymph node biopsies of patients with rheumatoid arthritis. *Rheumatology (Oxford, England).* (2019) 58:1075–85. doi: 10.1093/rheumatology/key428
183. Cianchini G, Corona R, Frezzolini A, Ruffelli M, Didona B, Puddu P. Treatment of severe pemphigus with rituximab: report of 12 cases and a review of the literature. *Arch Dermatol.* (2007) 143:1033–8. doi: 10.1001/archderm.143.8.1033
184. Quach TD, Rodriguez-Zhurbenko N, Hopkins TJ, Guo X, Hernandez AM, Li W, et al. Distinctions among circulating antibody-secreting cell populations, including B-1 cells, in human adult peripheral blood. *J Immunol.* (2016) 196:1060–9. doi: 10.4049/jimmunol.1501843
185. Jiang R, Fichtner ML, Hoehn KB, Stathopoulos P, Nowak RJ, Kleinstein SH, et al. Single-cell immune repertoire tracing identifies rituximab refractory B cells that emerge during relapse. *bioRxiv.* (2019) [Preprint]. doi: 10.1101/840389
186. Marino M, Basile U, Spagni G, Napodano C, Iorio R, Gulli F, et al. Long lasting rituximab-induced reduction of specific - but not of total- IgG4 in MuSK-positive myasthenia gravis. *Front Immunol.* (2020) doi: 10.3389/fimmu.2020.00613
187. Forsthuber TG, Cimbora DM, Ratchford JN, Katz E, Stuve O. B cell-based therapies in CNS autoimmunity: differentiating CD19 and CD20 as therapeutic targets. *Ther Adv Neurol Dis.* (2018) 11:1756286418761697. doi: 10.1177/1756286418761697
188. Chen D, Blazek M, Ireland S, Ortega S, Kong X, Meeuwissen A, et al. Single dose of glycoengineered anti-CD19 antibody (MEDI551) disrupts experimental autoimmune encephalomyelitis by inhibiting pathogenic adaptive immune responses in the bone marrow and spinal cord while preserving peripheral regulatory mechanisms. *J Immunol.* (1950) 2014:4823–32. doi: 10.4049/jimmunol.1401478
189. Chen D, Ireland SJ, Davis LS, Kong X, Stowe AM, Wang Y, et al. Autoreactive CD19+CD20- plasma cells contribute to disease severity of experimental autoimmune encephalomyelitis. *J Immunol.* (1950) 2016:1541–9. doi: 10.4049/jimmunol.1501376
190. Agius MA, Klodowska-Duda G, Maciejowski M, Potemkowski A, Li J, Patra K, et al. Safety and tolerability of inebilizumab (MEDI-551), an anti-CD19 monoclonal antibody, in patients with relapsing forms of multiple sclerosis: Results from a phase 1 randomised, placebo-controlled, escalating intravenous and subcutaneous dose study. *Mul Scl (Houndmills Basingstoke England).* (2019) 25:235–45. doi: 10.1177/1352458517740641
191. Schiopu E, Chatterjee S, Hsu V, Flor A, Cimbora D, Patra K, et al. Safety and tolerability of an anti-CD19 monoclonal antibody, MEDI-551, in subjects with systemic sclerosis: a phase I, randomized, placebo-controlled, escalating single-dose study. *Arthr Res Ther.* (2016) 18:131. doi: 10.1186/s13075-016-1021-2
192. Cree BA, Bennett JL, Sheehan M, Cohen J, Hartung HP, Aktas O, et al. Placebo-controlled study in neuromyelitis optica-Ethical and design considerations. *Mul Scl (Houndmills Basingstoke England).* (2016) 22:862–72. doi: 10.1177/1352458515620934
193. Alexander T, Sarfert R, Klotsche J, Kuhl AA, Rubbert-Roth A, Lorenz HM, et al. The proteasome inhibitior bortezomib depletes

plasma cells and ameliorates clinical manifestations of refractory systemic lupus erythematosus. *Ann Rheum Dis.* (2015) 74:1474–8. doi: 10.1136/annrheumdis-2014-206016
194. Gomez AM, Willcox N, Vrolix K, Hummel J, Nogales-Gadea G, Saxena A, et al. Proteasome inhibition with bortezomib depletes plasma cells and specific autoantibody production in primary thymic cell cultures from early-onset myasthenia gravis patients. *J Immunol.* (2014) 193:1055–63. doi: 10.4049/jimmunol.1301555
195. Teicher BA, Ara G, Herbst R, Palombella VJ, Adams J. The proteasome inhibitor PS-341 in cancer therapy. *Clin Cancer Res.* (1999) 5: 2638–45.
196. Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res.* (2001) 61:3071–6.
197. Ratnasingam S, Walker PA, Tran H, Kaplan ZS, McFadyen JD, Tran H, et al. Bortezomib-based antibody depletion for refractory autoimmune hematological diseases. *Blood Adv.* (2016) 1:31–5. doi: 10.1182/bloodadvances.2016001412
198. Gomez AM, Vrolix K, Martinez-Martinez P, Molenaar PC, Phernambucq M, van der Esch E, et al. Proteasome inhibition with bortezomib depletes plasma cells and autoantibodies in experimental autoimmune myasthenia gravis. *J Immunol.* (2011) 186:2503–13. doi: 10.4049/jimmunol.1002539
199. Liu RT, Zhang P, Yang CL, Pang Y, Zhang M, Zhang N, et al. ONX-0914, a selective inhibitor of immunoproteasome, ameliorates experimental autoimmune myasthenia gravis by modulating humoral response. *J Neuroimmunol.* (2017) 311:71–8. doi: 10.1016/j.jneuroim.2017.08.005
200. Kohler S, Marschenz S, Grittner U, Alexander T, Hiepe F, Meisel A. Bortezomib in antibody-mediated autoimmune diseases (TAVAB): study protocol for a unicentric, non-randomised, non-placebo controlled trial. *BMJ Open.* (2019) 9:e024523. doi: 10.1136/bmjopen-2018-024523
201. Samy E, Wax S, Huard B, Hess H, Schneider P. Targeting BAFF and APRIL in systemic lupus erythematosus and other antibody-associated diseases. *Int Rev Immunol.* (2017) 36:3–19. doi: 10.1080/08830185.2016.1276903
202. Ehrenstein MR, Wing C. The BAFFling effects of rituximab in lupus: danger ahead? *Nat Rev Rheumatol.* (2016) 12:367–72. doi: 10.1038/nrrheum.2016.18
203. Mackay F, Schneider P. Cracking the BAFF code. *Nat Rev Immunol.* (2009) 9:491–502. doi: 10.1038/nri2572
204. Kyrtsonis MC, Sarris K, Koulieris E, Maltezas D, Nikolaou E, Angelopoulou MK, et al. Serum soluble TACI, a BLyS receptor, is a powerful prognostic marker of outcome in chronic lymphocytic leukemia. *Biomed Res Int.* (2014) 2014:159632. doi: 10.1155/2014/159632
205. Thaler FS, Laurent SA, Huber M, Mulazzani M, Dreyling M, Kodel U, et al. Soluble TACI and soluble BCMA as biomarkers in primary central nervous system lymphoma. *Neuro Oncol.* (2017) 19:1618–27. doi: 10.1093/neuonc/nox097
206. Mulazzani M, Huber M, Borchard S, Langer S, Angele B, Schuh E, et al. APRIL and BAFF: novel biomarkers for central nervous system lymphoma. *J Hematol Oncol.* (2019) 12:102. doi: 10.1186/s13045-019-0796-4
207. Steri M, Orru V, Idda ML, Pitzalis M, Pala M, Zara I, et al. Overexpression of the cytokine BAFF and autoimmunity risk. *New Engl J Med.* (2017) 376:1615–26. doi: 10.1056/NEJMoa1610528
208. Stohl W, Metyas S, Tan SM, Cheema GS, Oamar B, Xu D, et al. B lymphocyte stimulator overexpression in patients with systemic lupus erythematosus: longitudinal observations. *Arthr Rheum.* (2003) 48:3475–86. doi: 10.1002/art.11354
209. Manzi S, Sanchez-Guerrero J, Merrill JT, Furie R, Gladman D, Navarra SV, et al. Effects of belimumab, a B lymphocyte stimulator-specific inhibitor, on disease activity across multiple organ domains in patients with systemic lupus erythematosus: combined results from two phase III trials. *Ann Rheum Dis.* (2012) 71:1833–8. doi: 10.1136/annrheumdis-2011-200831
210. Guptill JT, Yi JS, Sanders DB, Guidon AC, Juel VC, Massey JM, et al. Characterization of B cells in muscle-specific kinase antibody myasthenia gravis. *Neurol Neuroimmunol Neuroinflam.* (2015) 2:e77. doi: 10.1212/nxi.0000000000000077
211. Kim JY, Yang Y, Moon JS, Lee EY, So SH, Lee HS, et al. Serum BAFF expression in patients with myasthenia gravis. *J Neuroimmunol.* (2008) 199:151–4. doi: 10.1016/j.jneuroim.2008.05.010
212. Kang SY, Kang CH, Lee KH. B-cell-activating factor is elevated in serum of patients with myasthenia gravis. *Muscle Nerve.* (2016) 54:1030–3. doi: 10.1002/mus.25162
213. Hewett K, Sanders DB, Grove RA, Broderick CL, Rudo TJ, Bassiri A, et al. Randomized study of adjunctive belimumab in participants with generalized myasthenia gravis. *Neurology.* (2018) 90:e1425–34. doi: 10.1212/WNL.0000000000005323
214. Pers JO, Devauchelle V, Daridon C, Bendaoud B, Le Berre R, Bordron A, et al. BAFF-modulated repopulation of B lymphocytes in the blood and salivary glands of rituximab-treated patients with Sjogren's syndrome. *Arthr Rheum.* (2007) 56:1464–77. doi: 10.1002/art.22603
215. Sarantopoulos S, Stevenson KE, Kim HT, Washel WS, Bhuiya NS, Cutler CS, et al. Recovery of B-cell homeostasis after rituximab in chronic graft-versus-host disease. *Blood.* (2011) 117:2275–83. doi: 10.1182/blood-2010-10-307819
216. Isenberg D, Gordon C, Licu D, Copt S, Rossi CP, Wofsy D. Efficacy and safety of atacicept for prevention of flares in patients with moderate-to-severe systemic lupus erythematosus (SLE): 52-week data (APRIL-SLE randomised trial). *Ann Rheum Dis.* (2015) 74:2006–15. doi: 10.1136/annrheumdis-2013-205067
217. Merrill JT, Wallace DJ, Wax S, Kao A, Fraser PA, Chang P, et al. Efficacy and safety of atacicept in patients with systemic lupus erythematosus: results of a twenty-four-week, multicenter, randomized, double-blind, placebo-controlled, parallel-arm, phase IIb study. *Arthr Rheumatol.* (2018) 70:266–76. doi: 10.1002/art.40360
218. Bracewell C, Isaacs JD, Emery P, Ng WF. Atacicept, a novel B cell-targeting biological therapy for the treatment of rheumatoid arthritis. *Exp Opin Biol Therapy.* (2009) 9:909–19. doi: 10.1517/14712590903033919
219. Hartung HP, Kieseier BC. Atacicept: targeting B cells in multiple sclerosis. *Ther Adv Neurol Disord.* (2010) 3:205–16. doi: 10.1177/1756285610371146
220. Pont MJ, Hill T, Cole GO, Abbott JJ, Kelliher J, Salter AI, et al. gamma-Secretase inhibition increases efficacy of BCMA-specific chimeric antigen receptor T cells in multiple myeloma. *Blood.* (2019) 134:1585–97. doi: 10.1182/blood.2019000050
221. Kaplan M. Eculizumab (Alexion). *Curr Opin Investigat Drugs (London, England).* (2000) 2002:1017–23.
222. Hill A, Hillmen P, Richards SJ, Elebute D, Marsh JC, Chan J, et al. Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria. *Blood.* (2005) 106:2559–65. doi: 10.1182/blood-2005-02-0564
223. Hillmen P, Hall C, Marsh JC, Elebute M, Bombara MP, Petro BE, et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *New Engl J Med.* (2004) 350:552–9. doi: 10.1056/NEJMoa031688
224. Fakhouri F, Hourmant M, Campistol JM, Cataland SR, Espinosa M, Gaber AO, et al. Terminal complement inhibitor eculizumab in adult patients with atypical hemolytic uremic syndrome: a single-arm, open-label trial. *Am J Kidney Dis.* (2016) 68:84–93. doi: 10.1053/j.ajkd.2015.12.034
225. Menne J, Delmas Y, Fakhouri F, Licht C, Lommele A, Minetti EE, et al. Outcomes in patients with atypical hemolytic uremic syndrome treated with eculizumab in a long-term observational study. *BMC Nephrol.* (2019) 20:125. doi: 10.1186/s12882-019-1314-1
226. Legendre CM, Licht C, Muus P, Greenbaum LA, Babu S, Bedrosian C, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *New Engl J Med.* (2013) 368:2169–81. doi: 10.1056/NEJMoa1208981
227. Pittock SJ, Lennon VA, McKeon A, Mandrekar J, Weinshenker BG, Lucchinetti CF, et al. Eculizumab in AQP4-IgG-positive relapsing neuromyelitis optica spectrum disorders: an open-label pilot study. *Lancet Neurol.* (2013) 12:554–62. doi: 10.1016/s1474-4422(13)70076-0
228. Pittock SJ, Berthele A, Fujihara K, Kim HJ, Levy M, Palace J, et al. Eculizumab in aquaporin-4-positive neuromyelitis optica spectrum disorder. *New Engl J Med.* (2019) 381:614–25. doi: 10.1056/NEJMoa1900866
229. Howard JF Jr., Barohn RJ, Cutter GR, Freimer M, Juel VC, Mozaffar T, et al. A randomized, double-blind, placebo-controlled phase II study of eculizumab in patients with refractory generalized myasthenia gravis. *Muscle Nerve.* (2013) 48:76–84. doi: 10.1002/mus.23839

230. Howard JF Jr., Freimer M, O'Brien F, Wang JJ, Collins SR, Kissel JT, et al. QMG and MG-ADL correlations: Study of eculizumab treatment of myasthenia gravis. *Muscle Nerve.* (2017) 56:328–30. doi: 10.1002/mus.25529
231. Andersen H, Mantegazza R, Wang JJ, O'Brien F, Patra K, Howard JF Jr. Eculizumab improves fatigue in refractory generalized myasthenia gravis. *Qual Life Res.* (2019) 28: 2247–54. doi: 10.1007/s11136-019-02148-2
232. Howard JF Jr., Utsugisawa K, Benatar M, Murai H, Barohn RJ, Illa I, et al. Safety and efficacy of eculizumab in anti-acetylcholine receptor antibody-positive refractory generalised myasthenia gravis (REGAIN): a phase 3, randomised, double-blind, placebo-controlled, multicentre study. *Lancet Neurol.* (2017) 16:976–86. doi: 10.1016/S1474-4422(17)30369-1
233. Beecher G, Putko BN, Wagner AN, Siddiqi ZA. Therapies directed against B-cells and downstream effectors in generalized autoimmune myasthenia gravis: current status. *Drugs.* (2019) 79:353–64. doi: 10.1007/s40265-019-1065-0
234. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol.* (2007) 7:715–25. doi: 10.1038/nri2155
235. Zuercher AW, Spirig R, Baz Morelli A, Rowe T, Kasermann F. Next-generation Fc receptor-targeting biologics for autoimmune diseases. *Autoimmun Rev.* (2019) 18:102366. doi: 10.1016/j.autrev.2019.102366
236. Howard JF Jr., Bril V, Burns TM, Mantegazza R, Bilinska M, Szczudlik A, et al. Randomized phase 2 study of FcRn antagonist efgartigimod in generalized myasthenia gravis. *Neurology.* (2019) 92:e2661–73. doi: 10.1212/WNL.0000000000007600
237. Ling LE, Hillson JL, Tiessen RG, Bosje T, van Iersel MP, Nix DJ, et al. M281, an anti-FcRn antibody: pharmacodynamics, pharmacokinetics, and safety across the full range of IgG reduction in a first-in-human study. *Clin Pharmacol Ther.* (2019) 105:1031–9. doi: 10.1002/cpt.1276

**Conflict of Interest:** KO'C has received research support from Ra Pharma and is a consultant and equity shareholder of Cabaletta Bio. KO'C is the recipient of a sponsored research subaward from the University of Pennsylvania, the primary financial sponsor of which is Cabaletta Bio. MF has received research support from Grifols. RN has received research support from the Alexion Pharmaceuticals, Genentech, Grifols, and Ra Pharma.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AC declared a shared affiliation, with no collaboration, with one of the authors, AB, to the handling Editor at the time of the review.

frontiers in Immunology

REVIEW
published: 03 June 2020
doi: 10.3389/fimmu.2020.00917

# Complement Inhibitor Therapy for Myasthenia Gravis

*Khaled Albazli*[1], *Henry J. Kaminski*[1]* *and James F. Howard Jr.*[2]

[1] Department of Neurology, George Washington University, Washington, DC, United States, [2] Department of Neurology, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

Complement activation as a driver of pathology in myasthenia gravis (MG) has been appreciated for decades. The terminal complement component [membrane attack complex (MAC)] is found at the neuromuscular junctions of patients with MG. Animals with experimental autoimmune MG are dependent predominantly on an active complement system to develop weakness. Mice deficient in intrinsic complement regulatory proteins demonstrate a significant increase in the destruction of the neuromuscular junction. As subtypes of MG have been better defined, it has been appreciated that acetylcholine receptor antibody-positive disease is driven by complement activation. Preclinical assessments have confirmed that complement inhibition would be a viable therapeutic approach. Eculizumab, an antibody directed toward the C5 component of complement, was demonstrated to be effective in a Phase 3 trial with subsequent approval by the Federal Drug Administration of the United States and other worldwide regulatory agencies for its use in acetylcholine receptor antibody-positive MG. Second- and third-generation complement inhibitors are in development and approaching pivotal efficacy evaluations. This review will summarize the history and present the state of knowledge of this new therapeutic modality.

**Keywords: complement, C5, myasthenia (myasthenia gravis—MG), eculizumab, zilucoplan**

OPEN ACCESS

***Edited by:***
*Anna Rostedt Punga,*
*Uppsala University, Sweden*

***Reviewed by:***
*Ruksana Huda,*
*University of Texas Medical Branch at Galveston, United States*
*Rozen Le Panse,*
*Sorbonne Université, France*

***Correspondence:**
*Henry J. Kaminski*
*hkaminski@mfa.gwu.edu*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *22 December 2019*
***Accepted:*** *20 April 2020*
***Published:*** *03 June 2020*

***Citation:***
*Albazli K, Kaminski HJ and Howard JF Jr (2020) Complement Inhibitor Therapy for Myasthenia Gravis. Front. Immunol. 11:917. doi: 10.3389/fimmu.2020.00917*

## INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease in which the postsynaptic membrane is depleted of acetylcholine receptor (AChR) causing a compromise of neuromuscular transmission (1). Antibodies directed against the AChR are the primary driver of pathology in most patients. In those patients without detectable circulating AChR antibodies, the muscle-specific kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4) have been identified as pathological targets, and other neuromuscular junction proteins are under investigation (2, 3).

Myasthenic autoantibodies are polyclonal with variations in subclasses, epitope targets, binding avidity, and pathogenic mechanisms (4). The characteristics of the population of autoantibodies among individual patients vary and change over the course of the disease. The mechanisms of pathology are best understood for MuSK and AChR antibodies. The predominant subclass of MuSK autoantibodies is immunoglobulin G (IgG)4, which lacks the ability to activate the complement cascade and is considered to be functionally monovalent. MuSK is a receptor tyrosine kinase crucial for formation and maintenance of neuromuscular junction, and MuSK autoantibodies interfere with clustering of the AChR. Studies starting in the 1970s demonstrated the three pathogenic mechanisms for AChR antibodies: blockade of AChR channel function, cross-linking of AChR by the divalent AChR antibody (antigenic modulation), and complement activation (5–8). Antibody binding to a variety of determinants of the multimeric AChR may result in a functional block of

AChR channel function or prevent acetylcholine binding. Interestingly, most of the antibodies do not directly block the transmitter binding site on AChR. In antigenic modulation of AChR, binding of antibody and subsequent cross-linking lead to an increase in the natural degradation cycle of the receptors. The third, and likely most critical mechanism, is for AChR antibodies to activate complement with ultimate formation of the terminal complement component (TCC) causing damage to the muscle membrane (**Figure 1**) (3, 9). The role of complement activation in patients without AChR antibodies is poorly defined (10).

This review will provide a broad overview of the complement system, the preclinical data that support the role of complement in driving MG pathology, and application of complement inhibitors in the treatment of MG.

## THE COMPLEMENT CASCADE

Complement is part of the innate immune system and a key mediator of antibody function through the ultimate formation of the TCC, which serves to rupture bacterial and cellular membranes as well as signaling phagocytic cells to remove pathogens (11, 12). Over 30 proteins compose the complement cascade (**Figure 1**), which is activated by either antibody (classical pathway), spontaneously formed C3b (alternative), and binding of lectins found on bacterial cell surfaces (alternative). In human AChR Ab-positive MG, the classical pathway is initiated (activation step) when IgG1 or IgG3 (less so IgG2) autoantibodies attached to the AChR bind C1q. C1q binds the Fc domain of the antibody, leading to the autoactivation of C1r and the subsequent activation of C1s. C1s then cleaves C4 to C4a and the larger C4b. The C1s and C1r combine with C4b to form C14b. The amplification phase occurs when C14B enzymatically converts C2 to C2a and C2b. The C14B combines C2a to form C14b2a, which is also known as C3 convertase. Spontaneous hydrolysis of C3 may also occur, and the formation C3b combining with Factor B produces C3 convertase of the alternative pathway (**Figure 1**). C3 convertase enzymatically cleaves C3 into C3a and C3b. C3b with the C3 convertase forms C14b2a3b, which is the C5 convertase. The C5 convertase then cleaves C5 to C5a and C5b. The C5b combines with C6, C7, C8, and C9 to form C5b6789, which is the effector mechanism of the complement system. TCC formation produces focal lysis of the neuromuscular junction with loss of AChR and postsynaptic folds (**Figure 2**) (10).

