Journal of Neuroimmunology 396 (2024) 578464

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Pathogenic Th17 cells are a potential therapeutic target for tacrolimus in AChR-myasthenia gravis patients

Yingkai Li [a,b], Pei Chen [a], Xin Huang [a], Hao Huang [a], Qian Ma [a], Zhongqiang Lin [a], Li Qiu [a], Changyi Ou [a], Weibin Liu [a,*]

[a] Department of Neurology, National Key Clinical Department and Key Discipline of Neurology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China

[b] Neuromuscular division, Department of Neurology, Duke University Medical Center, Durham, NC 27710, USA

ARTICLE INFO

Keywords:
Tacrolimus
MG
Myasthenia gravis
Th17 cell
Calcineurin inhibitor

ABSTRACT

In our study, we investigated the impact of tacrolimus (TAC) on CD4+ T cell subsets in 41 AChR-MG patients over 12 weeks. Twenty-seven patients were classified as the response group (RG) (improved myasthenia gravis composite scores ≥3), while 14 were non-response. We found that TAC treatment significantly reduced Th17 and pathogenic Th17 cells, along with IL-17 levels in RG, while Th1 and Tfh cells slightly decreased without affecting Th2 or Treg subsets. This indicates that TAC's clinical benefits may be due to its inhibitory effect on the Th17 response, enhancing our insight into its immunomodulatory mechanisms in MG management.

1. Introduction

Myasthenia gravis (MG) is a chronic autoimmune neuromuscular junction disorder characterized by fluctuating muscle weakness resulting from autoantibodies against postsynaptic proteins, including acetylcholine receptor (AChR-Ab), muscle-specific tyrosine kinase (MuSK-Ab), and LRP4 (low density lipid receptor protein 4) (Lazaridis and Tzartos, 2020).

Conventional therapeutic treatment for MG includes symptomatic treatment (e.g., acetylcholinesterase inhibitors) and the use of immunosuppressants (ISs). Among ISs, corticosteroids are the most common agents for MG patients. However, numerous severe adverse events (AEs) limit long-term corticosteroid usage (Yasir and Sonthalia, 2022). In recent decades, non-steroidal ISs have been used to treat MG patients in place of corticosteroids. The non-steroid ISs are also known as steroid sparing agents, these include azathioprine (AZA), mycophenolate mofetil (MMF), and methotrexate (MTX). However, the clinical use of AZA, MMF and MTX for treating MG patients is also limited due to their relatively slow onset of action and side effects (Heckmann et al., 2011; Meriggioli et al., 2003; Witte et al., 1984).

Tacrolimus (TAC) is an effective therapy for MG patients. TAC is a macrolide calcineurin inhibitor that binds to the intracellular protein FKBP-12, preventing activity of Nuclear Factor of Activated T-cells (NFAT) and subsequent T cell activation (Monaghan et al., 2017). Many clinical studies have indicated that low-dose TAC treatment improves clinical symptoms in MG patients (Kawaguchi et al., 2004; Minami et al., 2011; Nagaishi et al., 2008; Nagane et al., 2005; Ponseti et al., 2005; Ponseti et al., 2008; Zhao et al., 2011; Zhou et al., 2017). Despite promising results in clinical trials, therapeutic response to TAC in MG patients is variable (Meng et al., 2020, Wang et al., 2019, Zhou et al., 2017) and unpredictable and no efficacy surrogates (biomarkers) currently exist to predict therapeutic response.

Accumulating experimental and clinical evidence suggest that helper T cells (CD4 T cell subsets) such as Th1 (produce interleukin-2 and interferon-γ) and Th17 (secrete cytokine interleukin-17) play a dominant role in MG pathogenesis (Aguilo-Seara et al., 2017; Ma et al., 2020;

Abbreviations: MG, myasthenia gravis; RG, response group; NRG, Non-response group; MGC, myasthenia gravis composite; Th, helper T cell; Tfh, follicular helper T cell; AChR-Ab, Acetylcholine receptor antibody; AChR-MG, Anti-acetylcholine receptor antibody-positive MG; MuSK-MG, Muscle-specific tyrosine kinase myasthenia gravis; NFAT, nuclear factor of activated T-cells; FKBP-12, FK506 binding protein 12; MGFA, Myasthenia gravis Foundation of America; MG-MMT, Manual muscle testing; PIS, post-intervention status; PBMCs, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; IONO, ionomycin; FBS, fetal bovine serum; HC, healthy control.

* Corresponding author at: The First Affiliated Hospital, Sun Yat-sen University, NO. 58, 2nd Road of Zhongshan, Yuexiu district, Guangzhou, Guangdong 510080, China.

E-mail address: liuwb@mail.sysu.edu.cn (W. Liu).

https://doi.org/10.1016/j.jneuroim.2024.578464
Received 18 April 2024; Received in revised form 4 October 2024; Accepted 6 October 2024
Available online 9 October 2024

Roche et al., 2011; Schaffert et al., 2014; Tabarkiewicz et al., 2015; Xie et al., 2016; Yi et al., 2014; Yi et al., 2020). We previously demonstrated that TAC inhibits Th1 and Th17 responses in vitro (Li et al., 2019). Several studies have found that TAC suppresses T cell proliferation and decreases the production of IL-2 and IFN-γ (Bao et al., 2019; Utsugisawa et al., 2003). We postulate that the wide variation in the therapeutic response to TAC is partly due to differences in the immunological states of MG patients. Thus, we aimed to compare the immunological changes observed in MG patients who were responsive vs non-responsive following TAC exposure.

2. Methods and materials

2.1. Participants

Forty-six MG patients were recruited from the MG Clinics in the Neurology Department, First Affiliated Hospital of Sun Yat-sen University, between October 2016 and January 2018; 1 patient withdrew due to symptom exacerbation, 1 patient withdrew due to increased blood sugar level, and 3 patients were dropouts due to poor compliance, leaving 41 patients. All had detectable anti-AChR antibodies obtained via commercially available testing (RSR Limited, Cardiff, UK) as well as clinical and electrodiagnostic symptoms consistent with MG. We applied the following inclusion criteria (clinical diagnosis of generalized MG, MG-Composite Score $\geq$ 3 points, and stable dose of steroids for at least 2 months) and exclusion criteria (contraindication to TAC according to the treating physician, an infection treated with antibiotics within the previous 2 weeks, treatment with IVIG or PE within the previous 3 months, other autoimmune diseases, HIV infection or unwillingness to comply with the study design). Clinical information including patient demographics, onset age, disease duration, Myasthenia Gravis Foundation of America (MGFA) severity class, MG-composite (MGC), Manual muscle testing (MG-MMT), Quantitative MG score (QMG) and Post-Intervention Status (PIS) were collected. This study was conducted following the guidelines of the World Medical Association's Declaration of Helsinki. This study was approved by First Affiliated Hospital of Sun Yat-sen University Review Boards and informed consent was obtained from each patient. Age and gender matched health controls were also recruited.

