Original Article
The immunosuppressant fingolimod ameliorates experimental autoimmune myasthenia gravis by regulating T-cell balance and cytokine secretion

Ya-Ting Luo, Ying-Fan Liang, Huan He, Meng-Ting Zhang, Rui Wang, Hong-Lin Li

Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai, PR China

Abstract: Myasthenia gravis is an autoimmune disease that affects skeletal muscle strength by impeding communication within the neuromuscular junction (NMJ). Research has shown that sphingosine-1-phosphate (S1P)/S1P receptor signaling may be involved in the process of neuromuscular diseases. Fingolimod is structurally similar to S1P, whose immunosuppressive effect has been recognized in many immune diseases. However, the mechanism underlying fingolimod's action on experimental autoimmune myasthenia gravis is still far from clear. The aim of this study was to investigate the efficacy and possible mechanism of fingolimod on experimental autoimmune myasthenia gravis. Our results showed that pretreatment with fingolimod improved experimental autoimmune myasthenia gravis symptoms in a dose-dependent manner, including decreased anti-acetylcholine receptor-2α autoantibody titer, reduced compound muscle action potential decrement, and increased acetylcholine receptor content. Further investigation indicated that fingolimod inhibited lymphocyte proliferation responses and also regulated the balance of Th1/Th2 cells and Treg/Th17 cells. Moreover, fingolimod suppressed the secretion of pro-inflammatory or inflammatory cytokines IL-17A, IL-6, and INF-γ, but did not noticeably alter the secretion of immunosuppressive cytokines TGF-β1 and IL-4. In conclusion, our results suggest that fingolimod has a preventive effect on experimental autoimmune myasthenia gravis by interfering with lymphocyte function.

Keywords: Myasthenia gravis, fingolimod, Th cells, cytokines, lymphocyte proliferation

Introduction

Myasthenia gravis (MG) is an autoimmune disease that affects skeletal muscle strength by impeding communication within the neuromuscular junction (NMJ). The characteristic symptoms of MG are muscle weakness and fatigue. Antibodies against the muscle nicotinic acetylcholine receptor (anti-AChR Abs) play an important role in the pathogenesis of MG. These antibodies, which are produced by autoreactive B cells, belong to either the IgG1 or IgG3 subclass. The anti-AChR Abs activate the classical complement cascade, resulting in the destruction of neuromuscular architecture and the failure of neuromuscular transmission [1, 2]. In fact, MG patients have abundant anti-AChR Th1 cells, which induce synthesis of complement-fixing IgG subclasses in the peripheral blood [3]. In addition, patients with thymic hyperplasia always display germinal centers (GCs) [4] that exhibit an overexpression of cytokine IL-17 [5, 6]. The frequency of regulatory T (Treg) cells decreases markedly in MG patients [7, 8]. However, the functions of Treg cells are also defective [9].

Current MG therapies mainly target the NMJ and the immune system [10, 11]. Acetylcholinesterase inhibitors (AChEI) increase the activity of acetylcholine (ACh) at the NMJ and compensate for reduced acetylcholine receptor (AChR) densities [12]. General immunosuppression and thymectomy are first-line immunosuppressive treatments for patients who do not adequately respond to AChEI therapy [12]. Additional immunotherapies are emerging, such as eculizumab, which is approved for refractory MG by the FDA, and efgartigimod, which is considered effective in phase II clinical trials [13].

The existing standard treatments for MG are not guaranteed to keep the disease well-controlled. Furthermore, they require a lifelong regimen and have significant side effects, such as risks of malignancy and infection. Therefore, there is an urgent need to overcome these shortcomings and find more effective treatments.

Fingolimod (FTY-720), a novel immunosuppressant, is structurally similar to sphingosine-1-phosphate (S1P). Current results show that FTY-720 regulates lymphocyte homing to lymphoid organs by binding to sphingosine-1-phosphate receptor 1 (S1PR1) and inducing its internalization and degradation [14-16]. The immunosuppressive effect of FTY-720, which results from its depletion of peripheral lymphocytes, has been recognized in organ transplantation and multiple sclerosis [14]. Moreover, FTY-720 mitigates the severity of chronic colitis and atherosclerosis by interfering with lymphocyte function [17, 18]. In addition to these therapeutic effects, FTY-720 is under consideration for use in treating other autoimmune diseases as well, such as rheumatoid arthritis [19] and systemic lupus erythematosus [20]. Therefore, we speculated that FTY-720 might be a prospective treatment for MG and experimental autoimmune myasthenia gravis (EAMG). Previous studies have shown that FTY-720 ameliorates EAMG [21, 22], although the mechanism of FTY-720 on MG is still far from clear. In this study, we focused on the possible mechanism of FTY-720 on EAMG. Particular attention was paid to the role of FTY-720 in lymphocyte function. The efficacy of FTY-720 against EAMG was determined by measuring autoantibody titer, repetitive nerve stimulation (RNS), and AChR content, followed by the study of possible mechanisms, including lymphocyte proliferation, balance of Th cells, and secretion of cytokines. Our data suggested that FTY-720 pretreatment ameliorated the severity of EAMG, and which was associated with lymphocyte proliferation suppression, regulation of Treg/Th17 and Th1/Th2 balance, and downregulation of proinflammatory or inflammatory cytokines, such as IL-17A, IL-6, and INF-γ. Research has shown that S1P/S1P receptor signaling may be involved in the processes of neuromuscular diseases, including Duchenne muscular dystrophy and MG [23]. FTY-720 is the first reported S1PR agonist with immunosuppressive activity. Since it is a nonspecific S1PR modulator, new similar compounds are expected to develop. Our data indicated that FTY-720 has a therapeutic potential for EAMG via interfering with lymphocyte function. These data may lay the foundation for new drug design in the treatment of autoimmune diseases, including MG.