The complement cascade may be spontaneously activated with potential devastating cell injury, which explains that need for inhibitory regulators that are found on nearly all cell surfaces (13). The decay accelerating protein [decay accelerating factor (DAF), CD55] and CD59 are the primary cell surface inhibitors in humans and can be found localized to the neuromuscular junction (14, 15). DAF is a membrane-bound protein that dissociates C3 and C5 convertases, while CD59 interferes with TCC formation. Interestingly, complement regulators are expressed at lower levels in extraocular muscle and could account for the differential involvement of these muscles by MG (16, 17).

## EVIDENCE OF COMPLEMENT AS AN EFFECTOR MECHANISM IN MYASTHENIA GRAVIS AND ITS ANIMAL MODELS

Several lines of evidence support activation of the complement system is critical to the pathology of human MG and animal models of MG. An important early observation was the identification of C3 and C9 localized to fragments of degenerated junction folds where identified in MG patient neuromuscular junctions (**Figure 2**) (8, 18–20). Depletion of serum complement components, C3 and C4, is observed in patients, terminal components of complement are present in MG patient sera, and sera induce complement-mediated lysis of cultured myotubes (21–23). The effectiveness of C5 inhibition in human trials, which will be discussed later in this review, offers further compelling evidence that complement is a critical mediator of MG.

The relative contribution of non-complement-mediated mechanisms to human MG is poorly defined. Administration into mice of even large quantities of human autoantibodies (equal to 50% of the total mouse circulating mouse IgG) produces only mild weakness (7, 24). Because human complement is not co-administered, weakness would likely only develop from antigenic modulation or impairment of AChR function. Miniature endplate potentials are not altered by infusion of human MG serum, when rats are rendered intolerant to human immunoglobulin (IG) (25). Studies using rodents are influenced by the ability of human AChR antibody to bind rodent AChR, leading to the potential to underestimate the effect of AChR blockade or antigenic modulation.

Two basic animal models are used to study MG. Passive transfer MG (PTMG) consists of administration of autoantibodies (9, 26). For AChR-antibody PTMG, monoclonal antibodies, syngeneic polyclonal serum, and highly concentrated human MG sera with a source of active complement have been used. Within 24–72 h of receiving antibody, rodents develop mild to severe weakness depending on species and antibody properties as well as having complement components deposited at the neuromuscular junction. A significant deficiency of PTMG is that it only interrogates the final antibody effector mechanism and not the loss of tolerance that leads to antibody production. Experimental autoimmune MG (EAMG) involves immunization with purified AChR or peptide fragments of the AChR. Over weeks to months depending on species, an antibody response develops directed toward the administered AChR and then toward the native protein. Complement components with antibody are then found at

**Abbreviations:** Ab, Antibody; AChR, Acetylcholine receptor; aHUS, Atypical hemolytic uremic syndrome; C, Complement; DAF, Decay accelerating factor; EAMG, Experimental autoimmune myasthenia gravis; gMG, Generalized myasthenia gravis; IVIg, Intravenous immunoglobulin; IST, Immunosuppressive therapy; LRP-4, Lipoprotein receptor-related protein 4; MAC, Membrane Attack Complex; MuSK, Muscle-specific kinase; MG, Myasthenia gravis; MGC, Myasthenia Gravis Composite; MG-ADL, Myasthenia Gravis Activities of Daily Living; MGFA, Myasthenia Gravis Foundation of America; MG-QoL 15, Myasthenia Gravis Quality of Life 15; MM, Minimal manifestation; MSE, Minimal symptom expression; FcRn, Neonatal Fc receptor; OLE, Open-label extension; PTMG, Passive transfer myasthenia gravis; PNH, Paroxysmal nocturnal hemoglobinuria; QMG, Quantitative Myasthenia Gravis; sCR1, Soluble complement receptor 1; TCC, Terminal complement component.

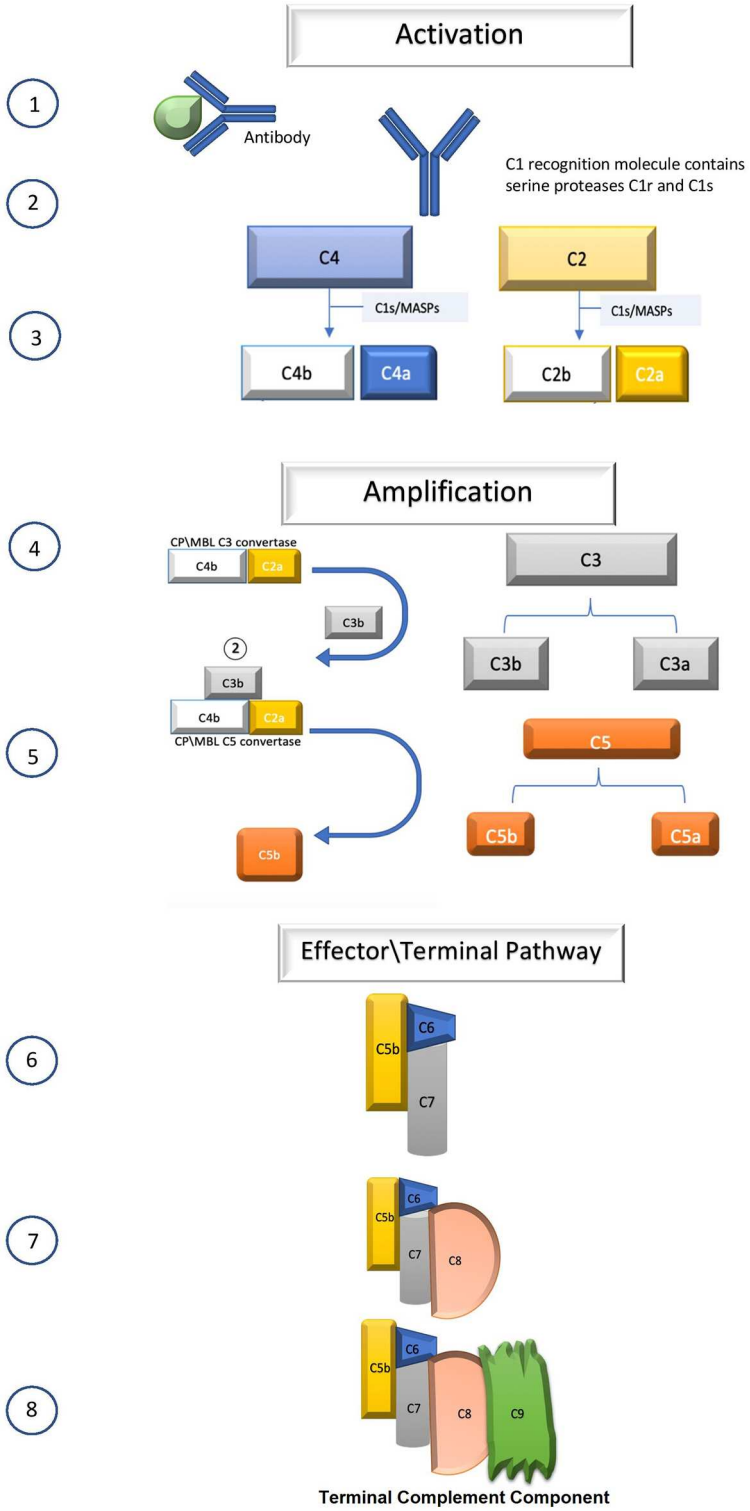



**FIGURE 1 |** Schematic of complement cascade (see text for details).

the neuromuscular junction, and animals develop weakness with neuromuscular transmission deficits consistent with a myasthenic phenotype.

Studies of complement depletion were the first approaches to assess complement mechanisms in the pathology of EAMG. Early experiments demonstrated that cobra venom injected, either

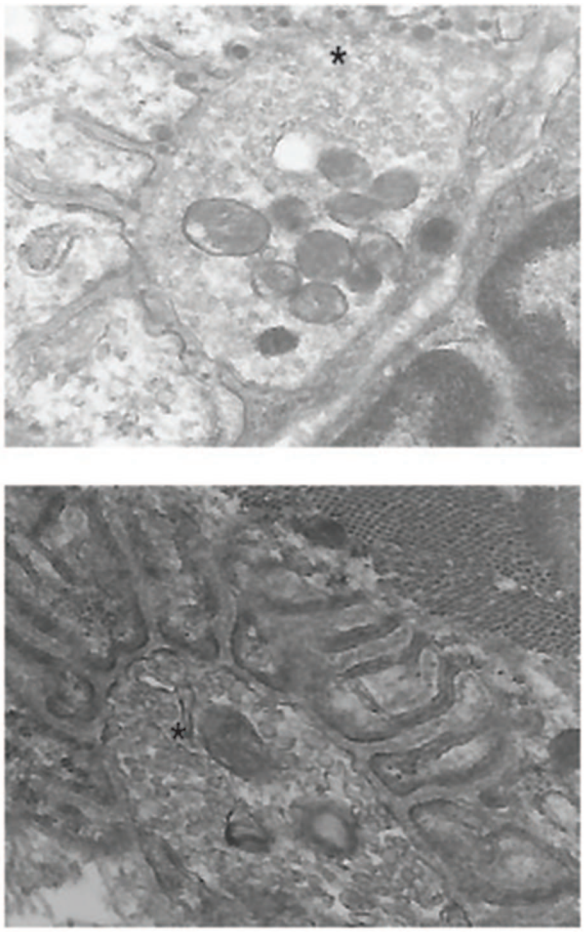



**FIGURE 2 |** Electron micrographs of mouse neuromuscular junction. **(A)** shows a junction from an experimental autoimmune myasthenia gravis (EAMG) animal. Note the electron-dense material in the synaptic cleft and the loss of regular postsynaptic folds that are seen in a normal junction in **(B)**. The* marks the nerve terminal. Previously unpublished images by one of the authors (HK).

during the acute phase of the active EAMG in rats or prior to AChR antibody administration in PTMG, leads to a reduction of disease severity (7, 27). C6-deficient rats do not demonstrate weakness or form TCC on the postsynaptic membrane in response to AChR antibody administration, while exogenous C6 restores TCC assembly and weakness (28). Engineered knockout of C3 and C4 in mice leads to a reduction of weakness from EAMG and preserved AChR density at the neuromuscular junctions (29). Interestingly, the animals show some reduction of complement fixing antibody subclasses, which suggests that the complement system influences cellular autoimmunity (30). In contrast, C5 knockout mice develop comparable levels of circulating AChR-specific antibodies, but C5 knockout mice have no weakness or junctional injury (31). Taken together, the data suggest that activation of complement is the overwhelming driver of EAMG pathology since weakness is not evident when complement activity is ablated. This is likely not to be the case in humans. In contrast to the preservation of function with removal of complement components, the engineered ablation of cell surface complement regulatory proteins leads to severe pathology when PTMG is induced (32–34).

## PRECLINICAL VALIDATION FOR COMPLEMENT INHIBITOR THERAPY IN MYASTHENIA GRAVIS

Exogenous provision of complement inhibitors as a potential therapy has a long history in MG. In 1989, a monoclonal antibody to C6 was administered to PTMG rats and reduced weakness, preserved body weight, and preserved normal electrophysiological properties (35). A soluble complement receptor 1 (sCR1) to Lewis rats at the time of PTMG induction found a reduction of weakness and retained AChR density (36). Anti-C5 antibody treatment was found to limit PTMG severity (37), which justified application of C5-focused treatments in humans (see below). Another C5 inhibitor is coversin (rEV576), which is a recombinant protein derived from ticks and effective in moderating disease severity of PTMG and EAMG (38). Coversin has moved to human clinical trials for paroxysmal nocturnal hemoglobinuria (PNH) but is not being developed for MG. Targeting complement inhibition to the site of pathology, the neuromuscular junction, reduces PTMG severity and has the advantage of limiting systemic complement inhibition (39, 40).

In addition to antibody-based targeting of complement components, siRNA therapies have been used to suppress C component expression. Reduction of the C2 component of complement reduced serum complement activity in mice with a resultant reduction of weakness, retention of AChR, and reduced TCC deposition in EAMG mice (41). Targeting of C5 expression by the liver leads to similar findings in rat EAMG (42). Thus, far, these approaches have not moved into human assessment.

## HUMAN STUDIES OF COMPLEMENT INHIBITION

Patients with generalized MG (gMG) have a large disease burden with an increased risk of disease exacerbation, hospitalization, intensive care stay, and intolerable side effects to the medications used in their treatment (43–45). In addition, 10–15% of patients with AChR antibody–positive (AChR+) gMG are refractory to the most common immunotherapeutic paradigms (46, 47). As such, there is a need for target-specific therapies with improved adverse event profiles. Progress has been made over the last 12 years with the development of new novel therapeutics that attempt to address these issues: the inhibition of complement targets and the neonatal Fc receptor (FcRn).

## CLINICAL TRIALS

To date, there have been three completed clinical trials of complement inhibition in gMG: two phase 2 trials and one phase 3 trial. All have targeted C5 with the goal of blocking terminal complement activation, preventing the pro-inflammatory effects of C5a and C5b and the subsequent formation of the terminal

complement component or membrane attack complex (C5b-9) (28). Each of these trials focused on AChR+ generalized MG as the predominant antibody subclass, IgG3, is a potent activator of complement.

## Phase 2 Trials

The initial phase 2 trial (NCT00727194), sponsored by Alexion Pharmaceuticals, was a prospective, double-blind, placebo-controlled crossover design of 14 AChR+, gMG treatment-refractory patients [Myasthenia Gravis Foundation of America (MGFA), Classes II–IVa)] initially treated for 16 weeks (Period 1) followed by a 5-week washout period and then crossed over (Period 2) to the other investigational product for an additional 16 weeks (48). Patients were required to have persistent weakness despite treatment with at least two immunosuppressive drugs for at least 1 year and could not have received intravenous immunoglobulin (IVIg), plasma exchange, rituximab, or thymectomy within 2, 3, 6, or 12 months of screening, respectively. This study used the full sized, humanized monoclonal antibody eculizumab that specifically binds to and inhibits cleavage of C5 into C5a and C5b (49). Standard of care was maintained through the duration of the study. Study subjects received either eculizumab 600 mg or a matching placebo infused IV for 4 consecutive weeks, followed by the administration of 900 mg IV of eculizumab or matching placebo every 2 weeks. Six of seven patients (86%), treated for 16 weeks, met the primary efficacy endpoint of a 3-point reduction in the Quantitative MG (QMG) score vs. 50% of placebo-treated patients. Four of seven (57%) of patients treated with eculizumab had an 8-point improvement in total QMG score compared to only one of seven (14%) who received placebo. Of note, eculizumab-treated patients did not return to their baseline QMG scores despite a 5-week washout prior to beginning Period 2 (**Figure 3**). This suggests a carryover effect, although the mechanism of such is not known.

The second phase 2 trial (ClinicalTrials.gov Identifier: NCT03315130), sponsored by Ra Pharmaceuticals was a prospective, double–blind, placebo-controlled study of 44 AChR+ gMG patients over 12 weeks followed by an open-label extension (OLE) trial that continues at this time (50). This study used zilucoplan, a small (3.5-kDa), 15-amino acid macrocyclic peptide, that binds to C5 with high affinity and specificity and also binds to the domain of C5 that corresponds to C5b and thereby also blocks binding of C5b to complement component C6 (51). Patients were randomized 1:1:1 to zilucoplan 0.1 mg/kg, zilucoplan 0.3 mg/kg, or matching placebo self-administered subcutaneously daily for 12 weeks, and eligible participants could enter the OLE. Entry criteria were like the Alexion phase 2 trial in age, disease severity, and baseline QMG scores, but there was no requirement to be treatment refractory. Standard of care was maintained throughout the study. Rapid, robust, and a sustained response was seen in the zilucoplan-treated group. The primary efficacy measure was the change in QMG score from baseline to week 12; a 6-point change in the 0.3-mg/kg zilucoplan group compared with −3.2 points in the placebo-treated group ($p = 0.05$). Onset of improvement was as early as 1 week (**Figure 4**). The 0.1-mg/kg zilucoplan dose demonstrated a slower onset of action and a less pronounced effect when compared to the higher zilucoplan dose although still a clinically meaningful response when compared to placebo. Similar findings were seen when comparing the change in MG Activities of Daily Living (MG-ADL) score from baseline to week 12 in both arms compared to placebo.

## Phase 3 Trials

REGAIN (NCT01997229), a phase 3 trial with an OLE (NCT02301624) also used the monoclonal antibody eculizumab (52, 53). This prospective, double–blind, placebo-controlled study enrolled 125 treatment-refractory AChR+ gMG patients of moderate to severe severity (MGFA Classes II–IV) at 72 centers in Asia, Europe, Latin America, and North America. Treatment refractory was defined as having persistent weakness despite treatment with at least two immunosuppressive therapies (ISTs) or one IST with the requirement of chronic plasma exchange or IVIg. Subjects were randomized 1:1 to either eculizumab or a matched control for 26 weeks. Eculizumab was administered IV; an induction dose of 900 mg weekly for four doses (day 1, weeks 1–3) and a maintenance dose of 1,200 mg every other week beginning on week 4. Subjects who completed the 26-week REGAIN study were eligible to participate in the OLE, and 117 patients elected to do so (53).

The primary efficacy endpoint was the change in the MG-ADL score from baseline to week 26 for eculizumab treated subjects compared to placebo measured by worst-rank analysis of covariance (ANCOVA) analysis. Multiple prespecified secondary endpoints included the change in QMG total score from baseline, responder analysis of the MG-ADL and QMG scores for those with at least a 3-point and 5-point improvement, respectively, and changes in the MG Composite (MGC) and MG Quality of Life 15 (MG-QoL15) scores from baseline.

The primary endpoint, the mean ranked difference in the change in MG-ADL score between baseline and placebo at week 26 was not significant despite significant change in 18 of 21 secondary measures (**Table 1**). Rapid, robust, and durable improvement was seen in the MG-ADL of eculizumab-treated patients compared to placebo (**Figure 5**). Improvement was noted during the week following their first infusion, was maximal around 12 weeks, and remained durable for the duration of the 130-week observation. A similar profile was seen with the QMG score (**Figure 5**), MGC, and MG-QoL15, although the latter has a slightly slower time course (data not shown). During the trial, 56% of patients achieved the clinical state of minimal manifestations. Additionally, exacerbation rates were reduced by 75% ($p = 0.0001$) from the year prior to study entry. Patients who received placebo during the REGAIN trial had a similar response when transitioned to eculizumab in the OLE. The speed and degree of improvement mimicked those seen in the REGAIN trial.

Currently, multinational phase 3 trials are underway with zilucoplan (NCT04115293) administered daily subcutaneously and ravulizumab (NCT03920293), a monoclonal antibody developed by Alexion Pharmaceuticals, administered IV every 8 weeks. The primary efficacy endpoint will be the change from baseline in the MG-ADL score at 12 and 26 weeks, respectively.

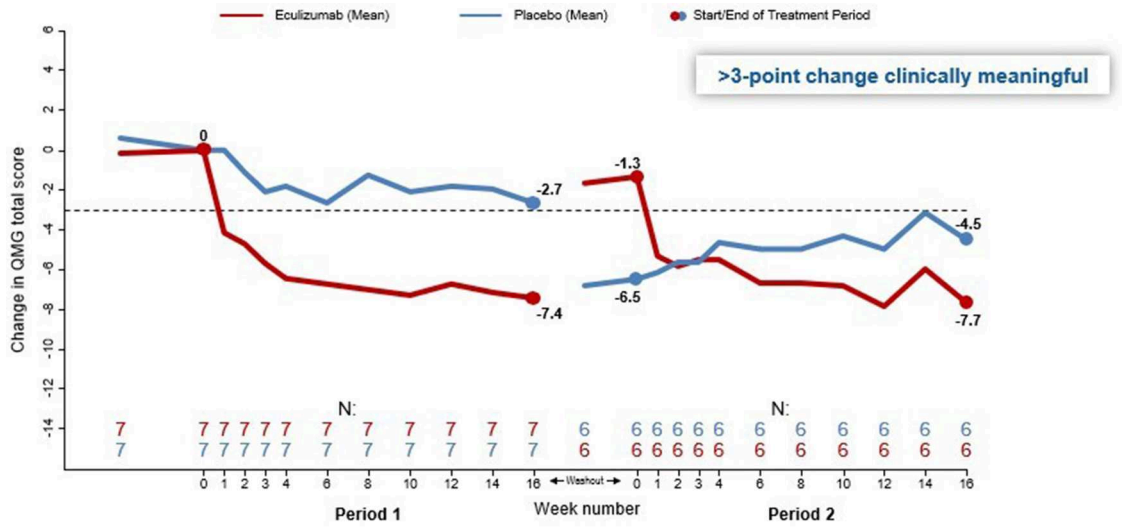



**FIGURE 3 |** Quantitative myasthenia gravis (QMG) score change from baseline over 12 weeks for eculizumab vs. placebo. Using patient data at the end of both period visits, overall change in mean QMG total score was significantly different between eculizumab and placebo (−7.92 vs. −3.67; paired t-test $p = 0.0144$). Using patient data at all visits, overall change in mean QMG total score was significantly different between eculizumab and placebo (−6.43 vs. −3.18; repeated-measures mixed model $p < 0.0001$). Modified from Howard et al. (48).