2.2. Study design and clinical evaluation

TAC capsules (Prograf; Astellas Pharma Inc., US) were administered orally at a dosage of 2 mg/day for at least 12 weeks. Patients were strongly encouraged to take TAC at least 4 h after dinner to minimize the impact on the drug's bioavailability. At baseline, 2, 4, 8 and 12 weeks, we conducted clinical evaluations, including MGC, QMG, MG-MMT and PIS status, and collected blood samples for TAC blood trough concentration testing and T cell analyses. Serum AChR-Ab titers collected at baseline and week 12 were tested by a commercial institution (Daan Gene, Guangzhou, China).

2.3. Isolation, storage and further processing of peripheral blood monocyte cells (PBMCs)

We collected 15 mL peripheral blood samples in ethylenediamine tetra acetic acid tubes and 5 mL peripheral blood in serum separator tube. The serum tube was kept on ice after 30 min coagulation and ethylenediamine tetra acetic acid tubes were stored at room temperature for further processing in 8 h. Blood samples were processed in accordance with previously published procedures (Li et al., 2020, Li et al., 2019).

2.4. Cytokine test in cell culture supernatant

After thawing PBMCs (Li et al., 2020) and washing twice with RPMI medium containing 10 % FBS (R10) (Gemini), cell number and viability were calculated by typan blue staining in microscopy (Strober, 2015). Cells optimal concentration based on titrations, a total of 2×10^5 PBMCs were plated in 96-well flat-bottom plates in R10. To stimulate the cells, 1 μg/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 0.25 μg/mL ionomycin (IONO, Sigma-Aldrich) were added to the cells in R10. After incubation for 48 h, we performed IFN-γ, IL-2, IL-4, IL-17 and IL-21 ELISA tests on the extracted supernatants according to the manufacturer's (Invitrogen, ThermoFisher) instructions.

2.5. Antibodies

The following antibodies from BioLegend (San Diego, CA) were used for flow cytometry (please refer panels in Supplemental Tables 2 and 3): APC-Cy7-conjugated anti-CD3, APC-conjugated anti-IL-2, APC-conjugated anti-CXCR5, AlexaFluor700-conjugated anti-CD8, PerCP-PC5.5-conjugated anti-CD4, FITC-conjugated anti-CD25, FITC-conjugated anti-IFN-γ, PerCP-conjugated anti-CD45RA, PerCP-PC5.5-conjugated anti-CD45RA, BV650-conjugated anti-PD-1, BV605-conjugated anti-ICOS, BV605-conjugated anti-IL-17 A, PE-Cy7-conjugated anti-CD4, PE-conjugated anti–FOXP3, PE-conjugated anti-IL-21, Live/Dead violet dye and PE-Cy7-conjugated anti-IL-4.

2.6. Cellular analysis and flow cytometry

After thawing PBMCs and washing twice with RPMI medium containing 10 % FBS (R10) (Gemini), cell number and viability were calculated (Strober, 2015). A total of 2×10^6 PBMCs were plated in 96-well round-bottom plates in R10. PBMCs were stimulated with 1 μg/mL PMA and 0.25 μg/mL IONO in the presence of 1 μg/mL Brefeldin A (BD Biosciences) for 5 h at 37 °C in 5 % CO_2 in a humidified incubator. After 300 *g* for 5 min centrifugation and removal of media, the cells were surface stained with LIVE/DEAD violet dye (Life Technologies, Grand Island, NY) for 15 min at room temperature, washed with 200 μL PBS, then stained with a surface stain cocktail mix for 30 min at 4 °C. Following cell surface staining, cells were treated with either FOXP3/Transcription Factor and Fixation/Permeabilization buffer for intranuclear staining following the manufacturer's recommendations (eBioscience, San Diego, CA) or, for intracellular staining, with Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA). Intranuclear or intracellular cytokine staining was then performed in accordance with manufacture instructions. Finally, cells were fixed with 1 % paraformaldehyde (Sigma-Alrich) and acquired on a Cytoflex flow cytometer (Beckman Coulter). The CD4 T cell subsets were defined according to the literature (Crotty, 2014; Sakaguchi et al., 2020; Zhu and Zhu, 2020) and listed as following: follicular helper T cell (Tfh) (CD4 + CD45RA-CXCR5+); follicular regulatory T cell (Tfr) (CD4 + CD45RA-CXCR5 + $FOXP_3$+); regulatory T cell (Treg)(CD3 + CD4 + CD25 + $FOXP_3$+); Th1 (CD4 + IFN-γ + or CD4 + IL-2+); Th2 (CD4 + IL-4+); Th17 (CD4 + IL-17 A+) and pathogenic Th17 (CD4 + IL-17 A + IFN-γ+) (Supplementary Fig. 1).

2.7. Statistical analysis

Flow data analysis was performed using FlowJo software (Tree Star, Ashland, OR). We used a chi-square test or Fisher's exact test for categorical variables, Student's *t*-tests to analyze normally distributed data for two group comparisons (one-way ANOVA for three or more groups), and the Wilcoxon rank-sum test for non-normally distributed data. A Pearson's correlation was computed to assess a linear association between two variables. Summary data are presented as mean values and the standard error of the mean. The *p*-values were calculated using Prism software (Graph Pad, LaJolla, CA).

3. Results

3.1. Demographic characteristics

Forty-one patients were included in our analysis (Supplement Table 1). Of these, 21 patients underwent thymectomy at least 2 years previously. Of the 41 patients, 27 whose MGC score decreased at least 3 points were classified as the Response Group MG (RG) and the remaining 14 patients were considered Non-Response Group (NRG) (Burns et al., 2010). The distribution of gender, age of onset, duration, thymectomy status and MGFA classification were not significantly different between the RG and NRG (Table 1), suggesting that these demographic factors did not affect the varied individual responses to TAC treatment.

3.2. Differences in blood trough TAC concentration and AChR-Ab titer in RG and NRG

We analyzed the blood trough concentration of TAC throughout the treatment period to determine whether variations in its level produced different clinical responses. The concentration of TAC remained nearly stable at 3.0 ± 3.0 μg/L from weeks 2–12 (Fig. 1B). TAC concentration in the response patients were not significantly different from those in non-response patients (Fig. 1D). The AChR-Ab titer also slightly decreased at the end of the TAC treatment compared to baseline (22.4 ± 23.6 nmol/L vs 20.23 ± 3.3 nmol/L, $p > 0.05$) (Fig. 1A). However, the antibody titers were not significantly different between the response and non-response patients (Fig. 1C). These results suggested that TAC concentration and antibody titer do not correlate with clinical response to TAC treatment.