Materials and methods

Animal

Female Lewis rats (weighing 150-180 g) were provided by the Qinglong Mountain Breeding Center (license no. SCXK (Su) 2017-0001, Nanjing, China) and 8- to 10-week-old male Sprague Dawley (SD) rats were purchased from Shanghai Sippr-BK Laboratory Animal Co. Ltd. The animals were kept in 12-h light/dark cycle conditions with food and water *ad libitum*. All experiments were performed in accordance with animal ethics rules.

Induction of EAMG and drug administration

Thirty Lewis rats were divided into five groups (six per group). All rats, except for the normal controls, were immunized with 300 μg of AChR2α (Bosgene Biotech, Nanjing, China), a purified recombinant 2 alpha subunit of human AChR, in complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) subcutaneously on the back and both feet on day 0. Subsequently, the rats were booster immunized with 300 μg of AChR2α in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, St. Louis, MO, USA) on day 20, and booster immunized with 300 μg of AChR2α in CFA on day 30 [24]. The control rats were injected with the same volume of PBS and adjuvant without AChR2α. In order to evaluate the therapeutic effect of FTY-720, rats were treated with either vehicle, 0.15 mg/kg, or 1.5 mg/kg of FTY-720 every day. Prednisone dosed at 3.5 mg/kg was treated as the positive control. All rats were weighed twice a week until they were euthanized on day 35.

Repetitive nerve stimulation

Compound muscle action potential (CMAP) decrement was tested using the BL-420F Data Acquisition & Analysis System (Techman, Chengdu, China). Rats were anesthetized, and their temperatures were maintained at 37°C. A stimulating electrode was then inserted near the sciatic nerve for supramaximal stimulation

at 5 Hz and 10 Hz. CMAPs were recorded with recording electrodes, which were placed in the gastrocnemius muscle. Decrement values were calculated as the percent of amplitude attenuation between the 1st and 4th CMAPs.

Detection of anti-AChR2α-IgG

Serum samples from Lewis rats were prepared in order to detect anti-AChR2α-IgG using ELISA in accordance with the manufacturer's instructions. In brief, 96-well plates, pre-coated with AChR2α, were incubated with diluted serum (1:5, 50 μL) for 35 min at 37°C, followed by washing three times with wash buffer and incubating with 100 μL enzyme-labeled antibodies for 30 min at 37°C. After additional washing, substrate A and substrate B were added and incubated for 15 min at 37°C. Finally, the reaction was stopped, and the optical density (OD) value at 450 nm was measured.

Immunohistochemical staining

In order to analyze the AChR content of the NMJ, 20-Mm-thick frozen sections of gastrocnemius muscle were prepared and washed with TBST (0.1% Tween 20). The sections were then blocked with 10% goat serum for 2 h at room temperature (RT), and 3 μg/mL α-Bungatotoxin-FITC (Alomone Labs, Jerusalem BioPark, Israel) and anti-neurofilament heavy polypeptide (Abcam, Cambridge, MA, USA) were added to detect AChR and endplate, respectively [25]. After washing, sections were incubated with goat anti-rabbit IgG-Alexa Fluor 594 (Proteintech, Wuhan, China) for 1 h at RT. Finally, sections were observed and photographed under a 200-fold fluorescence microscope, and a quantitative analysis of AChR fluorescence intensity was performed using ImageJ software.

Preparation of lymphocytes

Splenic lymphocytes of Lewis rats from the different treatment groups were prepared by grinding spleens through a cell strainer on day 35, after the first immunization. The freshly-prepared cells were then adjusted to $5×10^6$ cells/mL in RPMI 1640 incomplete medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Thornton, NSW, Australia). Splenic lymphocytes from SD rats were isolated using a Ficoll-Paque PREMIUM medium, and the cells were then cultured in an

RPMI 1640 medium including 20% FBS at $2×10^6$ cells/mL.