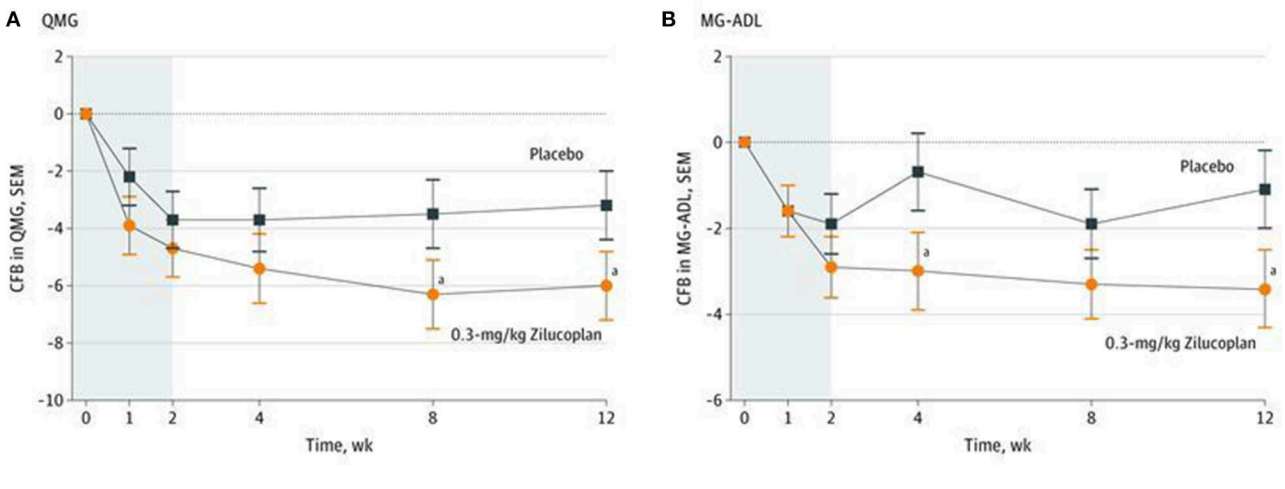



**FIGURE 4 |** Change from baseline over 12 weeks for 0.3 mg/kg zilucoplan vs. placebo. **(A)** Change from baseline to week 12 in Quantitative Myasthenia Gravis (QMG) Score. **(B)** Change from baseline to week 12 in MG Activities of Daily Living (MG-ADL) Score. Modified from Howard et al. (50). *$p < 0.10$.

## *Post hoc* Analyses

Numerous *post hoc* analyses have been performed on the REGAIN dataset. Eculizumab demonstrated rapid and significant improvement in ocular, bulbar, respiratory, and limb domains at week 26 compared to baseline and through 130 weeks of the OLE phase of the study. The improvements were observed at week 26 for both ocular and gross motor, by week 20 bulbar domains, and by week 12 for the respiratory domain and remain stable through 130 weeks (54).

The ability to reduce IST use during the OLE phase of the study was noted. At baseline, 115 of 117 (98.3%) study subjects were using at least one immunosuppressive drug (55). This was reduced to 103/117 (88%) at the time of their last assessment. Additionally, significant reductions in the total daily dose of corticosteroids, azathioprine, and mycophenolate mofetil were demonstrated.

The REGAIN study demonstrated that eculizumab-treated patients had significantly more improvement in the extended Neuro-QOL Fatigue scale, a patient-reported assessment of fatigue, when compared to the placebo arm. Eculizumab-treated patients had a 16.3-point change from baseline compared to a 7.7-point change in the placebo group at week 26 (56). This improvement was sustained through week 52 (data cut) of the open-label study. Strong correlations were observed between the

**TABLE 1 |** REGAIN totality and consistency of analyses.

| Outcome measure | Primary/secondary endpoints | | Sensitivity analyses | | |
|---|---|---|---|---|---|
| | Worst-Rank ANCOVA | Responder analysis | Repeated measures at week 26 (IST as covariate | Change from baseline at week 26 or LOCF ANCOVA | Wost-rank ANCOVA sensitivity |
| MG-ADL | 0.0698* | 0.0229 | 0.0058 (0.0077) | 0.0390 | 0.0800* |
| QMG | 0.0129 | 0.0018 | 0.0006 (0.0007) | 0.0032 | 0.0169 |
| MGC | 0.1026* | N/A | 0.0134 (0.0168) | 0.0406 | 0.1084* |
| MG-QOL15 | 0.0281 | N/A | 0.0010 (0.0009) | 0.0152 | 0.0328 |

*REGAIN Study results of 22 prespecified measures demonstrating the totality of the data supporting the positive effects of complement inhibition in the treatment of myasthenia gravis (MG). Analyses marked with an* reflect the bias imposed by the worst-rank analysis of covariance (ANCOVA) methodology. Adapted from Howard et al. (52).*

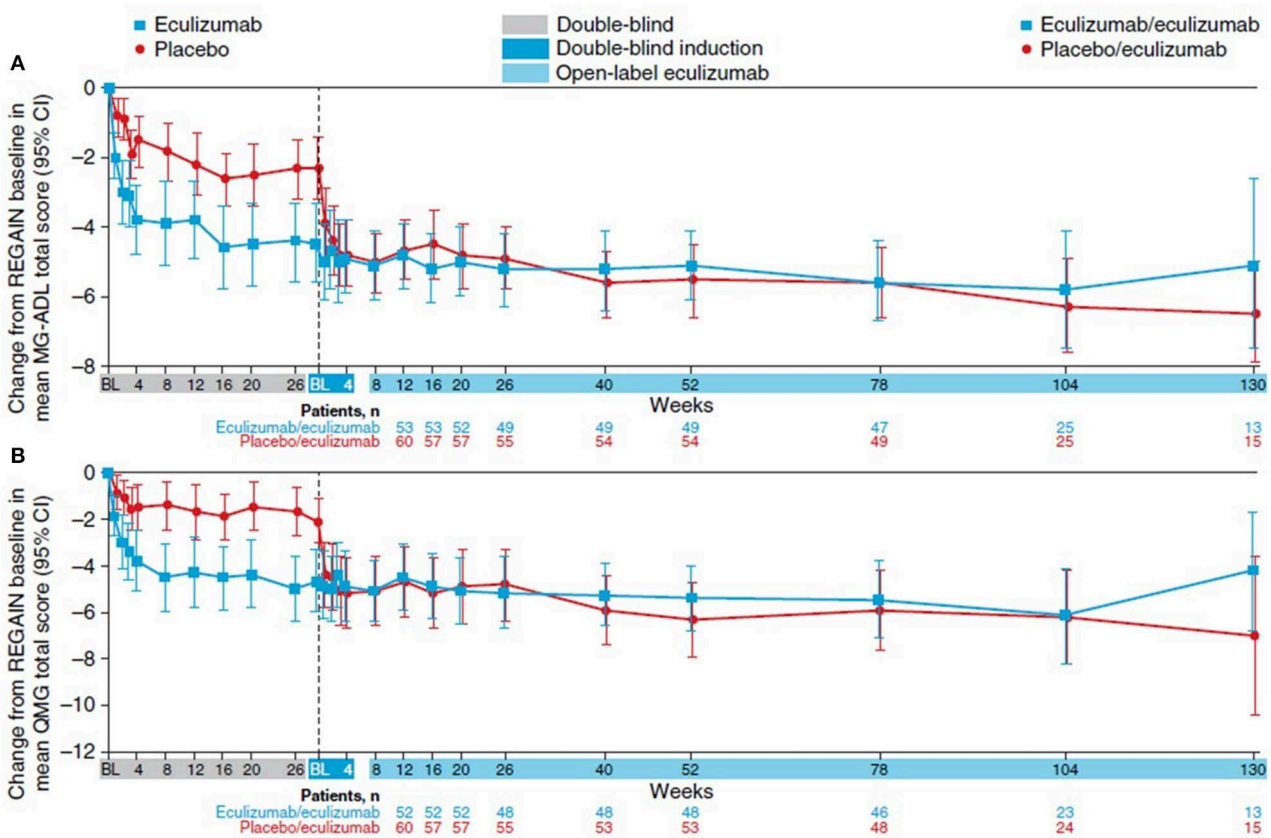



**FIGURE 5 |** REGAIN and open-label extension (OLE). **(A)** The change in MG-ADL score from REGAIN baseline to OLE week 130. **(B)** The change in QMG score from REGAIN baseline to OLE week 130. BL, baseline; CI, confidence interval; MG-ADL, Myasthenia Gravis Activities of Daily Living; QMG, Quantitative Myasthenia Gravis Score. Vertical dash line represents the transition from REGAIN to the OLE. Adapted from Muppidi et al. (53).

Neuro-QOL Fatigue scale and the MG-ADL, MG-QoL15, and QMG scores.

Subgroup analysis of the REGAIN population previously dependent upon IVIg demonstrated rapid and robust improvement in their MG outcome measures with fewer disease exacerbations when treated with eculizumab in both the blinded and open-label portions of the study (57). Treatment with eculizumab was associated with a 65% reduction ($p$ = 0.0057) in MG exacerbation rates during the REGAIN trial compared to the placebo arm (58). Further, there was a 66% reduction in hospitalization rates ($p = 0.0316$) as well as the need for rescue therapy ($p = 0.0072$).

The MGFA post intervention status of minimal manifestations (MMs), the presence of minimal nonfunctional weakness or no weakness on clinical examination, is the stated goal of several treatment guidelines (59–61). The REGAIN study demonstrated that at week 26, 25% of eculizumab-treated patients achieved a state of MM vs. 13.3% of those treated with placebo. A total of 57.1% of patients achieved this status through 130 weeks of OLE.

Current definitions of minimal symptoms rely on the physical examination and not that of the patient's impression of their disease. The concept of Minimal Symptom Expression (MSE) has been developed as a more meaningful way to assess patient response without the need for clinical examination. MSE is defined as an MG-ADL score of 0 or 1. Vissing et al. (62) have reported more patients receiving eculizumab than placebo met the criteria of MSE (21.4 vs. 1.7%, $p = 0.0069$) at week 26 of treatment during the REGAIN trial. This was maintained through 130 weeks of the OLE, and a similar number of patients in the placebo/eculizumab and the eculizumab/eculizumab groups achieved "minimal symptom expression" (MG-ADL: 22.9 and 27.8%, respectively, $p = 0.7861$) at week 130 of treatment. These findings suggest that MSE may be a potential endpoint for future clinical trials or in the clinic.

By week 12 in the REGAIN study, 67.3% of eculizumab-treated patients had at least a 3-point change in the MG-ADL score, and 56.1% had at least a 5-point change in the QMG score. At the end of OLE, 84.7% of eculizumab-treated patients reached the MG-ADL criteria, and 71.4% of patients reached the QMG criteria. Then, 15.3% and 28.6% of patients did not achieve MG-ADL or QMG response, respectively. These data would suggest that there is a subpopulation of patients whose response is much slower (63).

## SAFETY

The inhibition of C5 increases the risk of *Neisseria* infection. For this reason, patients treated with anti-C5 inhibitors must be vaccinated against *Neisseria* meningitides with both the quadrivalent and B-serotype vaccines according to the guidelines published by the CDC Advisory Committee on Immunization Practices. Information can be obtained from the Centers for Disease Control at https://www.cdc.gov/meningococcal/about/soliris-patients.html. Patients must be educated on the presenting symptoms of meningococcal meningitis, and all should carry an informational safety card to present at each health encounter.

Patients who are unable to be vaccinated at least 14 days prior to their initial dose of a C5 inhibitor must be treated with antibiotics. Treatment with C5 inhibitors should not be delayed during this period. Care must be taken to avoid the fluoroquinolone and macrolide classes as these have the potential to acutely worsen myasthenic weakness. The role of long-term prophylactic antibiotic therapy for MG patients treated with a complement inhibitor remains controversial. Pediatric myasthenic populations are not approved currently for the use of C5 inhibitors. Should this approval be forthcoming, additional vaccinations against *Streptococcus pneumoniae* and *Haemophilus influenzae* type b are recommended.

In all three trials, the safety profile for the three C5 inhibitors was like those seen in PNH and atypical hemolytic uremic syndrome (aHUS), most commonly headache and nasopharyngitis. No meningococcal infections occurred during the trials, and one occurred following the completion of the REGAIN OLE trial that was successfully treated.

Neutralizing antibodies to the C5 monoclonal antibody or the macrocyclic peptide have not been found to a degree that inhibited the therapeutic effects of the drug.

## HOW COMPLEMENT INHIBITORS INTEGRATE WITH STANDARD OF CARE THERAPIES

Currently, complement inhibition has been restricted by regulatory agencies in Europe and Japan for use in patients who have refractory AChR+ gMG. While the FDA did not place such a restriction for its use in the USA, the insurance industry has followed the parameters of the phase 3 REGAIN trial, limiting, for the most part, its use in a similar patient population. One hopes current trials of ravulizumab and zilucoplan will change this and allow their use in broader populations of patients and earlier in the disease course.

The accumulated data on adverse events related to complement inhibition in PNH, aHUS, and MG exceed 50,000 patient years. The adverse event profile is quite favorable when compared to adverse event profiles of current therapies, e.g., corticosteroids, purine inhibitors, cyclophilins, and other broad-spectrum immunosuppressants.

There is a single missense C5 heterozygous mutation, c.2654G→ A, that predicts the polymorphism p.Arg885His in 3.5% of the Japanese population and in a Han Chinese population. This mutation prevents binding and blockade of eculizumab at the C5 domain as shown in a Japanese study of this drug in PNH (64). The REGAIN trial did not enroll patients with this mutation. Zilucoplan, a macrocyclic peptide, has a different binding site on C5 and C5b and would be effective in patients with this mutation.

A clearer understanding of the pathophysiological processes that occur at the neuromuscular junction in response to complement inhibition is needed. For instance, is there repair of the postjunctional folds with long-term use of these drugs? If such were to occur, one would have a firm argument to initiate treatment much earlier than its current use and perhaps even as primary therapy. Further study is warranted, given the rapid onset of effect, to determine the role of complement inhibition as a rescue therapy during periods of acute deterioration or in myasthenic crisis. Its role in seronegative MG and those with antibody to LRP4 is yet to be determined. While IgG1 and IgG3 antibody subclasses are reported in MuSK MG, high levels of MuSK-specific IgG1 or IgG3 have not been identified in these patients (65, 66). These data suggest complement inhibition would not be effective in IgG4-predominant mediated MG, such as MuSK MG, as this immunoglobulin subclass activates complement weakly. It is to be determined what the role of combinational therapy will play in the management of MG. As detailed above, there is no question that complement inhibition targets the primary effector mechanism for the destruction of the postjunctional folds of the neuromuscular junction. However, circulating antibody remains available to target epitopes of the AChR complex, and non-complement mechanisms driving AChR loss are not

influenced. It is attractive to think of combinational treatment with an FcRn inhibitor as a means of accomplishing the task. Information regarding dosing, dosing intervals of each drug, and the predominance of one drug vs. the other will only come with further study.

The rapid, robust, and sustained improvement seen with C5 inhibition as evidenced by the clinical trials and subsequent analyses makes this treatment very favorable in patients with generalized AChR antibody-positive gMG. It has been transformational in the lives of many patients who have previously failed multiple therapies and has made significant strides in alleviating the burden of disease of chronic MG. Current trials will address its role in earlier management of the disease.

## AUTHOR CONTRIBUTIONS

KA, HK and JH contributed to the organization of the review and each wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

## FUNDING

HK and JH are supported by NIH grant U54 NS115054. The grant support the Rare Disease Clinical Research Network dedicated to myasthenia gravis (MGNet). Open access support is provided the Myasthenia Gravis Research fund of George Washington University.

## REFERENCES

1. Gilhus NE. Myasthenia gravis. *N Engl J Med.* (2017) 376:e25. doi: 10.1056/NEJMc1701027
2. Vincent A, Huda S, Cao M, Cetin H, Koneczny I, Rodriguez Cruz PM, et al. Serological and experimental studies in different forms of myasthenia gravis. *Ann N Y Acad Sci.* (2018) 1413:143–53. doi: 10.1111/nyas.13592
3. Howard JF Jr. Myasthenia gravis: the role of complement at the neuromuscular junction. *Ann N Y Acad Sci.* (2018) 1412:113–28. doi: 10.1111/nyas.13522
4. Koneczny I, Herbst R. Myasthenia gravis: pathogenic effects of autoantibodies on neuromuscular architecture. *Cells.* (2019) 8:671. doi: 10.3390/cells8070671
5. Drachman DB, Angus CW, Adams RN, Kao I. Effect of myasthenic patients' immunoglobulin on acetylcholine receptor turnover: selectivity of degradation process. *Proc Natl Acad Sci USA.* (1978) 75:3422–6. doi: 10.1073/pnas.75.7.3422
6. Drachman DB, Angus CW, Adams RN, Michelson JD, Hoffman GJ. Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. *N Engl J Med.* (1978) 298:1116–22. doi: 10.1056/NEJM197805182982004
7. Toyka KV, Drachman DB, Griffin DE, Pestronk A, Winkelstein JA, Fischbeck KH Jr, et al. Myasthenia gravis. Study of humoral immune mechanisms by passive transfer to mice. *N Engl J Med.* (1977) 296:125–31. doi: 10.1056/NEJM197701202960301
8. Sahashi K, Engel AG, Linstrom JM, Lambert EH, Lennon VA. Ultrastructural localization of immune complexes (IgG and C3) at the end-plate in experimental autoimmune myasthenia gravis. *J Neuropathol Exp Neurol.* (1978) 37:212–23. doi: 10.1097/00005072-197803000-00008
9. Kusner LL, Sengupta M, Kaminski HJ. Acetylcholine receptor antibody-mediated animal models of myasthenia gravis and the role of complement. *Ann N Y Acad Sci.* (2018) 1413:136–42. doi: 10.1111/nyas.13555
10. Cetin H, Vincent A. Pathogenic mechanisms and clinical correlations in autoimmune myasthenic syndromes. *Semin Neurol.* (2018) 38:344–54. doi: 10.1055/s-0038-1660500
11. Chamberlain JL, Huda S, Whittam DH, Matiello M, Morgan BP, Jacob A, et al. Role of complement and potential of complement inhibitors in myasthenia gravis and neuromyelitis optica spectrum disorders: a brief review. *J Neurol.* (2019). doi: 10.1007/s00415-019-09498-4. [Epub ahead of print].
12. Conti-Fine BM, Milani M, Kaminski HJ. Myasthenia gravis: past, present, and future. *J Clin Invest.* (2006) 116:2843–54. doi: 10.1172/JCI29894
13. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol.* (2009) 9:729–40. doi: 10.1038/nri2620
14. Kusner LL, Halperin JA, Kaminski HJ. Cell surface complement regulators moderate experimental myasthenia gravis pathology. *Muscle Nerve.* (2013) 47:33–40. doi: 10.1002/mus.23448
15. Navenot JM, Villanova M, Lucas-Héron B, Malandrini A, Blanchard D, Louboutin JP. Expression of CD59, a regulator of the membrane attack complex of complement, on human skeletal muscle fibers. *Muscle Nerve.* (1997) 20:92–6. doi: 10.1002/(SICI)1097-4598(199701)20:1<92::AID-MUS12>3.0.CO;2-3
16. Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR, et al. Extraocular muscle is defined by a fundamentally distinct gene expression profile. *Proc Natl Acad Sci USA.* (2001) 98:12062–7. doi: 10.1073/pnas.211257298
17. Kaminski H, et al. The complement hypothesis to explain preferential involvement of extraocular muscle by myasthenia gravis. In: Leigh R, Devereaux M, editors. *Advances in Understanding Mechanisms and Treatment of Infantile Forms of Nystagmus.* New York, NY: Oxford University Press (2008).
18. Nakano S, Engel AG. Myasthenia gravis: quantitative immunocytochemical analysis of inflammatory cells and detection of complement membrane attack complex at the end-plate in 30 patients. *Neurology.* (1993) 43:1167–72. doi: 10.1212/WNL.43.6.1167
19. Engel AG, Lambert EHFM. Howard, Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations. *Mayo Clin Proc.* (1977) 52:267–80.
20. Sahashi K, Engel AG, Lambert EH, Howard FM. Ultrastructural localization of the terminal and lytic ninth complement component (C9) at the motor end-plate in myasthenia gravis. *J Neuropathol Exp Neurol.* (1980) 39:160–72. doi: 10.1097/00005072-198003000-00005
21. Romi F, Kristoffersen EK, Aarli JA, Gilhus NE. The role of complement in myasthenia gravis: serological evidence of complement consumption in vivo. *J Neuroimmunol.* (2005) 158:191–4. doi: 10.1016/j.jneuroim.2004.08.002
22. Ashizawa T, Appel SH. Complement-dependent lysis of cultured rat myotubes by myasthenic immunoglobulins. *Neurology.* (1985) 35:1748–53. doi: 10.1212/WNL.35.12.1748
23. Barohn RJ, Brey RL. Soluble terminal complement components in human myasthenia gravis. *Clin Neurol Neurosurg.* (1993) 95:285–90. doi: 10.1016/0303-8467(93)90103-N
24. Toyka KV, Birnberger KL, Anzil AP, Schlegel C, Besinger U, Struppler A. Myasthenia gravis: further electrophysiological and ultrastructural analysis of transmission failure in the mouse passive transfer model. *J Neurol Neurosurg Psychiatry.* (1978) 41:746–53. doi: 10.1136/jnnp.41.8.746
25. Howard JF Jr, Sanders DB. Passive transfer of human myasthenia gravis to rats: 1. Electrophysiology of the developing neuromuscular block. *Neurology.* (1980) 30(7 Pt 1):760–4. doi: 10.1212/WNL.30.7.760
26. Kusner LL, Losen M, Vincent A, Lindstrom J, Tzartos S, Lazaridis K, et al. Guidelines for pre-clinical assessment of the acetylcholine receptor–specific passive transfer myasthenia gravis model-Recommendations for methods and experimental designs. *Exp Neurol.* (2015) 270:3–10. doi: 10.1016/j.expneurol.2015.02.025
27. Lennon VA, Seybold ME, Lindstrom JM, Cochrane C, Ulevitch R. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. *J Exp Med.* (1978) 147:973–83. doi: 10.1084/jem.147.4.973
28. Chamberlain-Banoub J, Neal JW, Mizuno M, Harris CL, Morgan BP. Complement membrane attack is required for endplate damage and clinical