3.3. IL-17 cytokine is inhibited in PBMC culture supernatants

As serum cytokine production was neither stable nor detectable in our preliminary research (data not shown), we tested IFN-γ and IL-2, IL-4, IL-17 and IL-21 (the major cytokines of T-cell subsets Th1, Th2, Th17 and Tfh, respectively) in PBMC culture supernatants (Barzago et al., 2016; Mosmann and Coffman, 1989; Romagnani, 1999; Saito et al., 2005; Seder and Paul, 1994). Only IL-17 (1198.3 ± 192.4 pg/mL vs 909.1 ± 133.3 pg/mL, $p = 0.014$) (Fig. 2A) and IL-2 (5389.9 ± 694.5 pg/mL vs 4256.2 ± 653.4 pg/mL, $p = 0.02$) levels (Supplementary Fig. 2C) were higher at baseline in MG patients compared with healthy controls (HC), but the RG showed a steeper decrease in IL-17 at 8-week (960.1 ± 120.2 pg/mL vs 1198.2 ± 130.1 pg/mL, $p > 0.05$) and 12-week (880.5 ± 136.5 pg/mL vs 1213.21 ± 160.1 pg/mL, $p < 0.05$). Additionally, IL-17 levels in the RG were significantly lower than in the NRG at the 12-week of post-treatment (880.5 ± 136.5 pg/mL vs 1213.2 ± 160.1 pg/mL, $p < 0.05$) (Fig. 2B). These findings indicated that IL-17 cytokines are robustly inhibited when patients respond well to TAC.

Table 1
Comparison of MG patients characteristics at timepoint of inclusion.

	Tacrolimus response ($N = 27$)	Tacrolimus non-response ($N = 14$)	p value
Gender	female(16, 59.3 %)	female(11, 78.6 %)	0.22
Age of onset(Y)	32.8 ± 3.6	36 ± 5.1	0.61
Duration (month)	71.3 ± 15.4	77.43 ± 29	0.84
Thymectomy	15 (55.6 %)	6 (42.9 %)	0.52
MGFA classification			
I	0(0 %)	1(7.1 %)	0.34
IIA	16(59.3 %)	9(64.4 %)	0.99
IIB	9(33.3 %)	3(21.4 %)	0.49
III	2(7.4 %)	1(7.1 %)	0.99

Footnote: MGFA classification, myasthenia gravis foundation of American clinical classification.

3.4. Th17 cells are inhibited in patients who respond well to TAC treatment

We used flow cytometry to determine CD4 T cell subset changes in our MG patients using the known classical cytokines or surface markers to these subsets (Golubovskaya and Wu, 2016), including Th1, Th2, Th17, Tfh and Treg cells. Of these, only Th17 cell levels were significantly higher in MG patients (pre-TAC-treatment) compared with HC (1.4 ± 0.8 % vs 0.9 ± 0.4 %, $p = 0.03$); after 12 weeks of TAC treatment, the frequencies of Th17 cells in MG patients decreased significantly to levels similar to those of HC (0.97 ± 0.59 % vs 0.87 ± 0.38 %, $p = 0.88$) (Fig. 3A, B) and were significantly lower in the RG than in the NRG (0.80 ± 0.06 % vs 1.09 ± 0.21 %, $p < 0.05$) (Fig. 3C). The frequencies of Th2 cells, Tfh cells, Tfr and Treg cells, which were defined as IL-4 positive CD4 T cells, CD45RA-CXCR5 + CD4 T cells, CD45RA-CXCR5 + $FOXP_3$ + CD4 T cells and CD25 + FOXP3 + CD4 T cells respectively, were neither significantly different in MG patients and HCs (Supplementary Fig. 3G-H, Supplementary Fig. 4 A-B, D-E, G-H), nor significantly different in RG and NRG (Supplementary Fig. 3I, Supplementary Fig. 4C, F, I). As the surface marker of CXCR5 downregulated after PMA/ION stimulation (data not shown), we gated on CD4 + CD45RA-IL-21+ to represent functional Tfh cells (Supplementary Fig. 1 B). This gating strategy is consistent with previous study (Schultz Bruce et al., 2016). We found that IL-2 positive Th1 cells (27.3 ± 11.9 % vs 37.7 ± 18.3 %, $p < 0.05$) not IFN- γ positive Th1 cells (20.6 ± 12.1 % vs 23.4 ± 12.0 %, $p = 0.56$), and functional Tfh cells (2.62 ± 2.19 % vs 4.03 ± 2.58 %, $p < 0.05$) decreased significantly in MG patients at the end of treatment compared to baseline levels (Supplementary Fig. 3 A-F, Fig. 3D-F). Unexpectedly, the levels of functional Tfh cells in the RG vs. NRG were not significantly different during the the first 8 weeks but were significantly different the end of 12 weeks of treatment (Fig. 3F). Thus, TAC was observed to strongly inhibit Th17 cells and slightly suppress Th1 and functional Tfh cells, but had no effect on Th2 and Treg cells.

3.5. Effects of TAC on the pathogenic Th17 subset

In our previous study, TAC was shown to inhibit pathogenic Th17 cells in vitro (Li et al., 2019). Based on the preliminary results, we further analyzed the changes in pathogenic Th17 cells, which were characterized by co-production of IFN-γ and IL-17 (Ito et al., 2019; Komuczki et al., 2019; Villegas et al., 2019), in clinical MG patients. The frequencies of pathogenic Th17 cell were significantly higher at baseline for MG patients than the HC group (0.33 ± 0.23 % vs 0.14 ± 0.05 %, $p < 0.05$)(Fig. 4A, B); at 12- week, pathogenic Th17 cells in MG patients were significantly reduced (0.33 ± 0.23 % vs 0.22 ± 0.17 %, $p < 0.05$), but there were no significant differences compared to HC group (0.22 ± 0.17 % vs 0.14 ± 0.05 %, $p > 0.05$) (Fig. 4B). Frequencies of pathogenic Th17 cell in the RG were significantly lower than those in the NRG at most of the follow-up timepoints (Fig. 4C). We found that changes in pathogenic Th17 cell levels were positively correlated with changes in MGC scores ($R^2 = 0.387$, $p < 0.05$) (Fig. 4D). Overall, TAC inhibits pathogenic Th17 cells more efficiently in the RG than in the NRG.

4. Discussion

In the present study, we showed that two thirds of the MG patients evaluated responded well to treatment with TAC. The varied responses to TAC among MG patients were not attributable to differences in TAC trough concentrations or AChR-Ab levels. Cytokines of IL-2 and IL-17 were increased at baseline in MG patients compared to HC, but only IL-17 was noticed significantly decreased at week 12 and in RG. We further found that Th17, IL-2 + Th1 and functional Tfh (defined as IL-21 positive CD4 T cell) (Schultz Bruce et al., 2016) subsets were significantly decreased in MG patients at the end of 12 weeks of TAC treatment. In addition, the Th17 cells, especially pathogenic Th17 cells were robustly inhibited in RG at 12 weeks. But Th2 and Treg subsets were not