Lymphocyte proliferation assay

A CCK-8 assay (Beyotime, Shanghai, China), in which WST-8 could be reduced to soluble formazan, was used to determine the effect of FTY-720 on lymphocyte proliferation. Briefly, 200 μL of cell suspension from SD rats, pre-treated with FTY-720 (0.5, 1, 5 μM) for 24 h at 37°C, was cultured in 96-well plates. The cells were then stimulated with or without concanavalin A (ConA, 5 μg/mL, Sigma, USA) for 24 and 48 h, respectively, followed by incubation with 10 μL CCK-8 for another 2 h. Cell culture supernatants were then collected for cytokine detection. OD values at 450 nm were measured using a microplate reader. The results were expressed in terms of the stimulation index (SI = $OD_{drug}/OD_{control}$).

Furthermore, $5×10^5$ cells from different treated rats were seeded into 96-well plates in the presence or absence of AChR2α (10 μg/mL). After 72 h of incubation, the cells were cultured for another 4 h with MTT. This was followed by the removal of supernatants and the addition of 100 μL DMSO to dissolve formazan, after which the OD values were tested at 490 nm. The results were expressed in terms of the stimulation index.

Cell cycle

In order to determine the effect of FTY-720 on the cell cycle, 2 mL of cell suspension from SD rats was cultured in six-well plates with or without FTY-720 (0.5, 1, 5 μM) for 24 h, after which the cells were collected and washed with PBS. After fixation with 1 mL 70% ethyl alcohol for 6 h at 4°C, the cells were stained with 0.5 mL propidium iodide (PI, Beyotime, Shanghai, China) at 37°C in the dark. Finally, the cells were detected using flow cytometry (Beckman Coulter, Brea, CA, USA), and the cell cycle was analyzed with FlowJo software.

Detection of Th cells

In order to detect the distribution of Th1/Th17/Th2 cells, 2 μL of PMA/ionomycin mixture and 2 μL of BFA/monensin mixture (MultiSciences, Hangzhou, China) were added to 500 μL of cell culture media and incubated for 6 h. The cells were then stained extracellularly with anti-CD3-

FITC (eBioscience, San Diego, CA, USA), and anti-CD8a-PE-Cy7 for 30 min at 4°C in the dark. After fixation for 15 min at RT, the cells were stained intracellularly with anti-INF-γ-eFluor 660, anti-IL-17A-PE, and anti-IL-4-eFluor 660 in the permeabilization solution, respectively. In order to detect Treg cells, surface markers on the splenocytes were analyzed by incubation with anti-CD4-FITC and anti-CD25-PE for 30 min at 4°C. After fixation and permeabilization, the cells were stained intracellularly with anti-Foxp3-APC for 30 min at 4°C. Finally, the samples were detected using flow cytometry.

Detection of cytokines

Sera from different treatment groups were prepared to detect TGF-β1 (R&D Systems, Minneapolis, MN, USA), IL-17A, and IL-6 (Bio-Rad, Hercules, CA, USA), and cell culture supernatants were harvested to detect TGF-β1, IL-4, IL-6, INF-γ, and IL-17A via Luminex assay in accordance with the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± SEM, and statistical differences among various groups were determined using a one-way ANOVA, with a post hoc test (LSD) performed for multiple comparisons. Statistical differences between two groups were determined using Student's *t*-test, with $P < 0.05$ indicating significant difference.

Results

FTY-720 alleviates AChR2α antigen-induced EAMG in Lewis rats

In order to determine the effect of FTY-720 on EAMG, the body weight, autoantibody titer, RNS, and AChR content of the rats were measured. During the experiment, the rats in different groups exhibited no significant difference in body weight (**Figure 1A**). The levels of anti-AChR IgG in the EAMG group were significantly increased compared with the levels in the control group ($P < 0.001$). Prednisone ($P < 0.05$) and FTY-720 at 1.5 mg/kg ($P = 0.052$) significantly reduced anti-AChR Abs titer compared with the EAMG group (**Figure 1B**). To determine the changes in AChR levels in the gastrocnemius muscle, frozen sections were double stained with BTX and anti-neurofilament heavy poly-

peptide antibody. The results revealed that AChR fluorescence intensity in the EAMG group was noticeably weaker than that in the control rats ($P < 0.05$). Treatment with FTY-720 and prednisone significantly strengthened the fluorescence intensity of AChR compared with the EAMG groups (**Figure 2A** and **2B**) ($P < 0.05$). In addition, gastrocnemius muscle function was tested using CMAP decrement. Consistent with all the above results, there was an obvious CMAP decrement in EAMG rats with supramaximal stimulation at 5 Hz and 10 Hz compared with the control group ($P < 0.001$ and $P < 0.01$, respectively). Compared with the EAMG group, both FTY-720 and prednisone noticeably reduced CMAP decrement, although this reduction did not reach statistical significance in the FTY-720 treated groups (**Figure 1E** and **1F**). All of these data suggest that FTY-720 could alleviate the severity of EAMG.