disease in passive experimental myasthenia gravis in Lewis rats. *Clin Exp Immunol.* (2006) 146:278–86. doi: 10.1111/j.1365-2249.2006.03198.x
29. Tüzün E, Scott BG, Goluszko E, Higgs S, Christadoss P. Genetic evidence for involvement of classical complement pathway in induction of experimental autoimmune myasthenia gravis. *J Immunol.* (2003) 171:3847–54. doi: 10.4049/jimmunol.171.7.3847
30. Arbore G, Kemper C, Kolev M. Intracellular complement - the complosome - in immune cell regulation. *Mol Immunol.* (2017) 89:2–9. doi: 10.1016/j.molimm.2017.05.012
31. Christadoss P. C5 gene influences the development of murine myasthenia gravis. *J Immunol.* (1988) 140:2589–92.
32. Kaminski HJ, Li Z, Richmonds C, Lin F, Medof ME. Complement regulators in extraocular muscle and experimental autoimmune myasthenia gravis. *Exp Neurol.* (2004) 189:333–42. doi: 10.1016/j.expneurol.2004.06.005
33. Morgan BP, Chamberlain-Banoub J, Neal JW, Song W, Mizuno M, Harris CL. The membrane attack pathway of complement drives pathology in passively induced experimental autoimmune myasthenia gravis in mice. *Clin Exp Immunol.* (2006) 146:294–302. doi: 10.1111/j.1365-2249.2006.03205.x
34. Lin F, Kaminski HJ, Conti-Fine BM, Wang W, Richmonds C, Medof ME. Markedly enhanced susceptibility to experimental autoimmune myasthenia gravis in the absence of decay-accelerating factor protection. *J Clin Invest.* (2002) 110:1269–74. doi: 10.1172/JCI0216086
35. Biesecker G, Gomez CM. Inhibition of acute passive transfer experimental autoimmune myasthenia gravis with Fab antibody to complement C6. *J Immunol.* (1989) 142:2654–9.
36. Piddlesden SJ, Jiang S, Levin JL, Vincent A, Morgan BP. Soluble complement receptor 1 (sCR1) protects against experimental autoimmune myasthenia gravis. *J Neuroimmunol.* (1996) 71:173–7. doi: 10.1016/S0165-5728(96)00144-0
37. Zhou Y, Gong B, Lin F, Rother RP, Medof ME, Kaminski HJ. Anti-C5 antibody treatment ameliorates weakness in experimentally acquired myasthenia gravis. *J Immunol.* (2007) 179:8562–7. doi: 10.4049/jimmunol.179.12.8562
38. Soltys J, Kusner LL, Young A, Richmonds C, Hatala D, Gong B, et al. Novel complement inhibitor limits severity of experimentally myasthenia gravis. *Ann Neurol.* (2009) 65:67–75. doi: 10.1002/ana.21536
39. Song C, Xu Z, Miao J, Xu J, Wu X, Zhang F, et al. Protective effect of scFv-DAF fusion protein on the complement attack to acetylcholine receptor: a possible option for treatment of myasthenia gravis. *Muscle Nerve.* (2012) 45:668–75. doi: 10.1002/mus.23247
40. Kusner LL, Satija N, Cheng G, Kaminski HJ. Targeting therapy to the neuromuscular junction: proof of concept. *Muscle Nerve.* (2014) 49:749–56. doi: 10.1002/mus.24057
41. Huda R, Tuzun E, Christadoss P. Complement C2 siRNA mediated therapy of myasthenia gravis in mice. *J Autoimmun.* (2013) 42:94–104. doi: 10.1016/j.jaut.2013.01.003
42. Kusner LL, Yucius K, Sengupta M, Sprague AG, Desai D, Nguyen T, et al. Investigational RNAi therapeutic targeting C5 is efficacious in pre-clinical models of myasthenia gravis. *Mol Ther Methods Clin Dev.* (2019) 13:484–92. doi: 10.1016/j.omtm.2019.04.009
43. Boscoe AN, Xin H, L'Italien GJ, Harris LA, Cutter GR. Impact of refractory myasthenia gravis on health-related quality of life. *J Clin Neuromuscul Dis.* (2019) 20:173–81. doi: 10.1097/CND.0000000000000257
44. Engel-Nitz NM, Boscoe A, Wolbeck R, Johnson J, Silvestri NJ. Burden of illness in patients with treatment refractory myasthenia gravis. *Muscle Nerve.* (2018) 58:99–105. doi: 10.1002/mus.26114
45. Schneider-Gold C, Hagenacker T, Melzer N, Ruck T. Understanding the burden of refractory myasthenia gravis. *Ther Adv Neurol Disord.* (2019) 12:1756286419832242. doi: 10.1177/1756286419832242
46. Suh J, Goldstein JM, Nowak RJ. Clinical characteristics of refractory myasthenia gravis patients. *Yale J Biol Med.* (2013) 86:255–60.
47. Silvestri NJ, Wolfe GI. Treatment-refractory myasthenia gravis. *J Clin Neuromuscul Dis.* (2014) 15:167–78. doi: 10.1097/CND.0000000000000034
48. Howard JF, Barohn RJ, Cutter GR, Freimer M, Juel VC, Mozaffar T, et al. A randomized, double-blind, placebo-controlled phase II study of eculizumab in patients with refractory generalized myasthenia gravis. *Muscle Nerve.* (2013) 48:76–84. doi: 10.1002/mus.23839
49. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol.* (2007) 25:1256–64. doi: 10.1038/nbt1344
50. Howard JF Jr, Nowak RJ, Wolfe GI, Benatar MG, Duda PW, MacDougall J, et al. Zilucoplan, a subcutaneously self-administered peptide inhibitor of complement component (C5), for the treatment of generalized myasthenia gravis: results of a phase 2 randomized, double-blind, placebo-controlled, multicenter clinical trial. *JAMA Neurol.* (2020). 77:582–92 doi: 10.1001/jamaneurol.2019.5125
51. Ricardo A, Arata M, DeMarco S, Dhamnaskar K, Hammer R, Fridkis-Hareli M, et al. Preclinical evaluation of RA101495, a potent cyclic peptide inhibitor of C5 for the treatment of paroxysmal nocturnal hemoglobinuria. *Blood.* (2015) 126:939. doi: 10.1182/blood.V126.23.939.939
52. Howard JF, Utsugisawa K, Benatar M, Murai H, Barohn RJ, Illa I, et al. Safety and efficacy of eculizumab in anti-acetylcholine receptor antibody-positive refractory generalised myasthenia gravis (REGAIN): a phase 3, randomised, double-blind, placebo-controlled, multicentre study. *Lancet Neurol.* (2017) 16:976–86. doi: 10.1016/S1474-4422(17)30369-1
53. Muppidi S, Utsugisawa K, Benatar M, Murai H, Barohn RJ, Illa I, et al. Long-term safety and efficacy of eculizumab in generalized myasthenia gravis. *Muscle Nerve.* (2019) 60:14–24. doi: 10.1002/mus.26447
54. Mantegazza R, Fujita K, O'Brien F, Howard J Jr. Eculizumab shows consistent improvement across quantitative myasthenia gravis test muscle groups. *Muscle Nerve.* (2018) 58:S97.
55. Nowak RJ, Muppidi S, Beydoun SR, O'Brien F, Yountz M, Howard JF. Changes in concomitant immunosuppressive therapy use during a phase 3 open-label study of eculizumab in adults with generalized myasthenia gravis: an interim analysis (P5.2-080). *Neurology.* (2019) 92 (15 Suppl.).
56. Andersen H, Mantegazza R, Wang JJ, O'Brien F, Patra K, Howard JF, et al. Eculizumab improves fatigue in refractory generalized myasthenia gravis. *Qual Life Res.* (2019) 28:2247–54. doi: 10.1007/s11136-019-02148-2
57. Jacob S, Murai H, Utsugisawa K, Nowak R, Wiendl H, Fujita K, et al. Response to eculizumab in myasthenia gravis patients recently treated with chronic IVIG. *Muscle Nerve.* (2018) 58.
58. Jacob S, Guptill J, Meisel A, Fujita K, Patra K, Howard J Jr, et al. Eculizumab reduces myasthenia gravis exacerbation rates. *Muscle Nerve.* (2018) 58:S98.
59. Jaretzki A, Barohn RJ, Ernstoff RM, Kaminski HJ, Keesey JC, Penn AS, et al. Myasthenia gravis: recommendations for clinical research standards. Task force of the medical scientific advisory board of the Myasthenia Gravis Foundation of America. *Neurology.* (2000) 55:16–23. doi: 10.1212/WNL.55.1.16
60. Sanders DB, Wolfe GI, Benatar M, Evoli A, Gilhus NE, Illa I, et al. International consensus guidance for management of myasthenia gravis: executive summary. *Neurology.* (2016) 87:419–25. doi: 10.1212/WNL.0000000000002790
61. Murai H, Utsugisawa K, Nagane Y, Suzuki S, Imai T, Motomura M. Rationale for the clinical guidelines for myasthenia gravis in Japan. *Ann N Y Acad Sci.* (2018) 1413:35–40. doi: 10.1111/nyas.13544
62. Vissing J, Jacob S, Fujita KP, O'Brien F, Howard JF. "Minimal symptom expression" with eculizumab in myasthenia gravis. *Muscle Nerve.* (2018) 58:S97.
63. Howard J, Karam C, Yountz M, O'Brien F, Mozaffar T. Long-term efficacy of eculizumab in refractory generalized myasthenia gravis: responder analyses. *Muscle Nerve.* (2019) 60:S133.
64. Nishimura J, Yamamoto M, Hayashi S, Ohyashiki K, Ando K, Brodsky AL, et al. Genetic variants in C5 and poor response to eculizumab. *N Engl J Med.* (2014) 370:632–9. doi: 10.1056/NEJMoa1311084
65. Huda S, Cao M, De Rosa A, Woodhall M, Rodriguez Cruz PM, Cossins J, et al. SHP2 inhibitor protects AChRs from effects of myasthenia gravis MuSK antibody. *Neurol Neuroimmunol Neuroinflamm.* (2020) 7:645. doi: 10.1212/NXI.0000000000000645
66. Takata K, Stathopoulos P, Cao M, Mané-Damas M, Fichtner ML, Benotti ES, et al. Characterization of pathogenic monoclonal autoantibodies derived from muscle-specific kinase myasthenia gravis patients. *JCI Insight.* (2019) 4:e127167. doi: 10.1172/jci.insight.127167

**Conflict of Interest:** HK is PI of the Rare Disease Network for Myasthenia Gravis (MGNet, NS115054) and received grant 508240 from the Muscular Dystrophy Association; is a consultant for Alnylam Pharmaceuticals, Ra Pharmaceuticals and UCB Pharmaceuticals; and is CEO and CMO of ARC Biotechnology, LLC based on US Patent 8,961,981. JH has received research support from Alexion Pharmaceuticals, Argenx BVBA, the Centers for Disease Control and Prevention

(Atlanta, GA, USA), the Muscular Dystrophy Association, the National Institutes of Health (including the National Institute of Neurological Disorders and Stroke and the National Institute of Arthritis and Musculoskeletal and Skin Diseases), Ra Pharmaceuticals; honoraria/consulting fees from Alexion Pharmaceuticals, argenx BVBA. Ra Pharmaceuticals and Viela Bio, Inc. and non-financial support from Alexion Pharmaceuticals, argenx BVBA, and Ra Pharmaceuticals.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with several of the authors HK and JH.

frontiers in Immunology

REVIEW
published: 10 June 2020
doi: 10.3389/fimmu.2020.01074

# Role of miRNAs in Normal and Myasthenia Gravis Thymus

*Mélanie A. Cron*[1†], *Émilie Guillochon*[1†], *Linda Kusner*[2*] *and Rozen Le Panse*[1*]

[1] Sorbonne University, INSERM, Association Institute of Myology, Center of Research in Myology, Paris, France, [2] Department of Pharmacology and Physiology, The George Washington University, Washington, DC, United States

The thymus, a primary lymphoid organ, provides a complex environment essential for the generation of the T-cell repertoire. Thymic alterations occur during life either in the context of thymic involution upon aging or the pathophysiological context of Myasthenia Gravis (MG). These changes involve complicated regulatory networks, in which microRNAs (miRNAs) are key players. Here, we analyzed the role of miRNAs in thymocyte maturation and differentiation sustained by thymic epithelial cells. We compared data from the literature regarding the role of mouse thymic miRNAs and original data obtained from a human thymic miRnome study. We identified a set of highly expressed miRNAs defined as ThymiRs and investigated miRNA expression in infants as compared to adults to determine those associated with human thymic involution. Thymic changes are also frequently observed in MG, an autoimmune disease which results in the production of anti-acetylcholine receptor (AChR) antibodies that lead to muscle weaknesses. Alterations such as thymoma in late-onset MG patients and hyperplasia with ectopic germinal centers (GCs) in early-onset (EOMG) patients are found. Thymic miRNA expression has been studied in AChR-MG patients both in thymoma-associated MG (TAMG) and EOMG, and their function through their mRNA targets investigated. Most of the dysregulated thymic miRNAs in EOMG are associated with GC development, such as miR-7, miR-24, miR-139, miR-143, miR-145, miR-146, miR-150, miR-452, miR-548 or thymic inflammation, such as miR-125b, miR-146, or miR-29. Understanding these pathways may provide therapeutic targets or biomarkers of disease manifestations.

**Keywords: autoimmunity, thymic involution, germinal center, early-onset myasthenia gravis, thymoma, thymocytes, thymic epithelial cells**

OPEN ACCESS

***Edited by:***
*Zhiguang Zhou,*
*Central South University, China*

***Reviewed by:***
*Renato Mantegazza,*
*Carlo Besta Neurological Institute (IRCCS), Italy*
*Tao Li,*
*National Center of Biomedical Analysis (NCBA), China*

***Correspondence:**
*Linda Kusner*
*lkusner@gwu.edu*
*Rozen Le Panse*
*rozen.lepanse@upmc.fr*

[†] *These authors have contributed equally to this work*

***Specialty section:***
*This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology*

***Received:*** *17 January 2020*
***Accepted:*** *04 May 2020*
***Published:*** *10 June 2020*

***Citation:***
*Cron MA, Guillochon É, Kusner L and Le Panse R (2020) Role of miRNAs in Normal and Myasthenia Gravis Thymus. Front. Immunol. 11:1074. doi: 10.3389/fimmu.2020.01074*

## OVERVIEW OF miRNAs

MicroRNAs (miRNAs) correspond to non-coding short single-stranded RNAs (∼22 nucleotides) that serve as post-transcriptional regulators. They are mainly transcribed and process through the canonical pathway as pri-miRNAs, cleaved into pre-miRNAs by DROSHA and DGCR8 (DiGeorge syndrome Critical Region gene 8) in the nucleus and exported to the cytoplasm by the protein exportin 5. Next, pre-miRNAs are cleaved by DICER and its partner TRBP1 into mature miRNAs. Mature miRNAs coupled with the RNA-induced silencing complex (RISC, a heterogeneous molecular complex) target mRNAs, leading to their degradation or the inhibition of their translation, according to the perfect or imperfect miRNA-mRNA matching, respectively (1). They are involved in physiological and pathophysiological processes, including autoimmune diseases (2).

Autoimmune diseases result from the dysfunction of the immune process. The breakdown of immunological tolerance leads to the presence of autoreactive immune cells which cause the destruction of self (3). miRNAs play crucial roles in the immune process through the development of the immune system, proliferation of key immunological cells, differentiation of cells into their lineage, and apoptosis at immunological checkpoints (4). Disturbance along the process by altered expression of miRNAs and their downstream function can initiate or maintain autoimmune conditions. Involvement of miRNAs, specifically key miRNAs (e.g. miR-21, miR-146, miR-155, miR-146, 125a-5p) is already well-documented in major autoimmune diseases such as lupus erythematosus, multiple sclerosis, diabetes or rheumatoid arthritis. Here, we highlight the significance of miRNAs in the development of the thymus and the ability for dysfunction to result in an autoimmune disease, myasthenia gravis.

## PHYSIOLOGICAL ROLE OF THE THYMUS

The thymus provides a complex environment essential for the generation of the T-cell repertoire. It is composed of various cell types, essentially thymocytes and thymic epithelial cells (TECs), but also fibroblasts, myoid cells, dendritic cells, macrophages, and B cells. Differentiation of thymocytes occurs through interactions with stromal cells while they are progressing in the different thymus compartments (5).

In their first differentiation steps, immature thymocyte precursors become progressively double positive (DP) for CD4 and CD8 co-receptors and acquire a complete T-cell receptor (TCR). Further successful differentiation of thymocytes depends on the quality and the specificity of the interaction of their T-cell receptor (TCR) with self-major histocompatibility complex (MHC) molecules. The large majority of thymocytes die either because the TCR-MHC interaction is too weak (death by neglect) or, in contrast, because the TCR-MHC interaction is too strong (negative selection, self-tolerance). In parallel, positively selected $CD4^+CD8^+$ thymocytes end up single positive (SP) for either CD4 or CD8 (lineage commitment) (5). Only a few thymocytes pass successfully selection and are exported to the periphery where they will differentiate into different T-cell subsets. However, within the thymic environment, some T cells can differentiate into natural regulatory T (Treg) cells (5).

TECs represent the main cell type amongst thymic stromal cells and include cortical and medullary TECs (cTECs and mTECs). mTECs play a central role in negative selection of thymocytes through their capacity to express a wide range of tissue-specific antigens (TSAs) and mediate depletion of autoreactive T cells. The ectopic expression of TSAs by mTECs is controlled by epigenetic factors and by transcription factors, the most well-known being the autoimmune regulator (AIRE) (6). The thymus is fully active during the neonatal period and undergoes an involution process early during life (after 1-year-old). Thymic involution is characterized by the alteration of thymic architecture and the loss of thymic function. In particular, involution is associated with a decrease in TECs, more prominent for mTECs as compared to cTECs, and replacement by fat tissue. Despite the decrease in cellular density, the adult thymus still contains thymocytes and maintains the proportion of the principal thymocyte subsets indicating that the thymus remains active during adult life (7).

**Abbreviations:** AChR, Acetylcholine receptor; AIRE, Autoimmune regulator (AIRE); DN, Double negative; DP, Double positive; EOMG, Early-onset MG; GC, Germinal center; IFN, Interferon; MG, Myasthenia gravis; miRNA, microRNA; NKT, Natural killer T cell; SP, Simple positive; TAMG, Thymoma-associated MG; TCR, T-cell receptor; TEC, Thymic epithelial cell; cTEC, Cortical thymic epithelial cell; mTEC, Medullary thymic epithelial cell; TLR, Toll-Like Receptor; Treg cell, regulatory T cell; TSA, Tissue-specific antigen.

## IMPLICATIONS OF miRNAs IN THE MOUSE AND HUMAN THYMUS

The role of enzymes involved in the biogenesis of miRNAs and specific miRNAs in T-cell lineage in the periphery has been largely documented (8, 9). Here, we will specifically review the role of miRNAs in the thymus, in particular in thymic epithelial cells (TECs) with their potential impact on thymic architecture, and on thymocyte differentiation.

Certain miRNAs are defined as immuno-miRs as they regulate many functions related to the immune system, or myomiRs, which are more particularly expressed in skeletal muscle. The most well-known immuno-miR are miR-146, miR-155 and the cluster miR-17∼92 (10). From original results detailed in **Figure 1** and **Table 1**, we analyzed the human thymus of infants for the most expressed miRNAs that could be defined as ThymiRs (**Figure 1**). The highly expressed human ThymiRs contained the most well-known immuno-miR, such as miR-146, miR-150, miR-155, miR-181 subtypes, some miRNAs from the miR-17∼92 cluster and the paralogous clusters miR-106b-25 and miR-106a-363. They also included six let-7 subtypes (**Table 1**).

### miRNAs and Thymic Epithelial Cells

Different studies have proved that molecules involved in the biogenesis of miRNAs play a central role in TEC differentiation.

Using *FoxN1 (Forkhead box protein N1)*-Cre knock-in mice to conditionally inactivate *Dgcr8* in TECs, Khan et al. demonstrated that *Dgcr8* is critical for maintaining a proper thymic architecture and that canonical miRNAs are required to support TEC cellularity and differentiation. In particular, they observed a progressive loss of $AIRE^+$ mTECs that could affect central tolerance and favor the development of autoimmune diseases (12). Embryonic loss of *Dicer* in TECs results in premature thymic involution, progressive disorganization of the thymic epithelium and the formation of epithelial voids. The normal differentiation and function of TECs are altered, impacting thymocyte development and inducing phenotypic changes in peripheral T cells. Loss of *Dicer* expression in TECs clearly affects T-cell development from the second week of life with an increase in DP and a decrease in $CD8^+$ and $CD4^+$ SP T cells (13). A higher number of double-negative (DN) cells is also observed, in part due to an increased number of immature B cells in the thymus of these *Dicer* deficient mice. They could develop *in situ*, possibly due to changes in the microenvironment as the thymic

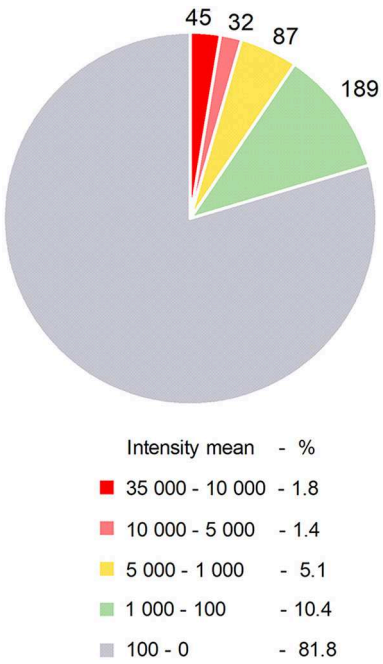



**FIGURE 1 |** Human ThymiRs. Repartition of ThymiRs according to the mean of intensities. Thymic biopsies were collected from female infants undergoing cardiovascular surgery at the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France) ($n = 6$, 3–12 months old). Studies on thymuses were approved by a local ethics committee (CPP, authorization number ID RCB 2010-A00250-39). Total RNA extraction and miRNA analyses were done as described in Cron et al. (11). Raw data were imported using R package pd.mirna.3.0 (v 3.12.0). Raw intensity values were background corrected and normalized with RMA (Robust Multi-array Average) function from oligo R package (1.48.0).

medulla progressively displayed more Lyve-1 lymphatic vessels, PNAd high endothelial venules, and CR1 follicular dendritic cells that are normally observed in secondary lymphoid tissues (13, 14). Besides, several publications showed a decrease in both *Aire*-dependent and -independent TSAs in *Dicer*-deficient mTECs (15); altered expression of TSAs in mice that lack *Dicer* expression in TECs could be correlated with multiorgan infiltrations (13).