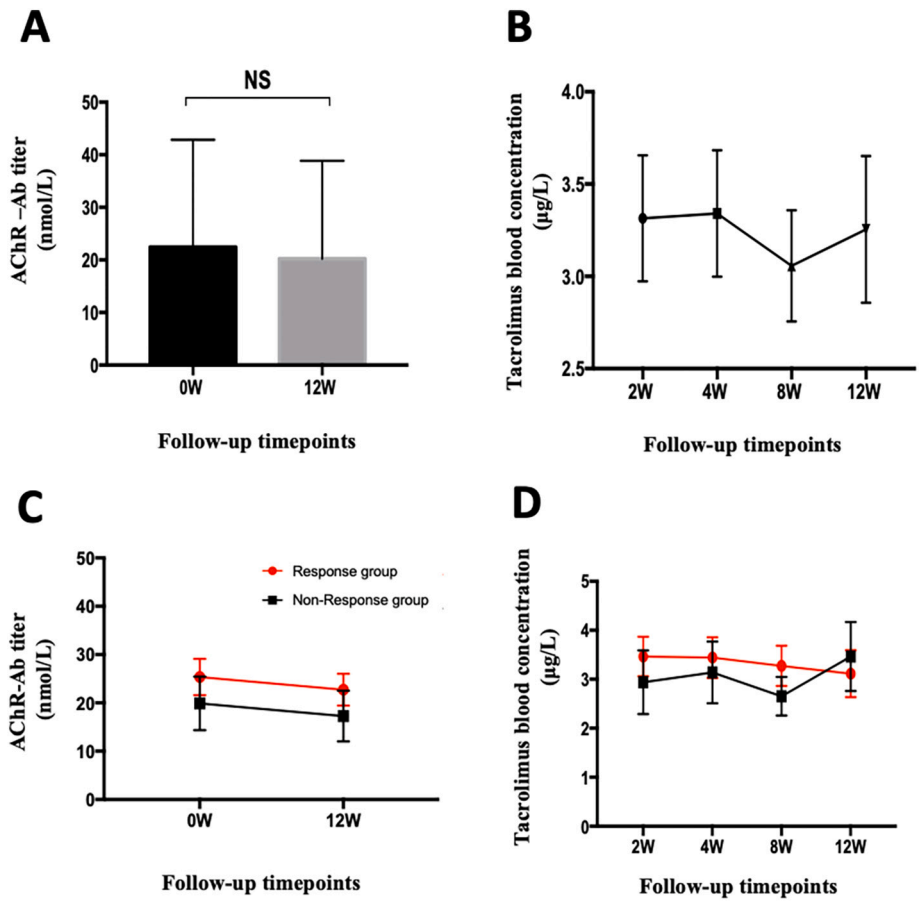

Fig. 1. Comparison of AChR-Ab and TAC blood concentration at different timepoints between RG and NRG. A. Comparison of AChR-Ab at baseline and at 12 weeks. B. Comparison of TAC blood concentrations at each follow-up (week 2, 4, 8, 12) time point. C. Comparison of AChR-Ab for RG and NRG at baseline and 12 weeks. D. Comparison of TAC blood concentrations for RG and NRG at 2, 4, 8, and 12 weeks. NS, not significant.

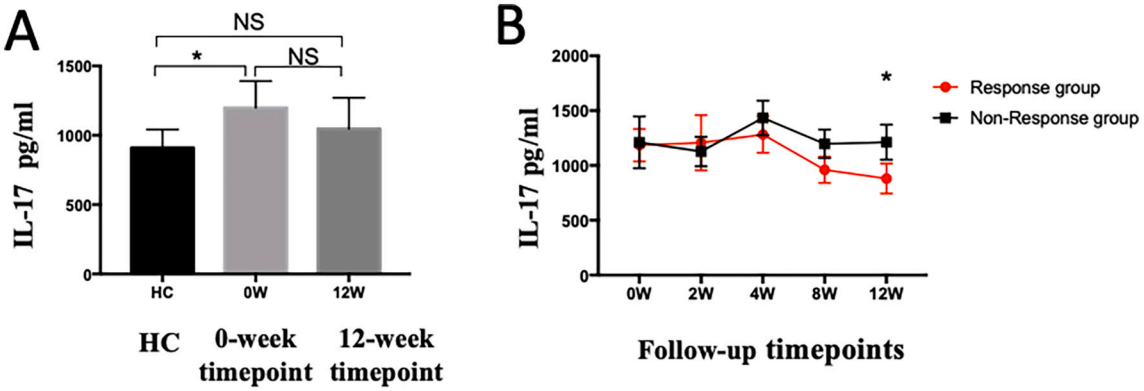

Fig. 2. Comparison of IL-17 in supernatant at different timepoints. A. Comparison of IL-17 in supernatant among HC and MG patients, the latter at both baseline and 12 weeks. B. Comparison of IL-17 between RG and NRG at 2, 4, 8, and 12 weeks. * $p < 0.05$; NS, not significant.

significantly affected.

We found that AChR-Ab titers were not significantly different between our RG and our NRG, which is in accordance with previous work (Vincent and Newsom-Davis, 1985), which demonstrated that AChR-Ab titers do not predict clinical responses. In our current study, blood TAC concentrations in RG and NRG patients were comparable, suggesting that TAC concentration does not determine response variation in MG patients; this is corroborated by previous findings (Meng et al., 2020, Nagane et al., 2010). However, our results are not in complete agreement with other studies due to differences in study population; for example, 31 % of the MG patients analyzed by Kanai et al. were MGFA class I and had decreases in MGC scores of less than 3 points (Kanai et al., 2017). These patients would not be counted as part of our RG, thus the differences in results could have resulted from our stricter inclusion standards.

We thoroughly examined the immunological changes to better understand the factors causing varied responses to TAC treatment in MG patients. First, we found that frequencies of Th1 cells and related cytokines IL-2 and IFN-γ were not significantly different between the RG and the NRG, which indicated that Th1 cells are not the major driving factor