FTY-720 inhibits lymphocyte proliferation

Lymphocyte proliferation was measured to determine whether the therapeutic effect of FTY-720 was related to lymphocyte responses. Splenic lymphocytes of SD rats were pretreated with FTY-720 (0.5, 1, 5 µM) for 24 h, then stimulated with ConA (5 µg/mL) and continuously incubated for 24 h or 48 h. The results revealed that FTY-720 dose-dependently inhibited lymphocyte proliferation (5 µM, $P < 0.001$; 1 µM, $P < 0.05$) (**Figure 3A** and **3B**). Furthermore, in order to determine the effect of FTY-720 on the antigen (AChR2α)-specific lymphocyte proliferation reaction, lymphocytes from different treatment groups were cultured with AChR2α (10 µg/mL) for 72 h. As can be seen in **Figure 3**, splenic lymphocytes from EAMG rats exhibited an obvious proliferation reaction in response to AChR2α compared with lymphocytes from control rats ($P < 0.001$). Lymphocyte proliferation was markedly decreased, however, in the prednisone group and the 1.5 mg/kg FTY-720 group ($P < 0.01$ and $P < 0.05$, respectively) compared with the control group (**Figure 3C**).

In order to examine how FTY-720 affects lymphocyte proliferation, primary isolated splenic lymphocytes were treated with FTY-720 (0.5, 1, 5 µM) for 24 h followed by PI staining. Flow cytometry was performed to determine the cell cycle phase distribution. The results indicated that FTY-720 significantly increased the per-
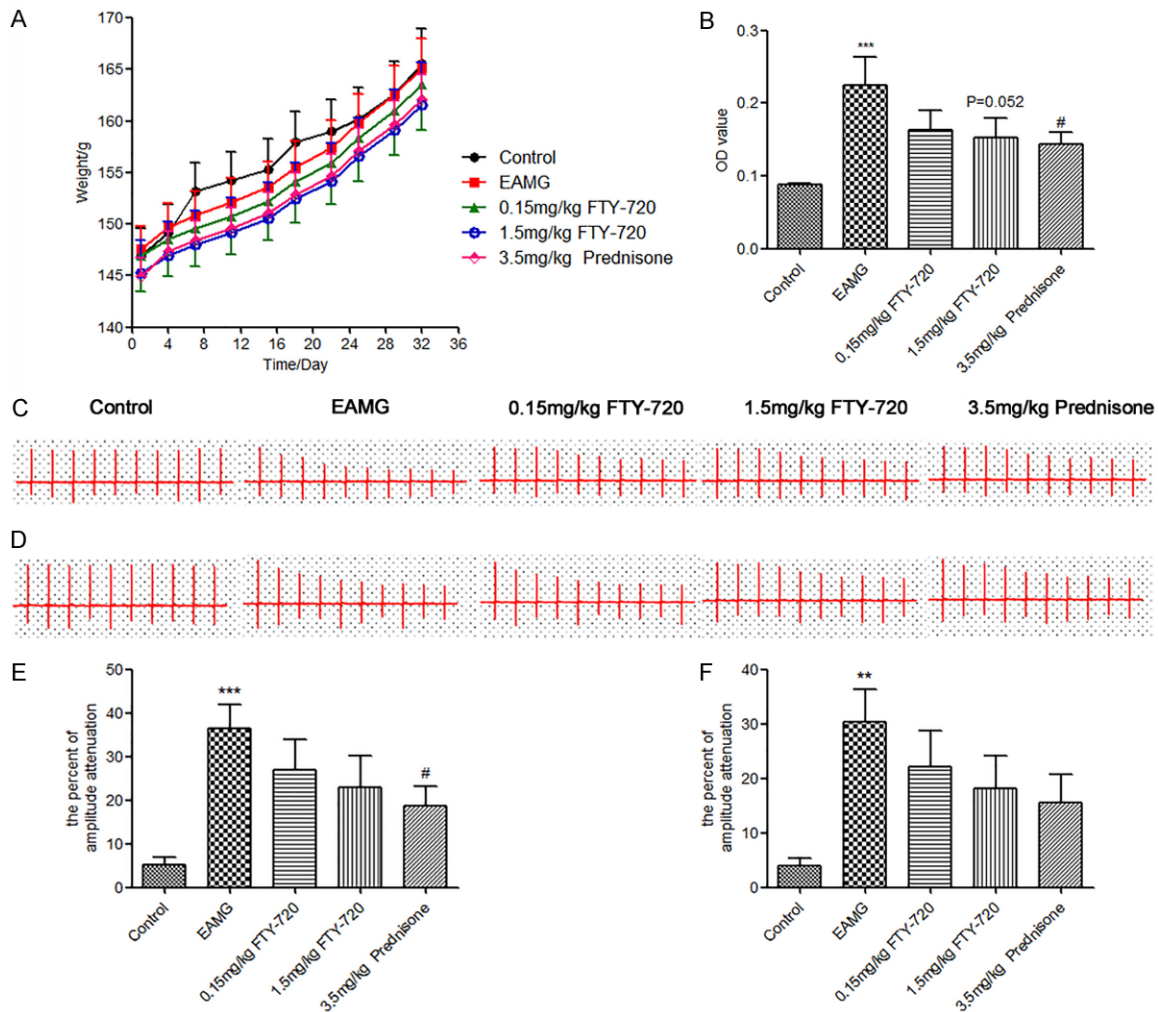