Papadopoulou et al. demonstrated that certain features of the premature thymic involution phenotype of *Dicer* mutants are recapitulated in mouse mutants lacking *miR-29a*; however, these animals do not display the same architectural disruption of the thymus, implying that other miRNAs regulate TEC maintenance (14). Besides, the absence of *miR-29a* selectively affects the *Aire*-dependent TSA gene expression (15). However, an increase in thymic *miR-29* subtypes is observed together with thymic involution in aging mice (16).

The changes observed in *Dicer* or *Dgcr8* deficient mice are usually attributed to the loss of miRNAs. However, we have to keep in mind that DICER can process other types of RNAs and regulate different cellular functions beyond its endonuclease activity (17). As for DGCR8, it is involved in maintaining heterochromatin organization and attenuating senescence, independently of its microRNA-processing activity (18).

**TABLE 1 |** Human ThymiRs.

| Name | Intensity mean | SEM |
|---|---|---|
| hsa-miR-16 | 42,177 | 995 |
| hsa-miR-181a | 39,264 | 987 |
| hsa-miR-26a | 36,825 | 751 |
| hsa-miR-17 | 32,468 | 245 |
| hsa-miR-106a | 30,695 | 134 |
| hsa-miR-20a | 29,018 | 241 |
| hsa-miR-3960 | 28,997 | 3,351 |
| hsa-miR-3665 | 26,827 | 2,385 |
| hsa-miR-92a | 26,734 | 560 |
| hsa-miR-181b | 26,425 | 657 |
| hsa-miR-103a | 23,522 | 459 |
| hsa-miR-4787-5p | 23,473 | 2,736 |
| hsa-miR-342-3p | 23,080 | 453 |
| hsa-let-7a | 22,697 | 1,680 |
| hsa-let-7b | 22,524 | 1,506 |
| hsa-miR-93 | 21,822 | 810 |
| hsa-miR-191 | 21,059 | 726 |
| hsa-let-7c | 20,657 | 573 |
| hsa-miR-205 | 20,509 | 806 |
| hsa-miR-107 | 20,000 | 367 |
| hsa-miR-125b | 19,037 | 722 |
| hsa-miR-106b | 17,449 | 652 |
| hsa-let-7d | 16,420 | 412 |
| hsa-miR-4497 | 16,199 | 903 |
| hsa-miR-20b | 15,256 | 473 |
| hsa-miR-150 | 15,058 | 415 |
| hsa-miR-19b | 14,736 | 1,521 |
| hsa-miR-155 | 14,724 | 401 |
| hsa-miR-4466 | 14,691 | 1,656 |
| hsa-miR-1915 | 13,850 | 1,881 |
| hsa-miR-4488 | 13,612 | 1,872 |
| hsa-miR-638 | 13,379 | 1,563 |
| hsa-miR-24 | 13,138 | 957 |
| hsa-miR-15b | 12,644 | 1,160 |
| hsa-miR-23a | 12,537 | 170 |
| hsa-let-7i | 12,007 | 1,014 |
| hsa-miR-3656 | 11,829 | 2,541 |
| hsa-miR-320b | 11,481 | 1,282 |
| hsa-miR-320a | 11,370 | 1,221 |
| hsa-miR-2861 | 10,996 | 1,367 |
| hsa-miR-100 | 10,871 | 313 |
| hsa-miR-200c | 10,783 | 630 |
| hsa-miR-15a | 10,574 | 1,133 |
| hsa-miR-4516 | 10,522 | 1,110 |
| hsa-miR-320c | 10,430 | 1,061 |

*List of the most highly expressed ThymiRs with a mean intensity over 10,000. The origin of data is detailed in **Figure 1**. Raw data were imported using R package pd.mirna.3.0 (v 3.12.0). Raw intensity values were background corrected and normalized with RMA (Robust Multi-array Average) function from oligo R package (1.48.0). Intensity means and standard error of the mean (SEM) were calculated from the normalized intensity values obtained using Limma R package (v 3.40.6).*

Ucar et al. analyzed the expression of miRNAs in isolated cTECs and mTECs in human and mouse thymuses. They demonstrated that certain miRNAs are differentially expressed in cTECs and mTECs, and even differentially expressed upon mTEC maturation. Using different approaches for selected miRNAs they observed that in mice some miRNAs are down-regulated in $CD80^+AIRE^+$mTECs as compared to $CD80^+AIRE^-$mTECs. This suggests that TEC differentiation could be associated with a decreased expression of certain miRNAs allowing, for example, a higher expression of AIRE. Inversely, AIRE regulates the expression of specific miRNAs. They showed in AIRE-deficient mice that some miRNAs can be either up or down-regulated in $CD80^+$mTECs as compared to wild type mice (15). This was confirmed by Macedo et al. that demonstrated that silencing *Aire* in mouse mTECs leads to the up- and down-regulation of specific miRNAs (19). The identified dysregulated miRNAs from these studies were different. However, we can hypothesize that in $AIRE^+$TECs, miRNAs that are decreased could lead to the specific expression of TSAs that are implicated in central tolerance mechanisms.

## miRNAs and Thymocyte Development

*Dicer* deletion from the double-positive stage of T-cell development compromises the survival of αβ lineage cells and results in a decreased thymic cellularity in DP and SP T cells. Surprisingly, *Dicer* seems to be dispensable for $CD4^+$ and $CD8^+$ T cell lineage commitment (20, 21). *Dicer* or *Drosha* deletion at a later stage in $CD4^+$ T cell does not alter the number and composition of thymocytes, though it results in a reduction in thymic $CD4^+CD25^+Foxp3^+$ natural Treg cells. *Dicer* depletion in $CD4^+$ thymocytes also results in the reduction of invariant natural killer T (iNKT) cells (22). Besides, mice with T-cell specific *Dicer* or *Drosha* deficiency develop immune pathologies, in particular, inflammatory bowel disease (23) and organ inflammation (24). Deletion of *Dicer* or *Drosha* results in the loss of mature miRNAs generated via the canonical biogenesis pathways in which not all miRNAs are deleted since other biogenesis pathways are involved (1). Nevertheless, these results demonstrate that miRNAs can modulate T-cell development. Different studies have shown that individual miRNAs are dynamically regulated during T-cell development and maturation as detailed below (25–27).

## Implication of miRNAs in Thymic Involution

Thymic involution is a natural process occurring with age and an adaptive process in response to stress situations. Thymic involution is characterized by morphological and functional changes and includes TEC-driven programmed thymic involution and thymocyte apoptosis. As miRNAs regulate numerous physiological and pathophysiological processes, they are probably key orchestrators of thymic involution.

### Thymic Involution With Aging

The expression of miRNAs is clearly modified in the aging mouse thymus (16). Guo et al. investigated thymic miRNA expression in 3 and 12 months old female and male mice as thymic involution is often described as being also sex-hormone dependent. Thymic atrophy is clearly observed in the first year of life, and even more in males as compared to females. Specific miRNAs exhibit age- or sex-differentially expression. Among them, *miR-2137* is increased in the aging thymus and particularly in mTECs (28). Some miRNAs seem dysregulated more specifically in female mice and to determine if the female-biased miRNAs were linked to estrogen, the expression of specific miRNAs was measured in the thymus either in ovariectomized or castrated mice treated with estradiol. Results show that *miR-27b* and *miR-378a* are estrogen-responsive miRNAs in mouse thymus (28).

miRNAs expression has also been investigated in isolated TECs from aging-mice and 17 miRNAs were showed to be down-regulated upon aging. In particular, miRNAs known to possess immune function, such as *miR-146*, *miR-150*, *miR 155*, *miR-181* subtypes and some miRNAs from the *miR-17∼92* cluster. The decreases observed in TECs are correlated with the age-related thymic weight loss suggesting that miRNA decreased expression in TECs precedes thymic involution (29).

In human thymus, we analyzed dysregulated thymic miRNAs in female adults (15–33 years old) compared to female infants (3–12 months old). Of the miRNAs assessed, 56 were up-regulated and 87 were down-regulated in the adult thymuses upon aging, respectively (**Tables 2A,B**). From these lists of dysregulated miRNAs in the course of thymic involution, among the up-regulated ones, those that seemed particularly interesting were those that reached in adults a fluorescence intensity mean above 1,000 (arbitrarily chosen). These results must be taken into account with caution due to the small sample size and the lack of validation. However, several of them, such as let-7b, miR-139, miR-193a, miR-214, miR-27b are also up-regulated in the thymus of male mice upon aging (28). miR-182 and miR-200b are also found up-regulated in isolated TECs from aging mice (30). miR-195a-5p is highly up-regulated in the TECs isolated from the aging mice inhibiting the proliferation of mTECs by directly targeting Smad7 (31). As for miR-451, its expression is up-regulated in the thymus of systemic lupus erythematosus mouse (32).

For the down-regulated miRNAs (**Table 2B**), we observed that miR-181 subtypes are significantly down-regulated with aging (see the miR-181 paragraph and **Figure 2A** below for more details). Besides, among miRNAs that had a fluorescence intensity mean above 1,000 in infants and that significantly decreased with aging, the majority of them belong to the miR-17∼92 cluster or the paralogous miR-106a∼363 cluster (33).

### Thymic Involution Linked to Stress

Thymic involution is also observed upon pathogen infections. This could be mediated via the increased expression of interferon (IFN)-I subtypes that will target thymic different cells. Loss of the miRNAs through the deletion of *Dicer* or the *miR-29a* cluster in TECs results in elevated sensitivity of TECs to Poly(I:C), a molecule mimicking dsRNA from viral infection, and premature thymic involution. In mice, *miR-29a* regulates the expression of IFNAR1 (one of the subunits of the IFN-I receptor) and consequently controls the ability of TECs to respond to

**TABLE 2 |** Dysregulated miRNAs during human thymic involution.

| miRNA ID | Intensity mean | | FC | *p*-value |
|---|---|---|---|---|
| | Infants | Adults | | |
| **(A) Up-regulated miRNAs in adults vs. infants (FC ≥ 1.5, $p \leq 0.05$)** | | | | |
| hsa-miR-383 | 9 | 182 | 20.32 | 9.88E-06 |
| **hsa-miR-486-5p** | 337 | 1,475 | 4.38 | 6.59E-03 |
| **hsa-miR-451** | 485 | 1,489 | 3.07 | 1.93E-02 |
| hsa-miR-486-3p | 14 | 41 | 3.01 | 4.25E-03 |
| **hsa-miR-3609** | 565 | 1,684 | 2.98 | 1.68E-03 |
| hsa-miR-34c-3p | 105 | 284 | 2.72 | 1.97E-03 |
| **hsa-miR-214** | 871 | 2,292 | 2.63 | 2.29E-02 |
| hsa-miR-375 | 124 | 309 | 2.48 | 1.64E-03 |
| **hsa-miR-206** | 749 | 1,794 | 2.39 | 1.85E-02 |
| hsa-miR-494 | 350 | 831 | 2.37 | 1.42E-02 |
| hsa-miR-139-3p | 35 | 82 | 2.32 | 7.48E-03 |
| **hsa-miR-149** | 865 | 2,000 | 2.31 | 7.31E-04 |
| hsa-miR-134 | 69 | 158 | 2.29 | 9.41E-03 |
| hsa-miR-650 | 16 | 37 | 2.26 | 1.99E-02 |
| hsa-miR-224* | 72 | 160 | 2.21 | 3.13E-03 |
| hsa-miR-139-5p | 356 | 781 | 2.20 | 1.14E-03 |
| hsa-miR-30c-2* | 10 | 22 | 2.17 | 6.75E-03 |
| **hsa-miR-182** | 2,116 | 4,534 | 2.14 | 5.98E-03 |
| hsa-miR-143* | 27 | 57 | 2.09 | 1.45E-02 |
| hsa-miR-3605-5p | 17 | 34 | 2.07 | 2.79E-03 |
| hsa-miR-664* | 141 | 291 | 2.06 | 1.62E-03 |
| **hsa-miR-195** | 4,561 | 9,012 | 1.98 | 4.33E-05 |
| hsa-miR-4788 | 18 | 35 | 1.94 | 3.03E-04 |
| hsa-miR-572 | 145 | 280 | 1.93 | 5.30E-03 |
| hsa-miR-10b | 308 | 593 | 1.93 | 5.24E-03 |
| hsa-miR-3607-5p | 102 | 197 | 1.92 | 6.24E-04 |
| hsa-miR-584 | 10 | 19 | 1.90 | 2.08E-02 |
| hsa-miR-34c-5p | 132 | 247 | 1.87 | 3.53E-03 |
| **hsa-miR-145** | 7,014 | 13,024 | 1.86 | 1.68E-03 |
| hsa-miR-125a-3p | 52 | 96 | 1.84 | 2.21E-02 |
| hsa-miR-4496 | 8 | 15 | 1.83 | 2.13E-02 |
| hsa-miR-193a-5p | 357 | 650 | 1.82 | 1.15E-02 |
| **hsa-let-7e** | 3,651 | 6,563 | 1.80 | 1.50E-02 |
| **hsa-let-7b** | 22,524 | 39,436 | 1.75 | 6.66E-05 |
| hsa-miR-200b* | 449 | 784 | 1.75 | 2.44E-03 |
| hsa-miR-30a* | 90 | 155 | 1.73 | 1.30E-02 |
| hsa-miR-492 | 7 | 12 | 1.73 | 4.09E-02 |
| hsa-miR-4324 | 36 | 62 | 1.72 | 4.38E-02 |
| hsa-miR-4657 | 10 | 17 | 1.71 | 4.90E-02 |
| **hsa-miR-455-3p** | 3,410 | 5,800 | 1.70 | 3.35E-02 |
| hsa-miR-4778-5p | 7 | 12 | 1.70 | 9.78E-03 |
| hsa-miR-1202 | 78 | 133 | 1.69 | 3.37E-02 |
| hsa-miR-4800-5p | 25 | 42 | 1.67 | 1.95E-02 |
| hsa-miR-379 | 180 | 299 | 1.66 | 1.91E-02 |
| **hsa-miR-4530** | 3,746 | 6,216 | 1.66 | 1.96E-02 |
| hsa-miR-4738-3p | 32 | 54 | 1.66 | 2.33E-03 |
| hsa-miR-4667-5p | 56 | 92 | 1.64 | 2.81E-03 |
| hsa-miR-29b-1* | 56 | 90 | 1.61 | 1.17E-02 |
| hsa-miR-23b* | 16 | 26 | 1.60 | 4.04E-02 |
| **hsa-miR-34a** | 1,415 | 2,225 | 1.57 | 9.55E-04 |
| hsa-miR-1 | 6 | 9 | 1.57 | 1.32E-03 |
| hsa-miR-339-3p | 409 | 636 | 1.56 | 4.02E-02 |
| **hsa-miR-27b** | 1,818 | 2,825 | 1.55 | 1.94E-02 |
| **hsa-miR-143** | 5,850 | 9,013 | 1.54 | 2.24E-02 |
| hsa-miR-3127-5p | 8 | 12 | 1.53 | 2.77E-02 |
| **hsa-miR-4505** | 867 | 1,311 | 1.51 | 4.28E-02 |
| **(B) Down-regulated miRNAs in adults vs. infants (FC ≤ −1.5, $p \leq 0.05$)** | | | | |
| hsa-miR-301a | 310 | 55 | −5.62 | 9.13E-03 |
| hsa-miR-449b | 70 | 12 | −5.61 | 1.03E-03 |
| hsa-miR-142-3p | 80 | 15 | −5.30 | 8.44E-04 |
| hsa-miR-449a | 70 | 14 | −4.90 | 1.65E-03 |
| hsa-miR-142-5p | 269 | 68 | −3.94 | 4.59E-03 |
| hsa-miR-297 | 69 | 19 | −3.57 | 4.02E-02 |
| hsa-miR-4793-3p | 76 | 22 | −3.41 | 4.74E-02 |
| hsa-miR-551a | 32 | 10 | −3.18 | 7.07E-04 |
| hsa-miR-590-5p | 36 | 12 | −2.96 | 4.83E-02 |
| hsa-miR-15a* | 52 | 18 | −2.91 | 3.14E-04 |
| hsa-miR-424 | 56 | 20 | −2.86 | 5.79E-03 |
| hsa-miR-502-5p | 32 | 12 | −2.71 | 6.54E-03 |
| hsa-miR-3907 | 24 | 9 | −2.71 | 7.61E-03 |
| hsa-miR-598 | 16 | 6 | −2.62 | 7.05E-03 |
| hsa-miR-550a* | 113 | 43 | −2.61 | 6.50E-04 |
| hsa-miR-542-5p | 139 | 54 | −2.55 | 6.75E-03 |
| **hsa-miR-19a** | 1,019 | 406 | −2.51 | 3.46E-03 |
| hsa-miR-19b-1* | 40 | 16 | −2.49 | 1.38E-02 |
| hsa-miR-449c | 37 | 15 | −2.48 | 4.13E-03 |
| hsa-miR-641 | 143 | 58 | −2.48 | 5.63E-04 |
| hsa-miR-4440 | 139 | 59 | −2.37 | 3.05E-02 |
| hsa-miR-301b | 10 | 4 | −2.29 | 4.94E-03 |
| **hsa-miR-18b** | 1,711 | 752 | −2.28 | 6.44E-06 |
| **hsa-miR-15a** | 10,574 | 4,666 | −2.27 | 1.66E-03 |
| hsa-miR-140-5p | 773 | 342 | −2.26 | 7.22E-03 |
| hsa-miR-3201 | 21 | 10 | −2.21 | 1.42E-02 |
| **hsa-miR-141** | 1,840 | 838 | −2.20 | 4.19E-03 |
| **hsa-miR-181a*** | 2,719 | 1,239 | −2.19 | 4.93E-05 |
| hsa-let-7g* | 62 | 28 | −2.17 | 3.14E-02 |
| hsa-miR-16-1* | 8 | 4 | −2.16 | 3.51E-03 |
| hsa-miR-3064-3p | 57 | 27 | −2.15 | 1.02E-03 |
| hsa-miR-589 | 15 | 7 | −2.14 | 1.68E-02 |
| hsa-miR-503 | 405 | 190 | −2.13 | 1.04E-02 |

*(Continued)*

**TABLE 2** | Continued

| miRNA ID | Intensity mean | | FC | *p*-value |
|---|---|---|---|---|
| | Infants | Adults | | |
| hsa-miR-122 | 67 | 31 | −2.13 | 3.20E-03 |
| hsa-miR-4286 | 147 | 70 | −2.10 | 1.52E-02 |
| hsa-miR-5096 | 27 | 13 | −2.09 | 3.94E-02 |
| hsa-miR-362-3p | 22 | 11 | −2.09 | 3.72E-02 |
| hsa-miR-431 | 17 | 8 | −2.05 | 8.44E-03 |
| hsa-miR-4786-5p | 37 | 18 | −2.03 | 4.76E-02 |
| hsa-miR-330-5p | 17 | 8 | −2.00 | 6.59E-03 |
| hsa-miR-3620 | 29 | 15 | −1.99 | 5.08E-03 |
| hsa-miR-185* | 25 | 13 | −1.94 | 2.05E-02 |
| hsa-miR-4787-3p | 19 | 10 | −1.93 | 1.41E-02 |
| hsa-miR-196b* | 264 | 137 | −1.92 | 2.52E-03 |
| hsa-miR-484 | 216 | 114 | −1.90 | 1.71E-04 |
| hsa-miR-1306 | 24 | 13 | −1.90 | 1.37E-02 |
| hsa-miR-637 | 45 | 24 | −1.89 | 4.67E-02 |
| hsa-miR-551b | 22 | 11 | −1.88 | 4.24E-02 |
| hsa-miR-612 | 18 | 10 | −1.88 | 8.73E-03 |
| hsa-let-7d* | 35 | 18 | −1.87 | 1.06E-02 |
| hsa-let-7i* | 67 | 36 | −1.85 | 2.79E-03 |
| hsa-miR-1915* | 39 | 21 | −1.83 | 4.31E-02 |
| hsa-miR-3157-5p | 37 | 20 | −1.82 | 4.62E-03 |
| **hsa-miR-128** | 6,129 | 3,418 | −1.79 | 7.24E-03 |
| **hsa-miR-181d** | 2,103 | 1,178 | −1.79 | 4.14E-03 |
| hsa-miR-130b* | 12 | 7 | −1.77 | 2.13E-02 |
| **hsa-miR-19b** | 14,736 | 8,370 | −1.76 | 1.26E-02 |
| **hsa-miR-210** | 1,280 | 731 | −1.75 | 1.82E-02 |
| hsa-miR-129-5p | 13 | 8 | −1.74 | 3.28E-02 |
| hsa-miR-760 | 25 | 14 | −1.74 | 4.16E-02 |
| hsa-miR-4493 | 6 | 4 | −1.73 | 1.87E-02 |
| hsa-miR-1229 | 13 | 8 | −1.72 | 1.76E-03 |
| **hsa-miR-363** | 4,120 | 2,399 | −1.72 | 3.07E-02 |
| hsa-miR-4763-5p | 34 | 20 | −1.72 | 9.15E-03 |
| **hsa-miR-18a** | 6,309 | 3,714 | −1.70 | 9.64E-03 |
| **hsa-miR-30e** | 2,171 | 1,282 | −1.69 | 1.91E-02 |
| hsa-miR-4323 | 31 | 18 | −1.69 | 5.58E-03 |
| hsa-miR-4746-3p | 15 | 9 | −1.67 | 1.97E-02 |
| **hsa-miR-106b** | 17,449 | 10,554 | −1.65 | 4.93E-03 |
| hsa-miR-3928 | 33 | 20 | −1.65 | 1.48E-02 |
| hsa-miR-675* | 15 | 9 | −1.64 | 9.47E-03 |
| hsa-miR-4284 | 262 | 163 | −1.61 | 1.46E-02 |
| hsa-miR-3610 | 19 | 12 | −1.61 | 1.72E-02 |
| hsa-miR-4457 | 8 | 5 | −1.60 | 2.84E-02 |
| hsa-miR-361-3p | 78 | 49 | −1.60 | 4.66E-02 |
| hsa-miR-18a* | 375 | 236 | −1.59 | 1.60E-03 |
| **hsa-miR-130b** | 7,565 | 4,770 | −1.59 | 3.91E-02 |
| hsa-miR-4478 | 42 | 27 | −1.58 | 4.53E-02 |
| hsa-miR-3180 | 438 | 280 | −1.57 | 3.58E-02 |
| **hsa-miR-17*** | 1,384 | 890 | −1.56 | 1.75E-03 |
| **hsa-miR-130a** | 5,672 | 3,709 | −1.53 | 2.13E-02 |
| hsa-miR-20b* | 229 | 150 | −1.52 | 9.11E-03 |
| hsa-miR-636 | 20 | 13 | −1.52 | 2.70E-02 |
| hsa-miR-3145-5p | 29 | 19 | −1.52 | 1.97E-02 |
| hsa-miR-1304 | 12 | 8 | −1.51 | 1.19E-02 |
| hsa-miR-769-5p | 285 | 188 | −1.51 | 2.86E-03 |
| hsa-miR-1226 | 10 | 7 | −1.51 | 2.61E-02 |

*Thymic biopsies were collected from female donors undergoing cardiovascular surgery at the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France): infants (n = 6, 3–12 months old) and adults (n = 6, 15-33 years old). Studies on thymuses were approved by a local ethics committee (CPP, authorization number ID RCB 2010-A00250-39). Total RNA extraction was done as described in Cron et al. (11). miRNA expression was measured using the Affymetrix GeneChip miRNA 3.0 Array (Santa Clara, USA) by the Genopolis consortium (Milano, Italy).*

*Raw data were imported using R package pd.mirna.3.0 (v 3.12.0). Raw intensity values were background corrected and normalized with RMA (Robust Multi-array Average) function from oligo R package (1.48.0). Differential expression analysis was performed using Limma R package (v 3.40.6). Limma fits a linear model to expression data for each miRNA and Empirical Bayes method was used to generate differential expression statistics. The tables list mature miRNAs up- (A) or down-(B) regulated in adults as compared to infants with a fold change (FC) set at 1.5 and a p <= 0.05. In bold miRNAs of particular interest regarding their mean intensity value. (A), miRNAs at least above 1,000 in adults. (B), miRNAs at least above 1,000 in infants. The mean intensity values are coded as follow: 40,000–10,000 (red), 10,000–5,000 (pink), 5,000–1,000 (orange), 1,000–100 (green), below 100 (gray).*

pathogen infections and IFN-I signalization (14). Linhares-Lacerda et al. observed the up-regulation of miRNAs in mouse TECs upon *Trypaosoma cruzi* infection but not that of *miR-29* subtypes (34).