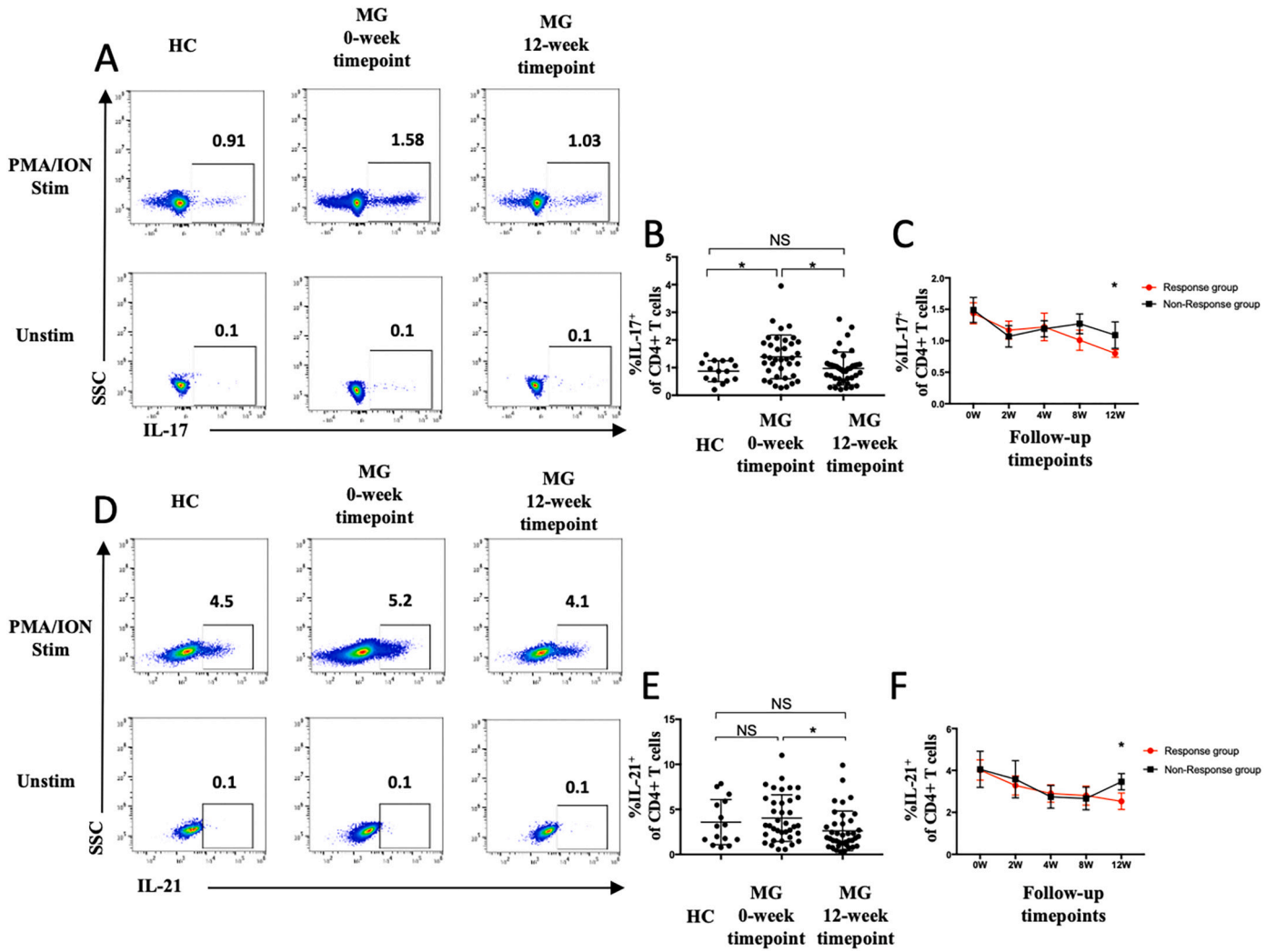

Fig. 3. Comparison of Th17 cells and IL-21+ CD4 T cells among HC and, at baseline and 12 weeks, RG and NRG. A. Flow cytometry pseudo-color plots of the comparison of Th17 cells among HC, RG, and NRG at baseline and 12 weeks. B. Plots of the comparison of Th17 cells among HC, RG and NRG at baseline and 12 weeks. C. Comparison of Th17 between RG and NRG at 2, 4, 8, and 12 weeks. D. Flow cytometry pseudo-color plots of the comparison of IL-21+ CD4 T cells among HC, RG and NRG at baseline and 12 weeks. E. Plots of the comparison of IL-21+ CD4 T cells among HC, RG and NRG at baseline and 12 weeks. F. Comparison of IL-21+ CD4 T cells between RG and NRG at 2, 4, 8, and 12 weeks. * $p < 0.05$; NS, not significant.

for the discrepancies in TAC responses. This observation aligns with our CD8 T cell data (Supplementary Fig. 5). In pathway analysis, Th1-determining factors, Signal Transducer and Activator Of Transcription 1(STAT1) could combine with NFAT, Activator protein 1(AP-1) and Neurogenic locus notch homolog protein 3/Recombination Signal Binding Protein For Immunoglobulin Kappa J Region/Mastermind-like 1 (Notch3/RBPJ/MAML1) signaling pathway to bind to T-box transcription factor 21(*tbx*21) promoter and induces the master transcription factor of Th1 cell (Hermann-Kleiter and Baier, 2010). In theory, TAC could suppress Th1 cells by inhibiting NFAT and downstream products (Tocci et al., 1989). However, our finding does not support this hypothesis and is not in line with our previous results demonstrating that TAC can inhibit Th1 cells in vitro (Li et al., 2019). This inconsistency further suggests that the inhibition of IL-2 production by TAC (Tocci et al., 1989) may be remedied by a rescue pathway. Second, we found that levels of Th2 cells and related cytokines were not significantly different between HC and MG patients (neither RG nor NRG), suggesting that Th2 cells, overactivated in allergy-related diseases or as a defense against parasites, are unlikely to be involved in the pathogenesis of myasthenia gravis. A previous pathway study demonstrated that NFAT inhibits Th2 differentiation (Hermann-Kleiter and Baier, 2010). This finding is consistent with our previous work, in which we found that Th2 cells could not be significantly suppressed by TAC in vitro (Li et al., 2019). Third, we demonstrate here that Treg cells were not significantly reduced in MG patients when exposed to TAC. This result is in contrast with our in vitro assay, which demonstrated that Treg cells were suppressed by TAC (Li et al., 2019). Thus, in vivo, Treg cells maintained their homeostatic status even when exposed to TAC. Fourth, studies indicate that Tfh cells promote antibodies production by facilitating B cells maturation in MG patients (Luo et al., 2013; Zhang et al., 2016). We found that TAC did not alter Tfh cell frequency, but TAC suppressed IL-21 production in Tfh. NFAT, together with STAT3 (Signal Transducer and Activator of Transcription 3) and c-MAF (musculoaponeurotic fibrosarcoma oncogene homolog), activates the transcription of *Il21* (Hermann-Kleiter and Baier, 2010). In our studies, IL-21 positive Tfh gradually decreased in both RG and NRG groups during the first 8 weeks of TAC treatment, after 12 weeks of treatment, IL-21 positive Tfh was significantly decreased only in RG and NRG. This finding still suggests that TAC may have an impact on Tfh, not by altering the number of cells but by impairing their ability to produce pathogenic cytokines.

Finally, Th17 cells are known to play an important role in the pathogenesis of autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and thyroiditis (Kuwabara et al., 2017,