Figure 1. Pretreatment with FTY-720 improved EAMG severity. EAMG was induced in Lewis rats after three immunizations with AChR2α on days 0, 20, and 30. During the experiment, the rats were treated with either vehicle, 0.15 mg/kg FTY-720, 1.5 mg/kg FTY-720, or 3.5 mg/kg prednisone (positive drug) every day. The control group and the EAMG group were supplied with placebo (n = 6 per group). Body weight and anti-AChR Ab titer are presented in (A and B); CMAPs stimulated with 5 Hz or 10 Hz are shown in (C and D), respectively; and the percent of amplitude attenuation stimulated by 5 Hz or 10 Hz are shown in (E and F). Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. EAMG); EAMG = experimental autoimmune myasthenia gravis.
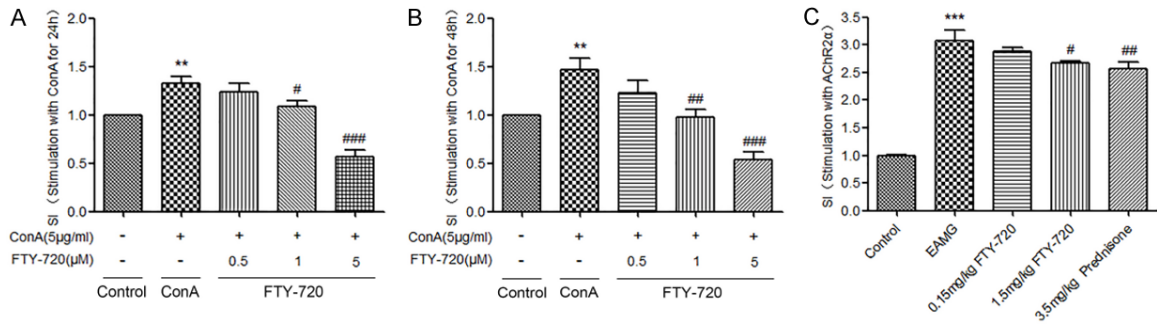
centage of S phase lymphocytes (5 µM, P < 0.001) (**Figure 4D**). Meanwhile, the percentage of G1 or G2 phase lymphocytes slightly decreased, and there was no statistical difference between the groups (**Figure 4C** and **4E**). These data suggest that FTY-720 could lead to S phase arrest followed by the inhibition of antigen-activated lymphocyte responses.

FTY-720 regulates the distribution of Th cells

In order to investigate the mechanisms of FTY-720 on EAMG, CD3$^+$CD8a$^-$INF-γ$^+$ Th1 cells,

CD3$^+$CD8a$^-$IL-4$^+$ Th2 cells, CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, and CD3$^+$CD8a$^-$IL-17A$^+$ Th17 cells from different groups were detected using flow cytometry. In the EAMG group, Th1 (P < 0.001), Th2 (P < 0.001), Treg (P < 0.05), and Th17 cells (P < 0.001) noticeably increased (**Figure 5E**, **5F**, **5H** and **5I**, respectively), which might be due to an immune system response to extrinsic antigens. In addition, we further analyzed the ratios of Th1/Th2 and Treg/Th17 in different groups and found a disorder related to Th cell distribution. The Th1/Th2 ratio markedly increased (P < 0.001) while the Treg/Th17 ratio decreased