Glucocorticoids can also induce thymic involution, mainly by triggering DP T cell apoptosis. Upon thymocytes exposure to glucocorticoids, a decrease expression of *Drosha*, *Dicer*, and *Dgcr8* is observed and subsequently a decreased expression of miRNAs such as the miRNAs of the *miR-17∼92* cluster (35, 36). Smith et al. demonstrated that *Dicer* depletion induces T-cell apoptosis while the overexpression of the *miR-17∼92* cluster reduces it (35).

## Focus on miRNAs Clearly Involved in the Thymic Functions

### Let-7 miRNAs

The let-7 miRNAs were among the first to be identified in mammals and they represent the most abundant miRNAs in the genome. The let-7 miRNAs consists of 12–14 members encoded on different chromosomes. They contain the identical seed region interacting with mRNAs. Mature functional let-7 miRNA expression is inhibited by the RNA binding protein LIN28 expressed in hematopoietic stem cells (37). An increase of several *let-7* family members is observed together with thymic involution in the thymus of aging mice (16). An increased expression was also observed for numerous let-7 members in female human thymus of adults as compared to infants (**Figure 2A**).

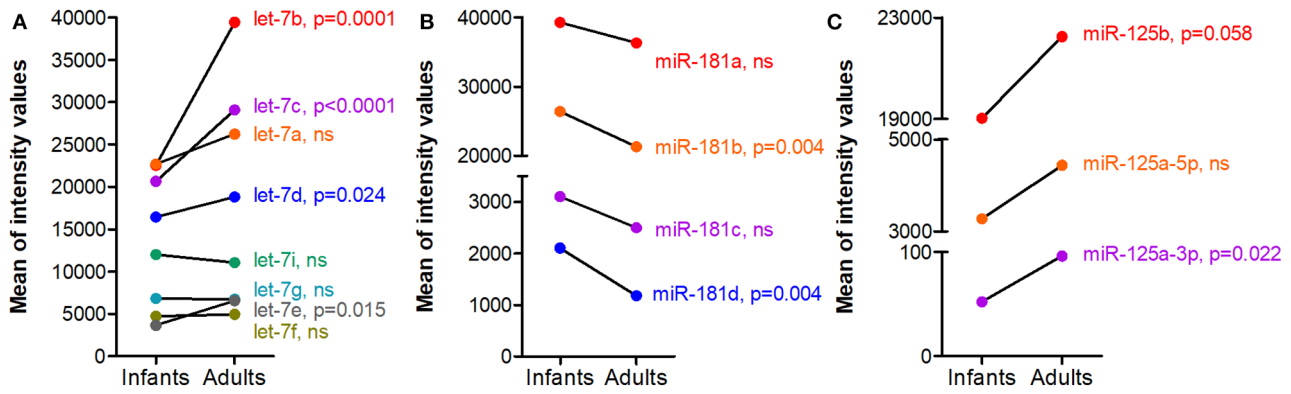



**FIGURE 2 |** Dysregulated miRNAs during human thymic involution. Thymic biopsies were collected and processed for miRNA analyses as detailed in **Figure 1**. Mean of normalized intensity values were calculated for each miRNA for infants ($n = 6$, 3–12 months old females) and adults ($n = 6$, 15–33 years old females) data. **(A)** let-7, **(B)** miR-181, and **(C)** miR-125 families. Differential expression analysis was performed using Limma R package (v 3.40.6). Limma fits a linear model to expression data for each miRNA and Empirical Bayes method was used to generate differential expression statistics: *p*-values and fold-change values.

Pobezinsky et al. investigated the role of *let-7* miRNAs in mouse thymic T-cell differentiation. They demonstrated that *let-7* miRNAs control the expression of the transcription factor Plzf (promyelocytic leukemia zinc finger) that regulates NKT-cell differentiation (38). Recently Xiao et al. demonstrated the role played by *let-7* miRNAs in the limitation of the number of B cells within the thymus. They used a mouse model, designated as Foxn1lacZ, in which the expression of the critical TEC-specific transcription factor *Foxn1* is normal at fetal stages, but down-regulated beginning at postnatal day 7, causing progressive reduction of total thymocytes and premature thymic involution. Early in life in *Foxn1* deficient mice, TECs control the up-regulation of *let-7b* and *let-7g* in hematopoietic stem cells, suppressing *Arid3a* expression in intrathymic B cell progenitors to limit their proliferation during the neonatal to adult transition (39).

In human, let-7a expression is highly up-regulated in mature peripheral $CD4^+$ and $CD8^+$ T lymphocytes compared to DP thymocytes. Microarray analyses showed the up-regulation of let7-e, let7-g and let-7f in $CD4^+$ and $CD8^+$ SP thymocytes compared to DP thymocytes and a differential expression for let-7e between $CD8^+$ and $CD4^+$ SP thymocytes (26).

## miR-181a

miR-181a is a member of miR-181 family that includes 6 miRNAs: miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c, and miR-181d (miR-181a-1 and miR-181a-2, as well as miR-181b-1 and miR-181b-2 being identical). In mice, *miR-181a* is highly expressed in DP thymocytes and controls the development of early thymocytes by targeting *TCR* and *CD69* (40, 41). Besides, *miR-181a* deletion impairs the development of NKT cells (42). Li et al. suggest that *miR-181a* acts as an intrinsic antigen sensitivity "rheostat" during T-cell development (40). Thymic *miR-181a* plays a central role in central tolerance and is important for the elimination of autoreactive T cells (41). In the *miR-181* family, *miR-181d* is one of the most stress-responsive miRNA identified in the thymus upon LPS injections. However, the different *miR-181* subtypes could have overlapping or compensatory functions, at least in response to stress (36). In human, Ghisi et al. showed that miR-181d and miR-181c were strongly down-regulated in $CD4^+$ as compared to $CD8^+$ SP thymocytes (26).

The expression of *miR-181a* and *miR-181b* is down-regulated in old mice compared to young ones (16) and all the miR-181 subtypes are also decreased in female human thymus of adults as compared to infants (**Figure 2B**). If *miR181a* deficiency disrupts thymocytes development, Stefanski et al. demonstrated that *miR-181a1* and *miR-181b1* are not required for maintenance of thymus integrity and that *miR-181a1* and *miR-181b1* are dispensable for TEC differentiation. They control thymocyte development and mature T-cell export and homeostasis within the periphery (43). However, Guo et al. showed that *miR-181a* is decreased in mTECs from aging mice in link with thymic involution. By transfecting mTECs (MTEC1) with a *miR-181a* mimic, they observed that it induces mTEC proliferation while its antagomiR reversed this effect. *miR-181a* was shown to target transforming growth factor β receptor (*Tgfbr1*). *Tgfbr1* expression increases with age in mice, which is consistent with the decreased level of *miR-181a* in addition to the ability of TGF-β to decrease the proliferation of mTECs (44). Recently, Cotrim-Sousa et al. suggest that *miR181b* and *miR-30b* modulate the expression of adhesion molecules involved in mTEC and thymocyte interactions (45).

## miR-150

First described in 2007 by Zhou et al. in hematopoietic stem cells (46), miR-150 is described as an immuno-miR regulating immune functions, such as proliferation, apoptosis and differentiation of NK, T and B cells. The role of miR-150 has especially being studied in peripheral blood cells.

In the thymus of mice, two different studies showed that *miR-150* expression decreases in mTECs from aging mice in link with thymic involution (28, 29). *miR-150* differentially controls the development of NK and iNKT cell lineages by targeting

*c-Myb* (47). In transgenic mice overexpressing *miR-150*, the development of thymocytes is partially blocked, especially the differentiation of DN3 to DN4, which ultimately leads to a decrease in the number of $CD4^+$ and $CD8^+$ SP thymocytes (48). An increased expression of *miR-150* is observed during the maturation of iNKT cells in the mouse thymus and *miR-150* deletion results in an interruption of iNKT cell final maturation in both thymus and periphery (49).

From transcriptomic analyses on purified human or mouse thymocytes and TECs, it was observed that miR-150 is highly expressed in thymocytes compared to mTECs or cTECs (15). In the human thymus, Ghisi et al. showed that miR-150 is upregulated as the maturation of T cell progresses. They showed that miR-150 targets NOTCH3, known to be important for T-cell differentiation, and that forced expression of miR-150 reduces NOTCH3 levels in T-cell lines affecting their proliferation and survival (26).

### miR-146

miR-146 family consists of two miRNAs with nearly identical sequences, miR-146a and miR-146b. They are potent immune-miRs and their role in peripheral blood cells has been largely investigated (50).

In the mouse thymus, *miR-146a* expression fluctuates in thymocytes at different developmental stages, increasing in $CD4^+$ and $CD8^+$ SP thymocytes compared DP thymocytes (51). The overexpression of *miR-146a* in transgenic mice impairs the process of positive selection during T-cell development and inhibits the further differentiation of selected SP (especially $CD8^+$ SP) thymocytes. The authors further identified 9 positive selection-associated genes, which are downregulated in *miR-146a* transgenic mice, such as genes encoding major histocompatibility complex class I/II molecules, *IL-7* receptor α chain, and *Gimap4* (52). However, in transgenic mice with a specific $CD4^+$-driven T cell overexpression, T-cell development occurs normally in the *mir-146a* transgenic mice and a decrease of $CD4^+$ T-cells is observed together with a reduction in surface expression of CD62L, which is normally upregulated upon thymic egress. Besides, *miR-146a* and *miR-146b* target *Traf6* and attenuate TCR signaling in the thymus (53).

FoxP3 is an indispensable transcription factor for the generation and the regulatory function of regulatory T cells. *miR-146a* does not seem critical to the formation of Treg cells in the thymus. Indeed, the role of *miR-146a* has been investigated in $FoxP3^+$ Treg cells in the thymus and peripheral lymphoid organs of *miR-146a*-deficient mice. These mice contain significantly increased numbers of $FoxP3^+$ Treg cells in the periphery, but not in the thymus (54).

In the human thymus, it was shown that miR-146a and miR-146b are the most up-regulated miRNAs as DP thymocytes differentiate in SP $CD4^+$ or $CD8^+$ thymocytes (26).

### miR-155

miR-155 is a highly studied immuno-miR and plays an important role in T-cell homeostasis as fully reviewed by Mashima (55).

In the mouse thymus, the absence of *miR-155* in hematopoietic cells tends to decrease the global representation of thymocytes but it is likely due to a defect occurring at an early stage of thymocyte development, as the proportions of DN, DP, and $CD4^+$ and $CD8^+$ SP cells are not affected. However, mouse thymic expression of *miR-155* seems to fluctuate in thymocytes at different developmental stages in the thymus (26, 51). In the absence of *miR-155*, a reduction of the number of Treg cells is observed both in the thymus and in the periphery in mice. miR-155 appears to be required for Treg cells development and its absence compromises their proliferation, survival and function in the periphery (56). It was demonstrated that an increased expression of miR-155 in both human and mouse $CD4^+$ T helper cells leads to a reduced susceptibility of Treg cells in mediating T-cell suppression, whereas a decreased expression of miR-155- results in a more pronounced suppression by natural Treg cells (57). The control of *miR-155* is also required for the proper development of iNKT cells in the mouse thymus with inhibition of *miR-155* expression along with iNKT cell differentiation (58).

The role of *miR-155* in TECs has not been investigated but its expression decreases with age-induced thymic involution in mice and this might be related to a decreased expression in TECs (29).

### miR-125

miR-125a and miR-125b sequences are highly conserved throughout diverse species. They have been found differentially regulated in several human disorders (50). They could be involved in inflammatory diseases. miR-125a regulates TNFAIP3 that is a negative regulator of the NF-κB signaling pathway (59). miR-125b targets TRAF6 and regulates IL-1β induced inflammatory genes (60).

*miR-215a* and *miR-125b* are up-regulated in the thymus of aging mice (16, 61) and also in the aging human thymus (**Figure 2C**). *miR-125a* by targeting *FoxN1*, decreases its expression associated with thymic involution (61). From transcriptomic analyses on purified human or mouse thymocytes and TECs, it was observed that miR-125a and miR-125b are highly expressed in TECs compared to thymocytes (15). Besides, in human thymocytes, miR-125b expression is higher in $CD4^+$ as compared to $CD8^+$ SP thymocytes (26).

## PATHOLOGICAL PROPERTIES OF THE THYMUS FROM MYASTHENIA GRAVIS PATIENTS

Myasthenia Gravis (MG) is an autoimmune disease due to antibodies against several components of the neuromuscular junction. Patients suffer from more or less invalidating muscle weaknesses leading to a generalized fatigability. The majority of patients (85%) displays antibodies against the acetylcholine receptor (AChR). Thymus abnormalities occur in two subtypes of $AChR^+$ MG patients, thymoma-associated (TAMG) and $AChR^+$ early-onset form (EOMG) (62).

The incidence of TAMG among the MG population is approximately 10-20% (62, 63). Thymomas are rare thymic epithelial cell neoplasms that develop usually in patients after 50

years old, both in women and men. The histological classification by the World Health Organization is based on the nature of the cortical or medullary epithelial cells involved in the tumor: type A for medullary thymoma, type B1 or B2 for respectively predominantly or entirely cortical thymoma, type AB for mixed thymoma (involving both cortical and medullary epithelial cells) and type B3 for cytonuclear atypia thymoma. Several paraneoplastic syndromes are associated with thymoma but the most common is MG with an incidence around 30% but variable from one study to another. Histologically, MG is associated more frequently with type B1, B2, or B3 tumors than with type A or AB tumors. The differences between thymomas and normal thymuses are detailed by Marx and collaborators. Of particular interest neoplastic TECs express less HLA-class II molecules, do not express AIRE in 95% of thymomas and contain reduced numbers of Treg cells (63). These alterations likely promote autoimmune disease occurrence. In addition, the activation of innate immune pathways and an IFN-I signature is observed in thymomas in TAMG (64).

In contrast, EOMG concerns usually female patients before 45–50 years old. Thymus in EOMG is the site of profound structural alterations. One of the main feature characterizing the thymus in AChR$^+$ MG is a lymphoproliferative hyperplasia characterized by increased number of B cells and ectopic germinal centers (GCs). The incidence of lymphoproliferative hyperplasia is approximately 70% of patients with AChR$^+$ MG, and thymectomy has proven to be efficacious (65). The hyperplastic MG thymus displays all the characteristics of tertiary lymphoid organs. Neoangiogenic processes with high endothelial venule and lymphatic vessel development are clearly observed. Several studies have demonstrated the overexpression or differential expression of chemokines, specifically key molecules involved in peripheral cell recruitment and GC development, such as CXCL13 and CCL21 (66). How the thymus turns into a tertiary lymphoid organ is not well-known but local inflammation seems mandatory. Numerous cytokines are overexpressed in the EOMG thymus. In particular, IFN-β orchestrates thymic changes associated with MG (67, 68) and favors the differentiation of pathogenic Th17 T cells (69). As well, different inflammatory signaling pathways are activated, such as Toll-Like Receptor (TLR) and NF-KB pathways that potentially orchestrate thymic changes.

The hallmarks of disease involve dysfunction of cellular pathways including changes in miRNA expression. In MG differentially expressed miRNAs have been noted in the circulating PBMCs (70–73). The concept of miRNA expression as initiation of disease or the maintenance of specific lymphocyte populations has led to the identification of potential biomarkers. The role of miRNAs in MG thymuses has also been studied as detailed below.

## miRNA PROFILE IN THYMOMA OF TAMG

Studies to assess the alterations of miRNAs in thymoma are limited. A study performed by Li, et al. on four thymoma samples compared to four control thymus samples demonstrated the differential expression of 137 miRNAs. Further analysis was performed on miR-125a-5p whose expression is increased in TAMG. They identify FoxP3 as one of the targets of miR-125a-5p (74) and FoxP3 expression is known to be altered in thymoma patients (75).

In their study, Li et al. also identified a decreased expression of miR-376a and miR-376c in TAMG patients. These miRNA could be of interest as their expression is decreased in AIRE-silenced TECs (19) and AIRE expression is known to be strongly down-regulated in TAMG (76).

In a separate study, the expression of miR-19b-5p, which targets thymic stromal lymphopoietin (TSLP), was elevated in MG-related thymomas. The negative correlation with TSLP mRNA contributes to T-cell imbalance and promotes MG-related thymoma development (77). Whereas, the levels of miR-20b were significantly decreased compared to those in adjacent non-tumor tissues, resulting in an increased proliferation of T cells through a NFAT5/CAMTA1 dependent pathway (78).

In an independent analysis of miR-150 expression, we observed a significant decreased of this miRNA expression in the thymus of TAMG patients (data not shown). Regarding its high expression in the thymus (**Table 1**), its role in thymoma should be further investigated.

Interestingly, a miRnome study was led on thymic epithelial tumors, including thymomas but it is not mentioned whether patients had a concomitant autoimmune MG. It could be interesting to know retrospectively which patients with a given miRNA profile later developed MG (79).

## miRNA PROFILE IN THE THYMUS OF EOMG PATIENTS

### Specific Dysregulated miRNAs in EOMG

Three miRnome studies have addressed the changes in miRNA expression in the thymus of EOMG patients (11, 80, 81). The multiple differences in the study designs include the thymus samples from EOMG, the designation of controls, the miRNA arrays, and the method of analysis.

The study by Cron et al. used thymus from untreated female EOMG patients compared to healthy age-matched female controls. The thymus samples from untreated EOMG patients displayed both low hyperplasia with 2 or fewer GCs per section and high hyperplasia with an increased number of GCs. The study was performed using the Affymetrix GeneChip miRNA 3.0 Array. 61 miRNAs are found dysregulated (24 up- and 37 down-regulated). The implication of miRNA clusters localized at the extremity of the X chromosome is also demonstrated. Among the dysregulated miRNAs, they focused their attention on the most down-regulated miRNAs: miR-7-5p. Its down-regulation in MG thymuses is confirmed, in particular in TECs, and an inverse correlation is observed between the expression of miR-7 and CCL21; a target mRNA for miR-7 (11). CCL21 is involved in the abnormal recruitment of B cells in the MG thymus and known to participate in GC formation (82).

As with TAMG studies, miR-125a-5p was elevated in the EOMG thymus samples compared to controls. miR-125a-5p by targeting FoxP3 could explain why the suppressive activity of regulatory T cells is severely impaired in EOMG patients and associated with a decreased expression of FoxP3 in $CD4^+$ T cells (83). miR-125a is also known to modulate inflammatory signaling pathways, such as TNFAIP3 (Tumor Necrosis Factor Alpha-Induced Protein 3) a key molecule in the negative regulation of NF-κB (Nuclear Factor κB) and TLR signaling pathways (59, 84), and WDR1 (WD repeat-containing protein 1) implicated in auto-inflammatory processes associated with IL-18 expression (11).

miR-29a is among the miRNAs down-regulated in $AIRE^+$ mTECs identified by Ucar et al. (15), and Cron et al. observed that all miRNAs of the miR-29 family were down-regulated in the thymus of EOMG patients (unpublished data). miR-29a is of particular interest regarding its role in IFN-I signaling sensitization (14) and the central role of IFN-β in the EOMG thymus (67).