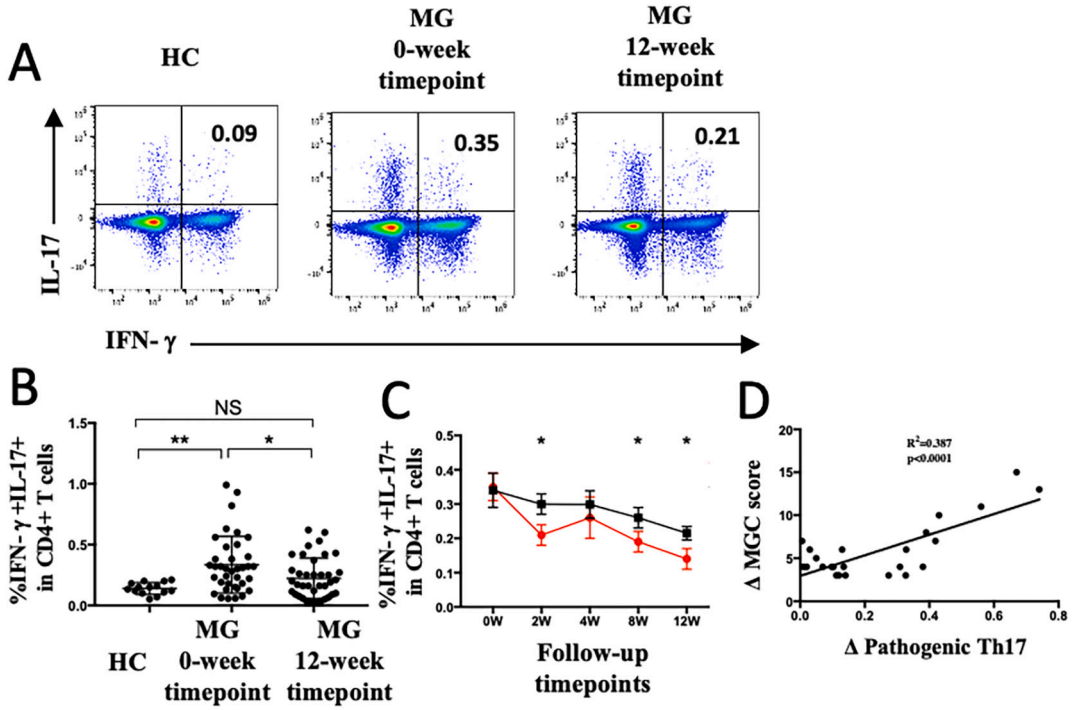

Fig. 4. Comparison of pathogenic Th17 cells among HC and, at baseline and 12 weeks, RG and NRG at baseline and 12 weeks. A. Flow cytometry pseudo-color plots of the comparison of pathogenic Th17 cells among HC, RG and NRG at baseline and 12 weeks. B. Plots of the comparison of pathogenic Th17 cells among HC, RG and NRG at baseline and 12 weeks. C. Comparison of pathogenic Th17 between RG and NRG at 2, 4, 8, and 12 weeks. D. Correlation of delta MGC and delta pathogenic Th17 in RG ($n = 24$). * $p < 0.05$; **, $p < 0.001$; NS, not significant.

Tabarkiewicz et al., 2015). Studies indicate that IL-17 levels are elevated in AChR-MG patients (Roche et al., 2011, Xie et al., 2016) and that IL-17 knockout mice in experimental autoimmune MG models do not develop clinical MG pathology (Aguilo-Seara et al., 2017). In our assay, we found that Th17 cells and IL-17 increased in AChR-MG when compared to HC and that Th17 cells were significantly reduced when they exposed to TAC treatment. Th17 cells can be classified as pathogenic or non-pathogenic according to their cytokine profiles (Lee et al., 2012; Stockinger and Omenetti, 2017). Experimental models have implicated pathogenic Th17 cells in autoimmunity in multiple sclerosis (Hu et al., 2017). This cell subset is induced by IL-23 and simultaneously co-produces IL-17 and IFN-γ (Gaublomme et al., 2015; Hirota et al., 2011). Pathogenic Th17 cells are known to promote B cell responses and could be a therapeutic target in autoimmune disease (Kuwabara et al., 2017; Lee et al., 2014; Yamagata et al., 2015). In our study, there was a statistically significant reduction in the overall number of Th17 cells as well as the IL-17^{+}IFN-γ^{+} pathogenic Th17 subset for RG vs NRG patients. From a signaling pathway perspective, NFAT, together with STAT3 and AP-1, binds to the promoter of Retineic-acid-receptor-related orphan nuclear receptor gamma (RORγt), which is the master transcription factor of Th17 cells. Subsequently, RORγt, in combination with NFAT, AP-1, STAT3, AhR (Aryl hydrocarbon Receptor), IRF4 (Interferon Regulatory Factor 4), and Runx1(Runt-related transcription factor 1), binds to the *Il17* promoter region to induce transcription (Hermann-Kleiter and Baier, 2010). Besides, NFAT combines with IRF4 and Smad2/3 [SMA("small" worm phenotype) and mothers against decapentaplegic 2/3] to drive the transcription of *Il23* (Hermann-Kleiter and Baier, 2010), the protein of which is an essential cytokine for Th17 differentiation (Toussirot, 2012), this is consistent with our previous study (Ma et al., 2020). NFAT plays a multi-role in Th17 differentiation, which may explain why TAC inhibits Th17 subsets in MG patients. In addition, NFAT also is one of the key components in Th1 differentiation, which may further explain why the pathogenic Th17 subset was significantly inhibited by TAC. When considering the role of pathogenic Th17 cells in MG pathogenesis and the role of NFAT in the Th17 differentiation, our finding that decreased pathogenic Th17 cells are correlated with symptom changes is critical because it may represent a potential biomarker for TAC treatment in MG patients.

Our study had several limitations. A limited number of MG patients were included in the study. This study did not include MG patients with severe symptoms (MGFA classification IV or V) and other less common subtypes of MG (muscle-specific tyrosine kinase myasthenia gravis, etc.). Furthermore, the evaluation of MG patients was limited to a clinical trial approach, and no investigation was conducted into the potential molecular or signaling pathway mechanisms of TAC. Future research expanding on these dimensions could provide a more comprehensive understanding of TAC's role in MG management. Finally, intracellular cytokines in CD4 T cell subsets were assessed following PMA/ION stimulation. The use of PMA/ION stimulants may accentuate or reveal differences when compared to unstimulated conditions in real-world patients. Future research employing more sensitive and high-resolution techniques, such as single-cell RNA sequencing, which can detect cytokine mRNA changes without the use of stimulants, could significantly advance the field of immune monitoring studies.

Overall, our findings indicate that TAC is effective in inhibiting Th17 and pathogenic Th17 responses in AChR-MG patients who respond well to TAC treatment. Additionally, TAC slightly suppresses functional Tfh cell and IL-2 positive Th1 cells. In conclusion, while there is still much to uncover the signaling pathways and immune cells interactions in the presence of TAC, our findings suggest that pathogenic Th17 cells can be a viable therapeutic target and a valuable biomarker for assessing the response to TAC in MG patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2024.578464.

Funding

Yingkai Li was supported by High-impact Pilot MGFA grant and

Myasthenia Gravis Network (MGNet) Scholar Program Research Funding (U54NS115054). The study was supported by the National Natural Science Foundation of China (No.30870850, No.81071002, No.81371386), the National Natural Science Foundation Key International (Regional) Cooperation Research Project (No.81620108010), the Clinical Trial of 5010 Planned Project Sun Yat-sen University (No.2010003), Science and Technology Planned Project of Guangdong in China (No.2007B16002039) and granted from the National Key Clinical Department and Key Discipline of Neurology, the Guangdong Provincial Key Laboratory for Diagnosis and Treatment of Major Neurological Diseases (No.2014B030301035), the Southern China International Cooperation Base for Early Intervention and Functional Rehabilitation of Neurological Diseases (No.2015B050501003), Guangdong Provincial Engineering Center for Major Neurological Disease Treatment, Science and Technology Planning Project of Guangzhou (No.201604020010).