A

B
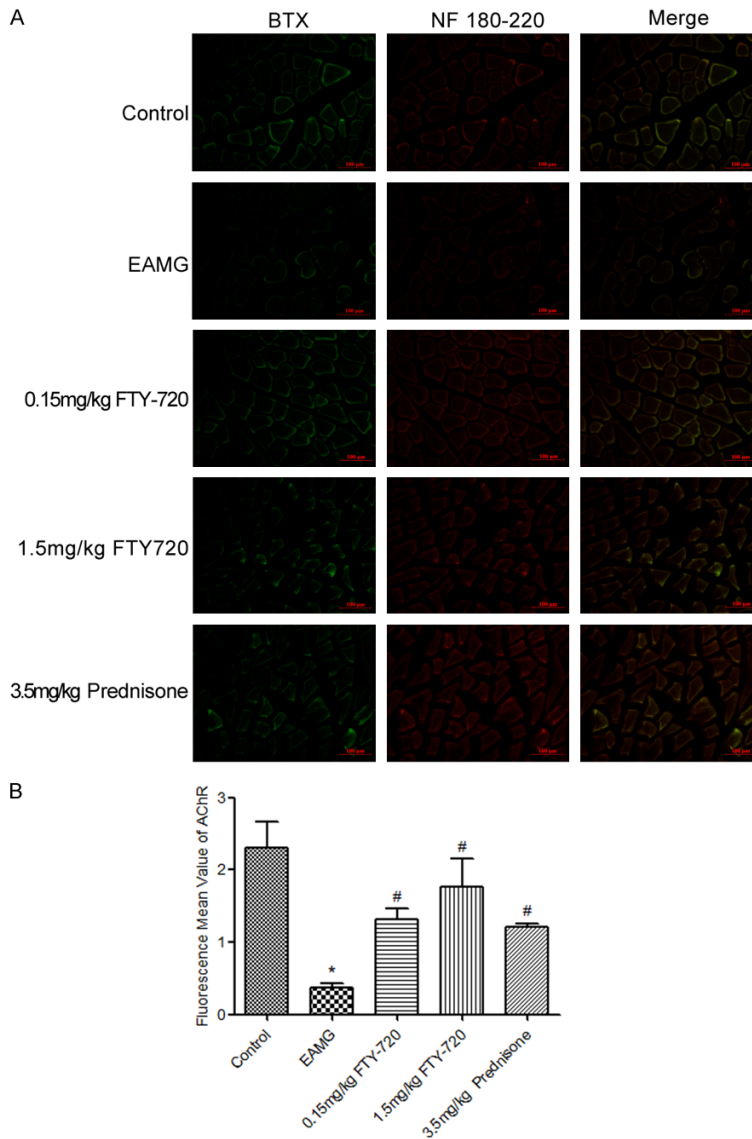
Figure 2. Gastrocnemius AChR levels in each group were detected. Frozen sections were blocked with 10% goat serum, incubated with BTX-FITC and anti-neurofilament heavy polypeptide overnight at 4°C, and then washed and incubated with goat anti-rabbit IgG-Alexa Fluor 594. Finally, the sections were observed and photographed with a fluorescence microscope, and quantitative analysis of AChR fluorescence intensity was performed using ImageJ software. The representative photographs and quantitative analyses are shown in (A and B), respectively. Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. EAMG).

clearly decreased in 1.5 mg/kg of FTY-720 treated rats (P < 0.05 and P < 0.01, respectively), but these cell levels in the 0.15 mg/kg FTY-720 group exhibited no significant difference when compared with levels in the EAMG group (**Figure 5F** and **5H**). Although FTY-720 could downregulate Th2 and Treg cells at high doses, the Th1/Th2 ratios in the 0.15 mg/kg and 1.5 mg/kg FTY-720 groups decreased significantly (P < 0.01 and P < 0.001, respectively), while the Treg/Th17 ratio in the 0.15 mg/kg FTY-720 group increased markedly (P = 0.053) compared with the EAMG groups (**Figure 5G** and **5J**). This indicates that FTY-720 shifted CD4$^+$ T cells from Th1 and Th17 cells to Th2 and Treg cells. These data suggest that FTY-720 pretreatment may improve EAMG by regulating Th1/Th2 and Treg/Th17 cell balance.

FTY-720 alters cytokine secretion in EAMG

To further investigate whether FTY-720 affects the secretion of cytokines, we first measured TGF-β1, IL-17A, and IL-6 levels in serum samples from different treatment groups. The level of IL-17A, a pro-inflammatory cytokine, increased in EAMG rats compared with the control group, although with no statistical significance, while the levels in the 1.5 mg/kg FTY-720 group markedly decreased compared with the EAMG group (**Figure 6B**) (P < 0.01). There was no significant difference in immunosuppressive cytokine TGF-β1 among all groups, although there was a slight decrease in the FTY-720 group (**Figure 6A**). Moreover, the IL-6 level significantly increased in the EAMG group (P < 0.001) and decreased in the 1.5 mg/kg FTY-720 group (P < 0.05) (**Figure 6C**). Second, we detected TGF-β1, IL-4,

in the EAMG groups compared with the control group (**Figure 5G** and **5J**), indicating that CD4$^+$ T cells differentiated towards Th1 and Th17 cells in EAMG rats. After drug administration, Th1 and Th17 cells markedly decreased in rats treated with FTY-720 in a dose-dependent manner (**Figure 5E** and **5I**) compared with the EAMG group. Similarly, Th2 cells and Treg cells