The study by Li et al. compared thymic miRNA expression in EOMG patients and controls using the Agilent Human miRNA array (V.18.0). This study was performed using four control and four MG thymuses. They extracted 33 dysregulated miRNAs with numerous down-regulated miRNAs from the miR-548 family. Next, they showed that miR-548k targets the 3′ UTR region of CXCL13 decreasing its expression in Jurkat cells (81). Knowing that CXCL13 is up-regulated in MG TECs and participate to B-cell recruitment and GC development (85), this miRNA could thus play a role in MG pathogenesis.

The study by Sengupta et al. used thymus from EOMG patients and separated the samples based on the presentation of GCs (80). For miRNA assessment 13 out of the 16 MG patients were treated with prednisolone (65). The experimental group contained samples with GCs, whereas, the control samples were thymus samples from EOMG patients that did not contain GCs in the thymus block. The Affymetrix GeneChip miRNA 4.0 Array identified 44 mature miRNAs that are altered in GC rich thymus samples from EOMG compared to EOMG patients with no GC expression. The dysregulated expression of 38 miRNAS is confirmed by RT-PCR (8 up- and 30 down-regulated). These miRNAs and targeted mRNAs are involved in regulatory pathways common to inflammation and immune response, cell cycle regulation and anti-apoptotic pathways. The Regulator of G protein Signaling 13 (RGS13), involved in GC regulation, is identified in EOMG thymuses with GCs and was paired with downregulation of miR-452-5p and miR-139-5p. The increased expression of miR-150-5p is found in EOMG samples with GCs which mirrored the miR-150-5p expression in sera of MG patients. miR-150 is also more expressed in the thymus of MG patients compared to healthy controls, and in particular in patients displaying a high degree of thymic hyperplasia (86). *In situ* hybridization analyses showed that miR-150 is strongly expressed by B cells of the mantle zone around GCs. The increased expression of miR-150 in hyperplastic MG thymuses seems thus linked to the abnormal presence of B cells and in particular to the development of GCs. By removal of the thymus, the sera levels of miR-150-5p are reduced (70) but no correlations between the degree of thymic hyperplasia and serum levels in MG patients are observed (86, 87). miR-150 overexpression in MG thymuses is also inversely correlated with the expression of MYB, the most well-known miR-150 mRNA target that displays four binding sites. MYB is a regulator factor essential for hematopoiesis and is highly expressed in the thymus (88). MYB is also characterized as an early regulator of T-cell associated diseases with an altered expression in autoimmune diseases (89). In the MG thymus, miR-150 could be secreted by B cells and alter MYB expression locally and consequently affect T cells, resulting in the significant alterations of the T-cell repertoire seen in MG (90).

miR-145, miR-24, and miR-143 are reduced in GC rich thymus samples as compared to samples with no GCs, and these miRNAs are also reduced in PBMCs of EAMG rats (91). Another group has also observed a decreased expression of miR-143 in the thymus of a MG mouse model in which mice were engrafted in hyperplastic thymic biopsies from EOMG patients and showed a link with CXCL13 expression in thymocytes (92).

Recently, miR-146, one of the most well-known immuno-miR, has been shown to be down-regulated in the thymus of EOMG patient as compared to age-matched donors. The decrease was localized in the thymic stroma and not linked to the presence of GCs. This deficiency was inversely correlated with increased expression of miR-146 targets such as IRAK1, c-REL and ICOS. Interestingly, the expression of miR-146 and these target genes were normalized in EOMG patients under corticosteroid treatment. Altogether, these results suggest that miR-146 could modulate TLR signaling via IRAK1 and cREL and GC formation via ICOS (93). In addition, miR-146 is of great interest in MG as it is known to control the activation of IFN-I signaling pathways (94). The decrease of miR-146 could be linked with the overexpression of IFN-β in MG thymuses (67). In addition a decrease expression of this miRNA could have a strong impact on T-cell differentiation in MG and could be associated with the defective function of Treg cells (83, 95) and/or the increase of pathogenic Th17 cells (69, 96).

## Pathway Analysis of miRNA Expression in EOMG

To progress in the understanding in the implication of miRNAs in EOMG thymuses beyond individual miRNAs, we provide functional relevance of the miRNAs differentially expressed in two miRnome studies (11, 80). Original results from these two publications have been used and miRNA lists were analyzed through miRNet (https://www.mirnet.ca/miRNet/home.xhtml) and targeted genes used to defined pathways. KEGG Pathway enrichment analyses were performed on dysregulated miRNAs identified in Cron et al. (**Table 3A**) and Sengupta et al. (**Table 3B**). Despite the difference in these two studies, one comparing thymuses from control donors vs. untreated EOMG patients (11) and the other one comparing thymuses rich in GCs vs. those with no GCs (80), interesting information

**TABLE 3 |** KEGG Pathway enrichment analyses were performed on dysregulated miRNAs identified in Cron et al. **(A)** and Sengupta et al. **(B)** using miRNet (https://www.mirnet.ca/miRNet/home.xhtml).

| Pathway | Hits | *P*-value |
|---|---|---|
| **A** | | |
| Pathways in cancer | 90 | 6.49E-29 |
| Prostate cancer | 35 | 2.74E-15 |
| Focal adhesion | 53 | 1.75E-14 |
| Pancreatic cancer | 30 | 2.21E-14 |
| Glioma | 28 | 2.38E-13 |
| Chronic myeloid leukemia | 29 | 8.4E-13 |
| Colorectal cancer | 23 | 5.21E-12 |
| Small cell lung cancer | 29 | 1.04E-11 |
| Regulation of actin cytoskeleton | 44 | 8.61E-11 |
| Neurotrophin signaling pathway | 35 | 1E-10 |
| Melanoma | 25 | 2.36E-10 |
| Acute myeloid leukemia | 22 | 1.26E-09 |
| Bacterial invasion of epithelial cells | 21 | 6.06E-09 |
| Non-small cell lung cancer | 20 | 8.82E-09 |
| Chemokine signaling pathway | 41 | 1.12E-08 |
| Adherens junction | 23 | 1.49E-08 |
| Renal cell carcinoma | 21 | 2.05E-08 |
| Toxoplasmosis | 26 | 5.40E-08 |
| ErbB signaling pathway | 25 | 5.50E-08 |
| Endometrial cancer | 17 | 1.16E-07 |
| Jak-STAT signaling pathway | 26 | 1.96E-07 |
| HTLV-I infection | 39 | 4.22E-07 |
| T cell receptor signaling pathway | 25 | 6.37E-07 |
| Insulin signaling pathway | 30 | 1.22E-06 |
| Chagas disease (American trypanosomiasis) | 23 | 1.55E-06 |
| p53 signaling pathway | 19 | 4.85E-06 |
| Cholinergic synapse | 23 | 5.12E-06 |
| B cell receptor signaling pathway | 20 | 5.17E-06 |
| Bladder cancer | 12 | 5.17E-06 |
| mTOR signaling pathway | 15 | 5.53E-06 |
| Epstein-Barr virus infection | 22 | 8.07E-06 |
| Hepatitis C | 23 | 1.14E-05 |
| Leukocyte transendothelial migration | 24 | 1.27E-05 |
| MAPK signaling pathway | 43 | 1.39E-05 |
| Apoptosis | 20 | 2.34E-05 |
| Progesterone-mediated oocyte maturation | 19 | 5.02E-05 |
| Type II diabetes mellitus | 14 | 6.24E-05 |
| Fc epsilon RI signaling pathway | 18 | 7.05E-05 |
| Fc gamma R-mediated phagocytosis | 21 | 7.34E-05 |
| Melanogenesis | 21 | 1.38E-04 |
| Thyroid cancer | 10 | 1.50E-04 |
| VEGF signaling pathway | 17 | 2.95E-04 |
| TGF-beta signaling pathway | 18 | 3.21E-04 |
| Hypertrophic cardiomyopathy (HCM) | 9 | 3.30E-04 |
| Axon guidance | 22 | 4.47E-04 |
| Tight junction | 22 | 4.47E-04 |
| Wnt signaling pathway | 25 | 4.97E-04 |
| Tuberculosis | 28 | 7.46E-04 |
| Aldosterone-regulated sodium reabsorption | 10 | 8.17E-04 |
| Carbohydrate digestion and absorption | 7 | 1.11E-03 |
| Alcoholism | 26 | 1.86E-03 |
| Cell cycle | 21 | 2.22E-03 |
| Dopaminergic synapse | 21 | 2.22E-03 |
| Measles | 18 | 3.27E-03 |
| Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 5 | 5.68E-03 |
| ECM-receptor interaction | 15 | 7.47E-03 |
| Osteoclast differentiation | 19 | 7.74E-03 |
| GnRH signaling pathway | 16 | 8.65E-03 |
| Herpes simplex infection | 17 | 8.84E-03 |
| Dilated cardiomyopathy | 14 | 9.31E-03 |
| Adipocytokine signaling pathway | 12 | 1.10E-02 |
| Toll-like receptor signaling pathway | 16 | 1.13E-02 |
| Salmonella infection | 13 | 1.18E-02 |
| Influenza A | 16 | 2.90E-02 |
| Basal cell carcinoma | 9 | 3.19E-02 |
| Cardiac muscle contraction | 4 | 3.59E-02 |
| Dorso-ventral axis formation | 4 | 3.59E-02 |
| NOD-like receptor signaling pathway | 9 | 4.00E-02 |
| Cytokine-cytokine receptor interaction | 30 | 4.15E-02 |
| Viral myocarditis | 6 | 4.15E-02 |
| **B** | | |
| Pathways in cancer | 87 | 3.17E-26 |
| Chronic myeloid leukemia | 33 | 4.40E-16 |
| HTLV-I infection | 55 | 9.17E-16 |
| Prostate cancer | 34 | 1.64E-14 |
| Colorectal cancer | 25 | 6.48E-14 |
| Pancreatic cancer | 29 | 1.88E-13 |
| Focal adhesion | 51 | 2.62E-13 |
| Melanoma | 27 | 6.84E-12 |
| Glioma | 26 | 1.40E-11 |
| Non-small cell lung cancer | 22 | 2.12E-10 |
| Endometrial cancer | 20 | 3.46E-10 |
| Bladder cancer | 16 | 8.40E-10 |
| Small cell lung cancer | 26 | 2.43E-09 |
| Cell cycle | 33 | 2.95E-09 |
| Thyroid cancer | 14 | 6.34E-08 |
| Osteoclast differentiation | 30 | 7.38E-08 |
| ErbB signaling pathway | 25 | 7.70E-08 |
| Renal cell carcinoma | 20 | 1.53E-07 |
| Toll-like receptor signaling pathway | 26 | 1.62E-07 |
| Regulation of actin cytoskeleton | 38 | 1.62E-07 |
| Toxoplasmosis | 25 | 2.78E-07 |
| Acute myeloid leukemia | 19 | 2.92E-07 |
| Adherens junction | 21 | 4.24E-07 |
| Chagas disease (American trypanosomiasis) | 24 | 4.36E-07 |

*(Continued)*

**TABLE 3** | Continued

| Pathway | Hits | *P*-value |
|---|---|---|
| p53 signaling pathway | 20 | 1.10E-06 |
| mTOR signaling pathway | 16 | 1.10E-06 |
| Bacterial invasion of epithelial cells | 18 | 1.10E-06 |
| MAPK signaling pathway | 46 | 1.25E-06 |
| Leukocyte transendothelial migration | 26 | 1.25E-06 |
| VEGF signaling pathway | 21 | 1.57E-06 |
| Epstein-Barr virus infection | 22 | 9.71E-06 |
| Neurotrophin signaling pathway | 26 | 1.63E-05 |
| B cell receptor signaling pathway | 19 | 2.30E-05 |
| Axon guidance | 25 | 2.31E-05 |
| TGF-beta signaling pathway | 20 | 3.37E-05 |
| Fc epsilon RI signaling pathway | 18 | 8.65E-05 |
| Apoptosis | 19 | 1.01E-04 |
| T cell receptor signaling pathway | 21 | 1.05E-04 |
| Chemokine signaling pathway | 31 | 3.46E-04 |
| Influenza A | 21 | 3.99E-04 |
| Hepatitis C | 20 | 4.38E-04 |
| Wnt signaling pathway | 25 | 6.63E-04 |
| Measles | 19 | 1.66E-03 |
| Insulin signaling pathway | 23 | 1.95E-03 |
| Salmonella infection | 15 | 1.95E-03 |
| Jak-STAT signaling pathway | 18 | 3.02E-03 |
| Type II diabetes mellitus | 11 | 4.79E-03 |
| Progesterone-mediated oocyte maturation | 15 | 5.83E-03 |
| Epithelial cell signaling in Helicobacter pylori infection | 9 | 8.37E-03 |
| Carbohydrate digestion and absorption | 6 | 8.44E-03 |
| Natural killer cell mediated cytotoxicity | 21 | 1.08E-02 |
| GnRH signaling pathway | 16 | 1.08E-02 |
| Amyotrophic lateral sclerosis (ALS) | 9 | 1.14E-02 |
| Fc gamma R-mediated phagocytosis | 16 | 1.44E-02 |
| Aldosterone-regulated sodium reabsorption | 8 | 1.65E-02 |
| ECM-receptor interaction | 14 | 2.21E-02 |
| Cholinergic synapse | 15 | 2.73E-02 |
| Long-term potentiation | 12 | 3.04E-02 |
| Amoebiasis | 9 | 3.26E-02 |
| Tight junction | 17 | 3.95E-02 |
| Dorso-ventral axis formation | 4 | 3.96E-02 |
| Legionellosis | 8 | 3.96E-02 |
| Hypertrophic cardiomyopathy (HCM) | 6 | 3.96E-02 |
| Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 4 | 3.96E-02 |
| One carbon pool by folate | 5 | 4.62E-02 |
| Viral myocarditis | 6 | 4.62E-02 |

*Pathways of interest are highlighted: cancer pathways (gray), pathogen infection pathways (blue), Toll-Like Receptor signaling pathway (green) and chemokine signaling pathway (yellow).*

results from these analyses. Numerous cancer pathways are represented but also pathways related to pathogen infection and TLRs. Pathogens are environmental factor that potentially drive/perpetuate autoimmunity (97) and the thymus is a common target organ for infectious diseases (98). Poliovirus-infected macrophages and the presence of Epstein-Barr virus (EBV)-infected B cells in MG thymus were described in MG thymus (99, 100). Besides, changes regarding TLR expression in MG thymus have been demonstrated (67, 101–103). The chemokine signaling pathway was also found dysregulated in both studies. This is not surprising regarding the role of chemokines in B-cell recruitment and GC development (82, 85, 104). Altogether, these studies provide novel clues to the potential pathways that occur in the hyperplastic thymus and the development of GCs.

## CONCLUSION

Thymic changes occur during life either in the context of thymic involution upon aging /stress or in MG. Here, we review the literature and used original data to describe specific miRNAs that could play a key role in these thymic changes. Among miRNAs that are dysregulated in EOMG or TAMG patients, miR-19b, miR-20b, miR-24, and miR-150 are listed as ThymiRs, and only miR-19b and miR-20b are actively involved in thymic involution. The literature often describes these miRNAs as playing a role in T-cell differentiation. The lack of correlation between miRNAs regulated upon thymic involution, and dysregulated in EOMG or TAMG thymuses suggests the immune response in the autoimmune condition is not directed at the dysregulation of the developmental pathway of the thymus. Most of the down-regulated thymic miRNAs in EOMG are associated with GC development, such as miR-7, miR-24, miR-139, miR-143, miR-145, miR-146, miR-150, miR-452, miR-548, or thymic inflammation, such as miR-125b, miR-146, or miR-29. Further investigations on these miRNAs could now help deciphering if they could represent therapeutic tools to normalized thymic inflammation and associated lymphofollicular hyperplasia in EOMG patients.

## AUTHOR CONTRIBUTIONS

MC performed the experiments and revised the manuscript. ÉG performed bioinformatics analyses and revised the manuscript. LK and RL wrote the manuscript.

## FUNDING

This work was supported by grants from the European Community (FIGHT-MG/HEALTH-92-242-210) and from the Association Française contre les Myopathies (AFM).

## ACKNOWLEDGMENTS

We recognize and thank Inovarion (https://www.inovarion.com/) as the employer of ÉG and the CNRS as the employer of RL. We thank Dr. Sonia Berrih-Aknin for reading the document and her suggestions.

## REFERENCES


1. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol*. (2009) 11:228–34. doi: 10.1038/ncb0309-228
2. Chen JQ, Papp G, Szodoray P, Zeher M. The role of microRNAs in the pathogenesis of autoimmune diseases. *Autoimmun Rev*. (2016) 15:1171–80. doi: 10.1016/j.autrev.2016.09.003
3. Theofilopoulos AN, Kono DH, Baccala R. The multiple pathways to autoimmunity. *Nat Immunol*. (2017) 18:716–24. doi: 10.1038/ni.3731
4. Mehta A, Baltimore D. MicroRNAs as regulatory elements in immune system logic. *Nat Rev Immunol*. (2016) 16:279–94. doi: 10.1038/nri.2016.40
5. Kurd N, Robey EA. T-cell selection in the thymus: a spatial and temporal perspective. *Immunol Rev*. (2016) 271:114–26. doi: 10.1111/imr.12398
6. Perniola R. Twenty Years of AIRE. *Front Immunol*. (2018) 9:98. doi: 10.3389/fimmu.2018.00098
7. Bertho JM, Demarquay C, Moulian N, Van Der Meeren A, Berrih-Aknin S, Gourmelon P. Phenotypic and immunohistological analyses of the human adult thymus: evidence for an active thymus during adult life. *Cell Immunol*. (1997) 179:30–40. doi: 10.1006/cimm.1997.1148
8. Dooley J, Linterman MA, Liston A. MicroRNA regulation of T-cell development. *Immunol Rev*. (2013) 253:53–64. doi: 10.1111/imr.12049
9. Xu M, Gan T, Ning H, Wang L. MicroRNA functions in thymic biology: thymic development and involution. *Front Immunol*. (2018) 9:2063. doi: 10.3389/fimmu.2018.02063
10. Kroesen BJ, Teteloshvili N, Smigielska-Czepiel K, Brouwer E, Boots AM, Van Den Berg A, et al. Immuno-miRs: critical regulators of T-cell development, function and ageing. *Immunology*. (2015) 144:1–10. doi: 10.1111/imm.12367
11. Cron MA, Maillard S, Deslile F, Samson N, Truffault F, Foti M, et al. Analysis of microRNA expression in the thymus of myasthenia gravis patients opens new research avenues *Autoimmunity Reviews*. (2018) 17:588–600. doi: 10.1016/j.autrev.2018.01.008
12. Khan IS, Taniguchi RT, Fasano KJ, Anderson MS, Jeker LT. Canonical microRNAs in thymic epithelial cells promote central tolerance. *Eur J Immunol*. (2014) 44:1313–9. doi: 10.1002/eji.201344079
13. Zuklys S, Mayer CE, Zhanybekova S, Stefanski HE, Nusspaumer G, Gill J, et al. MicroRNAs control the maintenance of thymic epithelia and their competence for T lineage commitment and thymocyte selection. *J Immunol*. (2012) 189:3894–904. doi: 10.4049/jimmunol.1200783
14. Papadopoulou AS, Dooley J, Linterman MA, Pierson W, Ucar O, Kyewski B, et al. The thymic epithelial microRNA network elevates the threshold for infection-associated thymic involution via miR-29a mediated suppression of the IFN-alpha receptor. *Nat Immunol*. (2012) 13:181–7. doi: 10.1038/ni.2193
15. Ucar O, Tykocinski LO, Dooley J, Liston A, Kyewski B. An evolutionarily conserved mutual interdependence between Aire and microRNAs in promiscuous gene expression. *Eur J Immunol*. (2013) 13:1769–78. doi: 10.1002/eji.201343343
16. Ye Y, Li D, Ouyang D, Deng L, Zhang Y, Ma Y, et al. MicroRNA expression in the aging mouse thymus. *Gene*. (2014) 547:218–25. doi: 10.1016/j.gene.2014.06.039
17. Song MS, Rossi JJ. Molecular mechanisms of dicer: endonuclease and enzymatic activity. *Biochem J*. (2017) 474:1603–18. doi: 10.1042/BCJ20160759
18. Deng L, Ren R, Liu Z, Song M, Li J, Wu Z, et al. Stabilizing heterochromatin by DGCR8 alleviates senescence and osteoarthritis. *Nat Commun*. (2019) 10:3329. doi: 10.1038/s41467-019-10831-8
19. Macedo C, Evangelista AF, Marques MM, Octacilio-Silva S, Donadi EA, Sakamoto-Hojo ET, et al. Autoimmune regulator. (Aire) controls the expression of microRNAs in medullary thymic epithelial cells. *Immunobiology*. (2013) 218:554–60. doi: 10.1016/j.imbio.2012.06.013
20. Cobb BS, Nesterova TB, Thompson E, Hertweck A, O'connor E, Godwin J, et al. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med*. (2005) 201:1367–73. doi: 10.1084/jem.20050572
21. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med*. (2005) 202:261–9. doi: 10.1084/jem.20050678
22. Fedeli M, Napolitano A, Wong MP, Marcais A, De Lalla C, Colucci F, et al. Dicer-dependent microRNA pathway controls invariant NKT cell development. *J Immunol*. (2009) 183:2506–12. doi: 10.4049/jimmunol.0901361
23. Cobb BS, Hertweck A, Smith J, O'connor E, Graf D, Cook T, et al. A role for Dicer in immune regulation. *J Exp Med*. (2006) 203:2519–27. doi: 10.1084/jem.20061692
24. Chong MM, Rasmussen JP, Rudensky AY, Littman DR. The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *J Exp Med*. (2008) 205:2005–17. doi: 10.1084/jem.20081219
25. Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev*. (2007) 21:578–89. doi: 10.1101/gad.1522907
26. Ghisi M, Corradin A, Basso K, Frasson C, Serafin V, Mukherjee S, et al. Modulation of microRNA expression in human T-cell development: targeting of NOTCH3 by miR-150. *Blood*. (2011) 117:7053–62. doi: 10.1182/blood-2010-12-326629
27. Hu L, Mao L, Liu S, Zhao J, Chen C, Guo M, et al. Functional Role of MicroRNAs in Thymocyte Development. *Int Arch Allergy Immunol*. (2019) 178:315–22. doi: 10.1159/000496093
28. Guo D, Ye Y, Qi J, Tan X, Zhang Y, Ma Y, et al. Age and sex differences in microRNAs expression during the process of thymus aging. *Acta Biochim Biophys Sin. (Shanghai)*. (2017) 49:409–19. doi: 10.1093/abbs/gmx029
29. Guo Z, Chi F, Song Y, Wang C, Yu R, Wei T, et al. Transcriptome analysis of murine thymic epithelial cells reveals ageassociated changes in microRNA expression. *Int J Mol Med*. (2013) 32:835–42. doi: 10.3892/ijmm.2013.1471
30. Jia HL, Zeng XQ, Huang F, Liu YM, Gong BS, Zhang KZ, et al. Integrated microRNA and mRNA sequencing analysis of age-related changes to mouse thymic epithelial cells. *IUBMB Life*. (2018) 70:678–90. doi: 10.1002/iub.1864
31. Guo D, Ye Y, Qi J, Xu L, Zhang L, Tan X, et al. MicroRNA-195a-5p inhibits mouse medullary thymic epithelial cells proliferation by directly targeting Smad7. *Acta Biochim Biophys Sin. (Shanghai)*. (2016) 48:290–7. doi: 10.1093/abbs/gmv136
32. Cheng J, Wu R, Long L, Su J, Liu J, Wu XD, et al. miRNA-451a targets IFN regulatory factor 8 for the progression of systemic lupus erythematosus. *Inflammation*. (2017) 40:676–87. doi: 10.1007/s10753-017-0514-8
33. Khuu C, Utheim TP, Sehic A. The three paralogous MicroRNA clusters in development and disease, miR-17-92, miR-106a-363, and miR-106b-25. *Scientifica*. (2016) 2016:1379643. doi: 10.1155/2016/1379643
34. Linhares-Lacerda L, Palu CC, Ribeiro-Alves M, Paredes BD, Morrot A, Garcia-Silva MR, et al. Differential expression of microRNAs in thymic epithelial cells from *Trypanosoma cruzi* acutely infected mice: putative role in thymic atrophy. *Front Immunol*. (2015) 6:428. doi: 10.3389/fimmu.2015.00428
35. Smith LK, Shah RR, Cidlowski JA. Glucocorticoids modulate microRNA expression and processing during lymphocyte apoptosis. *J Biol Chem*. (2010) 285:36698–708. doi: 10.1074/jbc.M110.162123
36. Belkaya S, Silge RL, Hoover AR, Medeiros JJ, Eitson JL, Becker AM, et al. Dynamic modulation of thymic microRNAs in response to stress. *PLoS ONE. (*2011) 6:e27580. doi: 10.1371/journal.pone.0027580
37. Roush S, Slack FJ. The let-7 family of microRNAs. *Trends Cell Biol*. (2008) 18:505–16. doi: 10.1016/j.tcb.2008.07.007
38. Pobezinsky LA, Etzensperger R, Jeurling S, Alag A, Kadakia T, Mccaughtry TM, et al. Let-7 microRNAs target the lineage-specific transcription factor PLZF to regulate terminal NKT cell differentiation and effector function. *Nat Immunol*. (2015) 16:517–24. doi: 10.1038/ni.3146
39. Xiao S, Zhang W, Manley NR. Thymic epithelial cell-derived signals control B progenitor formation and proliferation in the thymus by regulating Let-7 and Arid3a. *PLoS ONE. (*2018) 13:e0193188. doi: 10.1371/journal.pone.0193188
40. Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell*. (2007) 129:147–61. doi: 10.1016/j.cell.2007.03.008
41. Ebert PJ, Jiang S, Xie J, Li QJ, Davis MM. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. *Nat Immunol*. (2009) 10:1162–9. doi: 10.1038/ni.1797