CRediT authorship contribution statement

Yingkai Li: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pei Chen:** Writing – review & editing, Methodology, Data curation. **Xin Huang:** Writing – review & editing, Data curation. **Hao Huang:** Writing – review & editing, Data curation. **Qian Ma:** Writing – review & editing, Data curation. **Zhongqiang Lin:** Writing – review & editing, Data curation. **Li Qiu:** Writing – review & editing, Data curation. **Changyi Ou:** Writing – review & editing, Data curation. **Weibin Liu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgement

The authors thank the study participants.

References

Aguilo-Seara, G., Xie, Y., Sheehan, J., Kusner, L.L., Kaminski, H.J., 2017. Ablation of IL-17 expression moderates experimental autoimmune myasthenia gravis disease severity. Cytokine 96, 279–285.
Bao, J., Gao, S., Weng, Y., Zhu, J., Ye, H., Zhang, X., 2019. Clinical efficacy of tacrolimus for treating myasthenia gravis and its influence on lymphocyte subsets. Rev. Neurol. 175, 65–72.
Barzago, C., Lum, J., Cavalcante, P., Srinivasan, K.G., Faggiani, E., Camera, G., et al., 2016. A novel infection- and inflammation-associated molecular signature in peripheral blood of myasthenia gravis patients. Immunobiology 221, 1227–1236.
Burns, T.M., Conaway, M., Sanders, D.B., Composite, M.G., Group M-QS, 2010. The MG composite: a valid and reliable outcome measure for myasthenia gravis. Neurology 74, 1434–1440.
Crotty, S., 2014. T follicular helper cell differentiation, function, and roles in disease. Immunity 41, 529–542.
Gaublomme, J.T., Yosef, N., Lee, Y., Gertner, R.S., Yang, L.V., Wu, C., et al., 2015. Single-cell genomics unveils critical regulators of Th17 cell pathogenicity. Cell 163, 1400–1412.
Golubovskaya, V., Wu, L., 2016. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. Cancers 8.
Heckmann, J.M., Rawoot, A., Bateman, K., Renison, R., Badri, M., 2011. A single-blinded trial of methotrexate versus azathioprine as steroid-sparing agents in generalized myasthenia gravis. BMC Neurol. 11, 97.
Hermann-Kleiter, N., Baier, G., 2010. NFAT pulls the strings during CD4+ T helper cell effector functions. Blood 115, 2989–2997.
Hirota, K., Duarte, J.H., Veldhoen, M., Hornsby, E., Li, Y., Cua, D.J., et al., 2011. Fate mapping of IL-17-producing T cells in inflammatory responses. Nat. Immunol. 12, 255–263.
Hu, D., Notarbartolo, S., Croonenborghs, T., Patel, B., Cialic, R., Yang, T.H., et al., 2017. Transcriptional signature of human pro-inflammatory TH17 cells identifies reduced IL10 gene expression in multiple sclerosis. Nat. Commun. 8, 1600.
Ito, R., Katano, I., Otsuka, I., Hanazawa, A., Takahashi, T., Kawai, K., et al., 2019. Exacerbation of pathogenic Th17-cell-mediated cutaneous graft-versus-host-disease in human IL-1beta and IL-23 transgenic humanized mice. Biochem. Biophys. Res. Commun. 516, 480–485.
Kanai, T., Uzawa, A., Kawaguchi, N., Himuro, K., Oda, F., Ozawa, Y., Kuwabara, S., 2017. Adequate tacrolimus concentration for myasthenia gravis treatment. Eur. J. Neurol. 24, 270–275.
Kawaguchi, N., Yoshiyama, Y., Nemoto, Y., Munakata, S., Fukutake, T., Hattori, T., 2004. Low-dose tacrolimus treatment in thymectomised and steroid-dependent myasthenia gravis. Curr. Med. Res. Opin. 20, 1269–1273.
Komuczki, J., Tuzlak, S., Friebel, E., Hartwig, T., Spath, S., Rosenstiel, P., et al., 2019. Fate-mapping of GM-CSF expression identifies a discrete subset of inflammation-driving T helper cells regulated by cytokines IL-23 and IL-1beta. Immunity 50, 1289-304.e6.
Kuwabara, T., Ishikawa, F., Kondo, M., Kakiuchi, T., 2017. The role of IL-17 and related cytokines in inflammatory autoimmune diseases. Mediat. Inflamm. 2017, 3908061.
Lazaridis, K., Tzartos, S.J., 2020. Autoantibody specificities in myasthenia gravis; implications for improved diagnostics and therapeutics. Front. Immunol. 11, 212.
Lee, Y., Awasthi, A., Yosef, N., Quintana, F.J., Xiao, S., Peters, A., et al., 2012. Induction and molecular signature of pathogenic TH17 cells. Nat. Immunol. 13, 991–999.
Lee, Y., Collins, M., Kuchroo, V.K., 2014. Unexpected targets and triggers of autoimmunity. J. Clin. Immunol. 34 (Suppl. 1), S56–S60.
Li, Y., Guptill, J.T., Russo, M.A., Massey, J.M., Juel, V.C., Hobson-Webb, L.D., et al., 2019. Tacrolimus inhibits Th1 and Th17 responses in MuSK-antibody positive myasthenia gravis patients. Exp. Neurol. 312, 43–50.
Li, Y., Guptill, J.T., Russo, M.A., Howard Jr., J.F., Massey, J.M., Juel, V.C., et al., 2020. Imbalance in T follicular helper cells producing IL-17 promotes pro-inflammatory responses in MuSK antibody positive myasthenia gravis. J. Neuroimmunol. 345, 577279.
Luo, C., Li, Y., Liu, W., Feng, H., Wang, H., Huang, X., et al., 2013. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. J. Neuroimmunol. 256, 55–61.
Ma, Q., Ran, H., Li, Y., Lu, Y., Liu, X., Huang, H., et al., 2020. Circulating Th1/17 cells serve as a biomarker of disease severity and a target for early intervention in AChR-MG patients. Clin. Immunol. 218, 108492.
Meng, H.Y., Li, X., Jin, W.L., Yan, C.K., Dong, X.H., Xu, Q., et al., 2020. Multiple genetic factors affecting the pharmacokinetic and pharmacodynamic processes of tacrolimus in Chinese myasthenia gravis patients. Eur. J. Clin. Pharmacol. 76, 659–671.
Meriggioli, M.N., Ciafaloni, E., Al-Hayk, K.A., Rowin, J., Tucker-Lipscomb, B., Massey, J. M., Sanders, D.B., 2003. Mycophenolate mofetil for myasthenia gravis: an analysis of efficacy, safety, and tolerability. Neurology 61, 1438–1440.
Minami, N., Fujiki, N., Doi, S., Shima, K., Niino, M., Kikuchi, S., Sasaki, H., 2011. Five-year follow-up with low-dose tacrolimus in patients with myasthenia gravis. J. Neurol. Sci. 300, 59–62.
Monaghan, P., Leneghan, D.B., Shaw, W., Bell, A., 2017. The antimalarial action of FK506 and rapamycin: evidence for a direct effect on FK506-binding protein PfFKBP35. Parasitology 144, 869–876.
Mosmann, T.R., Coffman, R.L., 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7, 145–173.
Nagaishi, A., Yukitake, M., Kuroda, Y., 2008. Long-term treatment of steroid-dependent myasthenia gravis patients with low-dose tacrolimus. Intern. Med. 47, 731–736.
Nagane, Y., Utsugisawa, K., Obara, D., Kondoh, R., Terayama, Y., 2005. Efficacy of low-dose FK506 in the treatment of Myasthenia gravis–a randomized pilot study. Eur. Neurol. 53, 146–150.
Nagane, Y., Suzuki, S., Suzuki, N., Utsugisawa, K., 2010. Factors associated with response to calcineurin inhibitors in myasthenia gravis. Muscle Nerve 41, 212–218.
Ponseti, J.M., Azem, J., Fort, J.M., Lopez-Cano, M., Vilallonga, R., Buera, M., et al., 2005. Long-term results of tacrolimus in cyclosporine- and prednisone-dependent myasthenia gravis. Neurology 64, 1641–1643.
Ponseti, J.M., Gamez, J., Azem, J., Lopez-Cano, M., Vilallonga, R., Armengol, M., 2008. Tacrolimus for myasthenia gravis: a clinical study of 212 patients. Ann. N. Y. Acad. Sci. 1132, 254–263.
Roche, J.C., Capablo, J.L., Larrad, L., Gervas-Arruga, J., Ara, J.R., Sanchez, A., Alarcia, R., 2011. Increased serum interleukin-17 levels in patients with myasthenia gravis. Muscle Nerve 44, 278–280.
Romagnani, S., 1999. Th1/Th2 cells. Inflamm. Bowel Dis. 5, 285–294.
Saito, R., Onodera, H., Tago, H., Suzuki, Y., Shimizu, M., Matsumura, Y., et al., 2005. Altered expression of chemokine receptor CXCR5 on T cells of myasthenia gravis patients. J. Neuroimmunol. 170, 172–178.
Sakaguchi, S., Mikami, N., Wing, J.B., Tanaka, A., Ichiyama, K., Ohkura, N., 2020. Regulatory T cells and human disease. Annu. Rev. Immunol. 38, 541–566.
Schaffert, H., Meisel, A., Thiel, A., Kohler, S., 2014. CD4+ T-cell produced IL17 is essential for loss of B-cell tolerance in experimental autoimmune myasthenia gravis (EAMG). Eur. J. Neurol. 21, 40.
Schultz Bruce, T., Teigler Jeffrey, E., Pissani, F., Oster Alexander, F., Kranias, G., Alter, G., et al., 2016. Circulating HIV-specific Interleukin-21+CD4+ T cells represent peripheral Tfh cells with antigen-dependent helper functions. Immunity 44, 167–178.
Seder, R.A., Paul, W.E., 1994. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. 12, 635–673.
Stockinger, B., Omenetti, S., 2017. The dichotomous nature of T helper 17 cells. Nat. Rev. Immunol. 17, 535–544.