Figure 3. FTY-720 suppresses lymphocyte proliferation. Splenic lymphocytes from SD rats were pretreated with FTY-720 (0.5, 1, 5 μM) for 24 h, and then stimulated with ConA for 24 h or 48 h. Cell viability was determined by CCK-8 assay. In order to perform the antigen-specific lymphocyte proliferation reaction, lymphocytes from Lewis rats of different groups were stimulated with AChR2α for 72 h. Results are expressed as SI, where SI = $OD_{drug}/OD_{Control}$. The results of stimulation with ConA for 24 h and 48 h are shown in (A and B); the results of stimulation with AChR2α for 72 h are shown in (C). Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. ConA or EAMG).
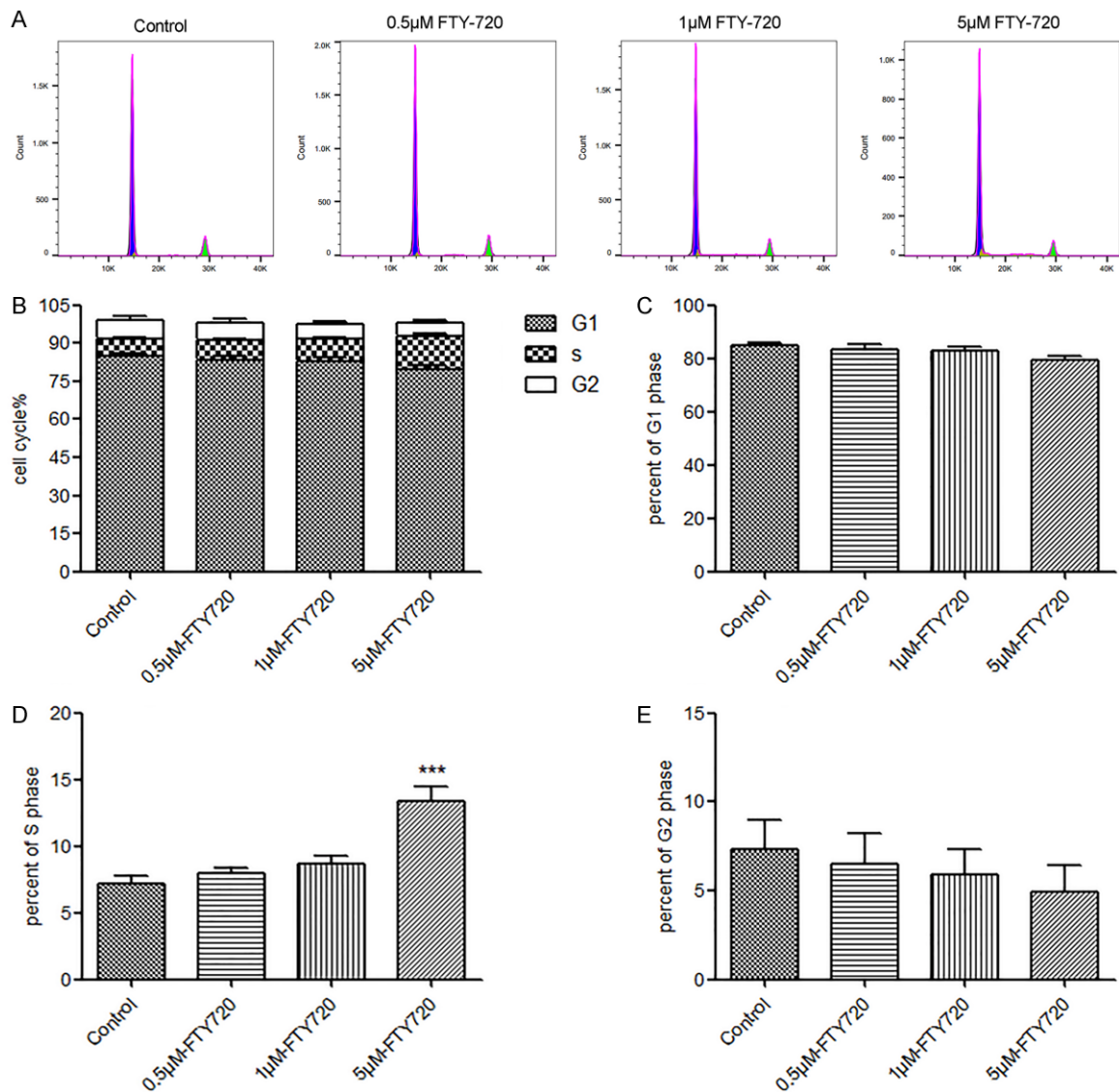
Figure 4. Effect of FTY-720 on cell cycle. Splenic lymphocytes were treated with FTY-720 (0.5, 1, 5 μM) for 24 h, stained with propidium iodide, detected using flow cytometry, and analyzed with FlowJo software. The representative fitting graphs of the cell cycle are shown in (A); the cell cycle statistics are shown in (B); and the percent of cells in the G1, S, or G2 phase in the cell cycle are shown in (C-E), respectively. Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control).