42. Henao-Mejia J, Williams A, Goff LA, Staron M, Licona-Limon P, Kaech SM, et al. The microRNA miR-181 is a critical cellular metabolic rheostat essential for NKT cell ontogenesis and lymphocyte development and homeostasis. *Immunity*. (2013) 38:984–97. doi: 10.1016/j.immuni.2013.02.021
43. Stefanski HE, Xing Y, Taylor PA, Maio S, Henao-Meija J, Williams A, et al. Despite high levels of expression in thymic epithelial cells, miR-181a1 and miR-181b1 are not required for thymic development. *PLoS ONE*. (2018) 13:e0198871. doi: 10.1371/journal.pone.0198871
44. Guo D, Ye Y, Qi J, Zhang L, Xu L, Tan X, et al. MicroRNA-181a-5p enhances cell proliferation in medullary thymic epithelial cells via regulating TGF-beta signaling. *Acta Biochim Biophys Sin. (Shanghai)*. (2016) 48:840–9. doi: 10.1093/abbs/gmw068
45. Cotrim-Sousa L, Freire-Assis A, Pezzi N, Tanaka PP, Oliveira EH, Passos GA. Adhesion between medullary thymic epithelial cells and thymocytes is regulated by miR-181b-5p and miR-30b. *Mol Immunol*. (2019) 114:600–11. doi: 10.1016/j.molimm.2019.09.010
46. Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci USA*. (2007) 104:7080–5. doi: 10.1073/pnas.0702409104
47. Bezman NA, Chakraborty T, Bender T, Lanier LL. miR-150 regulates the development of NK and iNKT cells. *J Exp Med*. (2011) 208:2717–31. doi: 10.1084/jem.20111386
48. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. (2007) 131:146–59. doi: 10.1016/j.cell.2007.07.021
49. Zheng Q, Zhou L, Mi QS. MicroRNA miR-150 is involved in Valpha14 invariant NKT cell development and function. *J Immunol*. (2012) 188:2118–26. doi: 10.4049/jimmunol.1103342
50. Lee HM, Kim TS, Jo EK. MiR-146 and miR-125 in the regulation of innate immunity and inflammation. *BMB Rep*. (2016) 49:311–8. doi: 10.5483/BMBRep.2016.49.6.056
51. Kirigin FF, Lindstedt K, Sellars M, Ciofani M, Low SL, Jones L, et al. Dynamic microRNA gene transcription and processing during T cell development. *J Immunol*. (2012) 188:3257–67. doi: 10.4049/jimmunol.1103175
52. Li Z, Zhang S, Wan Y, Cai M, Wang W, Zhu Y, et al. MicroRNA-146a Overexpression Impairs the Positive Selection during T Cell Development. *Front Immunol*. (2018) 8:2006. doi: 10.3389/fimmu.2017.02006
53. Burger ML, Xue L, Sun Y, Kang C, Winoto A. Premalignant PTEN-deficient thymocytes activate microRNAs miR-146a and miR-146b as a cellular defense against malignant transformation. *Blood*. (2014) 123:4089–100. doi: 10.1182/blood-2013-11-539411
54. Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, et al. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell*. (2010) 142:914–29. doi: 10.1016/j.cell.2010.08.012
55. Mashima R. Physiological roles of miR-155. *Immunology*. (2015) 145:323–33. doi: 10.1111/imm.12468
56. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol*. (2009) 182:2578–82. doi: 10.4049/jimmunol.0803162
57. Stahl HF, Fauti T, Ullrich N, Bopp T, Kubach J, Rust W, et al. miR-155 inhibition sensitizes CD4+ Th cells for TREG mediated suppression. *PLoS ONE*. (2009) 4:e7158. doi: 10.1371/journal.pone.0007158
58. Burocchi A, Pittoni P, Tili E, Rigoni A, Costinean S, Croce CM, et al. Regulated Expression of miR-155 is Required for iNKT Cell Development. *Front Immunol*. (2015) 6:140. doi: 10.3389/fimmu.2015.00140
59. Kim SW, Ramasamy K, Bouamar H, Lin AP, Jiang D, Aguiar RC. MicroRNAs miR-125a and miR-125b constitutively activate the NF-κB pathway by targeting the tumor necrosis factor alpha-induced protein 3. (TNFAIP3, A20). *Proc Natl Acad Sci USA*. (2012) 109:7865–70. doi: 10.1073/pnas.1200081109
60. Rasheed Z, Rasheed N, Abdulmonem WA, Khan MI. MicroRNA-125b-5p regulates IL-1beta induced inflammatory genes via targeting TRAF6-mediated MAPKs and NF-kappaB signaling in human osteoarthritic chondrocytes. *Sci Rep*. (2019) 9:6882. doi: 10.1038/s41598-019-42601-3
61. Xu M, Sizova O, Wang L, Su DM. A fine-tune role of Mir-125a-5p on Foxn1 During age-associated changes in the thymus. *Aging Dis*. (2017) 8:277–86. doi: 10.14336/AD.2016.1109
62. Gilhus NE. Myasthenia gravis. *N Engl J Med*. (2016) 375:2570–81. doi: 10.1056/NEJMra1602678
63. Marx A, Porubsky S, Belharazem D, Saruhan-Direskeneli G, Schalke B, Ströbel P, et al. Thymoma related myasthenia gravis in humans and potential animal models. *Exp Neurol*. (2015) 270:55–65. doi: 10.1016/j.expneurol.2015.02.010
64. Cufi P, Soussan P, Truffault F, Fetouchi R, Robinet M, Fadel E, et al. Thymoma-associated myasthenia gravis: on the search for a pathogen signature. *J Autoimmun*. (2014) 52:29–35. doi: 10.1016/j.jaut.2013.12.018
65. Wolfe GI, Kaminski HJ, Aban IB, Minisman G, Kuo HC, Marx A, et al. Randomized trial of thymectomy in myasthenia gravis. *N Engl J Med*. (2016) 375:511–22. doi: 10.1056/NEJMoa1602489
66. Cron MA, Maillard S, Villegas J, Truffault F, Sudres M, Dragin N, et al. Thymus involvement in early-onset myasthenia gravis. *Ann N Y Acad Sci*. (2018) 1412:137–45. doi: 10.1111/nyas.13519
67. Cufi P, Dragin N, Weiss JM, Martinez-Martinez P, De Baets MH, Roussin R, et al. Implication of double-stranded RNA signaling in the etiology of autoimmune myasthenia gravis. *Ann Neurol*. (2013) 73:281–93. doi: 10.1002/ana.23791
68. Cufi P, Dragin N, Ruhlmann N, Weiss JM, Fadel E, Serraf A, et al. Central role of interferon-beta in thymic events leading to myasthenia gravis. *J Autoimmun*. (2014) 52:44–52. doi: 10.1016/j.jaut.2013.12.016
69. Villegas JA, Bayer AC, Ider K, Bismuth J, Truffault F, Roussin R, et al. Il-23/Th17 cell pathway: a promising target to alleviate thymic inflammation maintenance in myasthenia gravis. *J Autoimmun*. (2019) 98:59–73. doi: 10.1016/j.jaut.2018.11.005
70. Punga T, Le Panse R, Andersson M, Truffault F, Berrih-Aknin S, Punga AR. Circulating miRNAs in myasthenia gravis: miR-150-5p as a new potential biomarker. *Ann Clin Transl Neurol*. (2014) 1:49–58. doi: 10.1002/acn3.24
71. Nogales-Gadea G, Ramos-Fransi A, Suarez-Calvet X, Navas M, Rojas-Garcia R, Mosquera JL, et al. Analysis of serum miRNA profiles of myasthenia gravis patients. *PLoS ONE*. (2014) 9:e91927. doi: 10.1371/journal.pone.0091927
72. Chunjie N, Huijuan N, Zhao Y, Jianzhao W, Xiaojian Z. Disease-specific signature of serum miR-20b and its targets IL-8 and IL-25, in myasthenia gravis patients. *Eur Cytokine Netw*. (2015) 26:61–6. doi: 10.1684/ecn.2015.0367
73. Zhang Y, Guo M, Xin N, Shao Z, Zhang X, Zhang Y, et al. Decreased microRNA miR-181c expression in peripheral blood mononuclear cells correlates with elevated serum levels of IL-7 and IL-17 in patients with myasthenia gravis. *Clin Exp Med*. (2016) 16:413–21. doi: 10.1007/s10238-015-0358-1
74. Li J, Qiu D, Chen Z, Du W, Liu J, Mo X. Altered expression of miR-125a-5p in thymoma-associated myasthenia gravis and its down-regulation of foxp3 expression in Jurkat cells. *Immunol Lett*. (2016) 172:47–55. doi: 10.1016/j.imlet.2016.02.005
75. Strobel P, Rosenwald A, Beyersdorf N, Kerkau T, Elert O, Murumagi A, et al. Selective loss of regulatory T cells in thymomas. *Ann Neurol*. (2004) 56:901–4. doi: 10.1002/ana.20340
76. Ströbel P, Murumägi A, Klein R, Luster M, Lahti M, Krohn K, et al. Deficiency of the autoimmune regulator AIRE in thymomas is insufficient to elicit autoimmune polyendocrinopathy syndrome type 1 (APS-1). *J Pathol*. (2007) 211:563–71. doi: 10.1002/path.2141
77. Wang Z, Chen Y, Xu S, Yang Y, Wei D, Wang W, et al. Aberrant decrease of microRNA19b regulates TSLP expression and contributes to Th17 cells development in myasthenia gravis related thymomas. *J Neuroimmunol*. (2015) 288:34–9. doi: 10.1016/j.jneuroim.2015.08.013
78. Xin Y, Cai H, Lu T, Zhang Y, Yang Y, Cui Y. miR-20b inhibits T cell proliferation and activation via NFAT signaling pathway in thymoma-associated myasthenia gravis. *Biomed Res Int*. (2016) 2016:9595718. doi: 10.1155/2016/9595718
79. Ganci F, Vico C, Korita E, Sacconi A, Gallo E, Mori F, et al. MicroRNA expression profiling of thymic epithelial tumors. *Lung Cancer*. (2014) 85:197–204. doi: 10.1016/j.lungcan.2014.04.008

80. Sengupta M, Wang BD, Lee NH, Marx A, Kusner LL, Kaminski HJ. MicroRNA and mRNA expression associated with ectopic germinal centers in thymus of myasthenia gravis. *PLoS ONE.* (2018) 13:e0205464. doi: 10.1371/journal.pone.0205464
81. Li J, Qiu D, Chen Z, Du W, Liu J, Mo X. miR-548k regulates CXCL13 expression in myasthenia gravis patients with thymic hyperplasia and in Jurkat cells. *J NeuroImmunol. (*2018) 320:125–32. doi: 10.1016/j.jneuroim.2018.03.021
82. Berrih-Aknin S, Ruhlmann N, Bismuth J, Cizeron-Clairac G, Zelman E, Shachar I, et al. CCL21 overexpressed on lymphatic vessels drives thymic hyperplasia in myasthenia. *Ann Neurol*. (2009) 66:521–31. doi: 10.1002/ana.21628
83. Balandina A, Lecart S, Dartevelle P, Saoudi A, Berrih-Aknin S. Functional defect of regulatory CD4(+)CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis. *Blood*. (2005) 105:735–41. doi: 10.1182/blood-2003-11-3900
84. Hamerman JA, Pottle J, Ni M, He Y, Zhang ZY, Buckner JH. Negative regulation of TLR signaling in myeloid cells–implications for autoimmune diseases. *Immunol Rev*. (2016) 269:212–27. doi: 10.1111/imr.12381
85. Méraouna A, Cizeron-Clairac G, Le Panse R, Bismuth J, Truffault F, Talaksen C, et al. The chemokine CXCL13 is a key molecule in autoimmune Myasthenia Gravis. *Blood*. (2006) 108:432–40. doi: 10.1182/blood-2005-06-2383
86. Cron MA, Maillard S, Truffault F, Gualeni AV, Gloghini A, Fadel E, et al. Causes and Consequences of miR-150-5p dysregulation in myasthenia gravis. *Front Immunol*. (2019) 10:539. doi: 10.3389/fimmu.2019.00539
87. Punga AR, Andersson M, Alimohammadi M, Punga T. Disease specific signature of circulating miR-150-5p and miR-21-5p in myasthenia gravis patients. *J Neurol Sci*. (2015) 356:90–6. doi: 10.1016/j.jns.2015.06.019
88. Wang X, Angelis N, Thein SL. MYB - A regulatory factor in hematopoiesis. *Gene*. (2018) 665:6–17. doi: 10.1016/j.gene.2018.04.065
89. Gustafsson M, Gawel DR, Alfredsson L, Baranzini S, Bjorkander J, Blomgran R, et al. A validated gene regulatory network and GWAS identifies early regulators of T cell-associated diseases. *Sci Transl Med*. (2015) 7:313ra178. doi: 10.1126/scitranslmed.aad2722
90. Truffault F, Cohen-Kaminsky S, Khalil I, Levasseur P, Berrih-Aknin S. Altered intrathymic T-cell repertoire in human myasthenia gravis. *Ann Neurol*. (1997) 41:731–41. doi: 10.1002/ana.410410609
91. Wang J, Zheng S, Xin N, Dou C, Fu L, Zhang X, et al. Identification of novel MicroRNA signatures linked to experimental autoimmune myasthenia gravis pathogenesis: down-regulated miR-145 promotes pathogenetic Th17 cell response. *J Neuroimmune Pharmacol*. (2013) 8:1287–302. doi: 10.1007/s11481-013-9498-9
92. Wu DM, Wen X, Han XR, Wang S, Wang YJ, Shen M, et al. Micro-RNA-143 inhibits proliferation and promotes apoptosis of thymocytes by targeting CXCL13 in a myasthenia gravis mouse model. *Am J Physiol Cell Physiol*. (2019) 316:C70–80. doi: 10.1152/ajpcell.00090.2018
93. Bortone F, Scandiffio L, Marcuzzo S, Bonanno S, Frangiamore R, Motta T, et al. miR-146a in myasthenia gravis thymus bridges innate immunity with autoimmunity and is linked to therapeutic effects of corticosteroids. *Front Immunol*. (2020) 11:142. doi: 10.3389/fimmu.2020.00142
94. Tang Y, Luo X, Cui H, Ni X, Yuan M, et al. MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. *Arthritis Rheum*. (2009) 60:1065–75. doi: 10.1002/art.24436
95. Zhou Q, Haupt S, Kreuzer JT, Hammitzsch A, Proft F, Neumann C, et al. Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis. *Ann Rheum Dis*. (2015) 74:1265–74. doi: 10.1136/annrheumdis-2013-204377
96. Li B, Wang X, Choi IY, Wang YC, Liu S, Pham AT, et al. miR-146a modulates autoreactive Th17 cell differentiation and regulates organ-specific autoimmunity. *J Clin Invest.* (2017) 127:3702–16. doi: 10.1172/JCI94012
97. Munz C, Lunemann JD, Getts MT, Miller SD. Antiviral immune responses: triggers of or triggered by autoimmunity? *Nature Reviews: Immunology*. (2009) 9:246–58. doi: 10.1038/nri2527
98. Savino W. The thymus is a common target organ in infectious diseases. *PLoS Pathogens*. (2006) 2:e62. doi: 10.1371/journal.ppat.0020062
99. Cavalcante P, Barberis M, Cannone M, Baggi F, Antozzi C, Maggi L, et al. Detection of poliovirus-infected macrophages in thymus of patients with myasthenia gravis. *Neurology*. (2010) 74:1118–26. doi: 10.1212/WNL.0b013e3181d7d884
100. Cavalcante P, Serafini B, Rosicarelli B, Maggi L, Barberis M, Antozzi C, et al. Epstein-Barr virus persistence and reactivation in myasthenia gravis thymus. *Ann Neurol*. (2010) 67:726–38. doi: 10.1002/ana.21902
101. Bernasconi P, Barberis M, Baggi F, Passerini L, Cannone M, Arnoldi E, et al. Increased toll-like receptor 4 expression in thymus of myasthenic patients with thymitis and thymic involution. *Am J Pathol.* (2005) 167:129–39. doi: 10.1016/S0002-9440(10)62960-4
102. Cavalcante P, Galbardi B, Franzi S, Marcuzzo S, Barzago C, Bonanno S, et al. Increased expression of Toll-like receptors 7 and 9 in myasthenia gravis thymus characterized by active Epstein-Barr virus infection. *Immunobiology*. (2016) 221:516–27. doi: 10.1016/j.imbio.2015.12.007
103. Robinet M, Maillard S, Cron MA, Berrih-Aknin S, Le Panse R. Review on toll-like receptor activation in myasthenia gravis: application to the development of new experimental models. *Clin Rev Allergy Immunol*. (2017) 52:133–47. doi: 10.1007/s12016-016-8549-4
104. Cordiglieri C, Marolda R, Franzi S, Cappelletti C, Giardina C, Motta T, et al. Innate immunity in myasthenia gravis thymus: pathogenic effects of Toll-like receptor 4 signaling on autoimmunity. *J Autoimmun*. (2014) 52:74–89. doi: 10.1016/j.jaut.2013.12.013


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# Advantages of publishing in Frontiers

**OPEN ACCESS**
Articles are free to read for greatest visibility and readership

**FAST PUBLICATION**
Around 90 days from submission to decision

**HIGH QUALITY PEER-REVIEW**
Rigorous, collaborative, and constructive peer-review

**TRANSPARENT PEER-REVIEW**
Editors and reviewers acknowledged by name on published articles

**Frontiers**
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

**Visit us:** www.frontiersin.org
**Contact us:** info@frontiersin.org | +41 21 510 17 00

**REPRODUCIBILITY OF RESEARCH**
Support open data and methods to enhance research reproducibility

**DIGITAL PUBLISHING**
Articles designed for optimal readership across devices

**FOLLOW US**
@frontiersin

**IMPACT METRICS**
Advanced article metrics track visibility across digital media

**EXTENSIVE PROMOTION**
Marketing and promotion of impactful research

**LOOP RESEARCH NETWORK**
Our network increases your article's readership