Strober, W., 2015. Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol. 111, A3.B.1.
Tabarkiewicz, J., Pogoda, K., Karczmarczyk, A., Pozarowski, P., Giannopoulos, K., 2015. The role of IL-17 and Th17 lymphocytes in autoimmune diseases. Arch. Immunol. Ther. Exp. 63, 435–449.
Tocci, M.J., Matkovich, D.A., Collier, K.A., Kwok, P., Dumont, F., Lin, S., et al., 1989. The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. J. Immunol. 143, 718–726.
Toussirot, E., 2012. The IL23/Th17 pathway as a therapeutic target in chronic inflammatory diseases. Inflamm. Allergy Drug Targets 11, 159–168.
Utsugisawa, K., Nagane, Y., Yonezawa, H., Obara, D., Kondoh, R., Tohgi, H., 2003. Effects of FK506 on myasthenia gravis patients with high interleukin-2 productivity in peripheral blood mononuclear cells. Muscle Nerve 27, 245–248.
Villegas, J.A., Bayer, A.C., Ider, K., Bismuth, J., Truffault, F., Roussin, R., et al., 2019. Il-23/Th17 cell pathway: a promising target to alleviate thymic inflammation maintenance in myasthenia gravis. J. Autoimmun. 98, 59–73.
Vincent, A., Newsom-Davis, J., 1985. Acetylcholine receptor antibody as a diagnostic test for myasthenia gravis: results in 153 validated cases and 2967 diagnostic assays. J. Neurol. Neurosurg. Psychiatry 48, 1246–1252.
Wang, L., Xi, J., Zhang, S., Wu, H., Zhou, L., Lu, J., et al., 2019. Effectiveness and safety of tacrolimus therapy for myasthenia gravis: a single arm meta-analysis. J. Clin. Neurosci. 63, 160–167.
Witte, A.S., Cornblath, D.R., Parry, G.J., Lisak, R.P., Schatz, N.J., 1984. Azathioprine in the treatment of myasthenia gravis. Ann. Neurol. 15, 602–605.
Xie, Y., Li, H.F., Jiang, B., Li, Y., Kaminski, H.J., Kusner, L.L., 2016. Elevated plasma interleukin-17A in a subgroup of myasthenia gravis patients. Cytokine 78, 44–46.
Yamagata, T., Skepner, J., Yang, J., 2015. Targeting Th17 effector cytokines for the treatment of autoimmune diseases. Arch. Immunol. Ther. Exp. 63, 405–414.
Yasir, M.G.A., Sonthalia, S., 2022. Corticosteroid Adverse Effects. StatPearls Publishing.
Yi, J.S., Guidon, A., Sparks, S., Osborne, R., Juel, V.C., Massey, J.M., et al., 2014. Characterization of CD4 and CD8 T cell responses in MuSK myasthenia gravis. J. Autoimmun. 52, 130–138.
Yi, J.S., Russo, M.A., Raja, S., Massey, J.M., Juel, V.C., Shin, J., et al., 2020. Inhibition of the transcription factor ROR-γ reduces pathogenic Th17 cells in acetylcholine receptor antibody positive myasthenia gravis. Exp. Neurol. 325, 113146.
Zhang, C.J., Gong, Y., Zhu, W., Qi, Y., Yang, C.S., Fu, Y., et al., 2016. Augmentation of circulating follicular helper T cells and their impact on autoreactive B cells in myasthenia gravis. J. Immunol. 197, 2610–2617.
Zhao, C.B., Zhang, X., Zhang, H., Hu, X.Q., Lu, J.H., Lu, C.Z., Xiao, B.G., 2011. Clinical efficacy and immunological impact of tacrolimus in Chinese patients with generalized myasthenia gravis. Int. Immunopharmacol. 11, 519–524.
Zhou, L., Liu, W., Li, W., Li, H., Zhang, X., Shang, H., et al., 2017. Tacrolimus in the treatment of myasthenia gravis in patients with an inadequate response to glucocorticoid therapy: randomized, double-blind, placebo-controlled study conducted in China. Ther. Adv. Neurol. Disord. 10, 315–325.
Zhu, X., Zhu, J., 2020. CD4 T Helper Cell Subsets and Related Human Immunological Disorders. Int. J. Mol. Sci. 21.