IL-6, INF-γ, and IL-17A levels in cell culture supernatants using Luminex assay. Compared to control cells, ConA significantly increased INF-γ level. The enhanced INF-γ, however, was suppressed in a concentration-dependent manner by FTY-720 (5 μM, P < 0.01) (**Figure 6D**). Similarly, the levels of IL-17A and IL-6 were noticeably upregulated in the presence of ConA (P < 0.01 and P < 0.05, respectively), but downregulated by FTY-720 in a concentration-dependent manner, although with no significant difference (**Figure 6G** and **6H**). In addition, the results in **Figure 6E** and **6F** suggested that FTY-720 has no marked effect on the immunosuppressive cytokines IL-4 and TGF-β1.

Discussion

MG is an autoimmune disease characterized by skeletal muscle weakness and fatigue. Currently, there are few effective treatments, which encouraged us to explore and discover new therapies. In the present study, we evaluated the efficacy and mechanism of immunosuppressant FTY-720, a novel selective functional antagonist of S1PR1, on EAMG rats. The rats pretreated with FTY-720 had lower anti-AChR Abs titers, higher AChRs, and less CMAP decrement. Since CD4+ helper T-cell subtypes play a crucial role in the pathogenesis of EAMG, we focused our research on the relationship between the therapeutic effects of FTY-720 and the alteration of CD4+ helper T-cell subtypes in rat EAMG. We speculated that FTY-720 might improve the symptoms of EAMG by suppressing lymphocyte proliferation, regulating Treg/Th17 and Th1/Th2 balance, and altering the secretion of cytokines.

As is well known, anti-AChR Abs participate in the pathogenesis of MG. These antibodies induce pathogenicity by activating the classical complement cascade, which leads to membrane lysis and serious damage to the postsynaptic apparatus, including AChR loss, which impedes communication in the neuromuscular junction [2, 10, 26]. Anti-AChR Abs produced by antigen-specific B-cell subsets require activated AChR-specific CD4+ T cells and B cells [27].

In our study, FTY-720 was found to inhibit ConA-induced lymphocyte proliferation in a dose-dependent manner and inhibit AChR2α antigen-specific lymphocyte proliferation. To further investigate the mechanisms of splenic lymphocyte proliferation suppression, we examined the effect of FTY-720 on the cell cycle. Our data suggested that FTY-720 induced the S phase of cell cycle arrest, which might subsequently affect other cellular functions, including proliferation. Specific lymphocytes primarily contain T cells and B cells, and the interaction of activated CD4+ T cells and B cells induce autoantibody production [27]. Taken together, our results indicated that the protective effect of FTY-720 on EAMG may be due to the proliferation inhibition of activated AChR2a-CD4+ T cells and B cells.

Furthermore, naïve CD4+ T cells differentiate into several subtypes when stimulated by dendritic cells or antigens, and both the Th subtypes and cytokines they secrete influence the pathogenesis of the autoimmune response. Th1 cells and their cytokine INF-γ facilitate antigen presentation, activate anti-AChR CD4+ T cells, and induce complement-fixing IgG subclasses [28, 29]. Meanwhile, Th17 cells and IL-17A promote GC formation [30-32] and increase in the IgG1 and IgG3 subclasses [33], whereas Th2 cells and IL-4 suppress the activation of antigen-presenting cells (APCs) and Th1 cells [34]. Treg cells could directly kill antigen-presenting and autoantibody-producing B cells in a cell contact-dependent manner [35], and their cytokine TGF-β could skew the production of antibodies toward non-inflammatory Ig [36].

Lili Mu et al. found that in EAMG rats, the frequencies of Th1 cells and Th17 cells significantly increased, and the frequencies of Th2 and Treg cells markedly decreased compared with control rats [37]. These findings suggested that naïve CD4+ T cells differentiate toward Th1 and Th17 cells in EAMG rats, and the disequilibrium of Th1/Th2 and Treg/Th17 cells promotes the development of EAMG. Similarly, our data revealed that naïve CD4+ T cells of EAMG rats differentiated towards Th1 and Th17 cells,
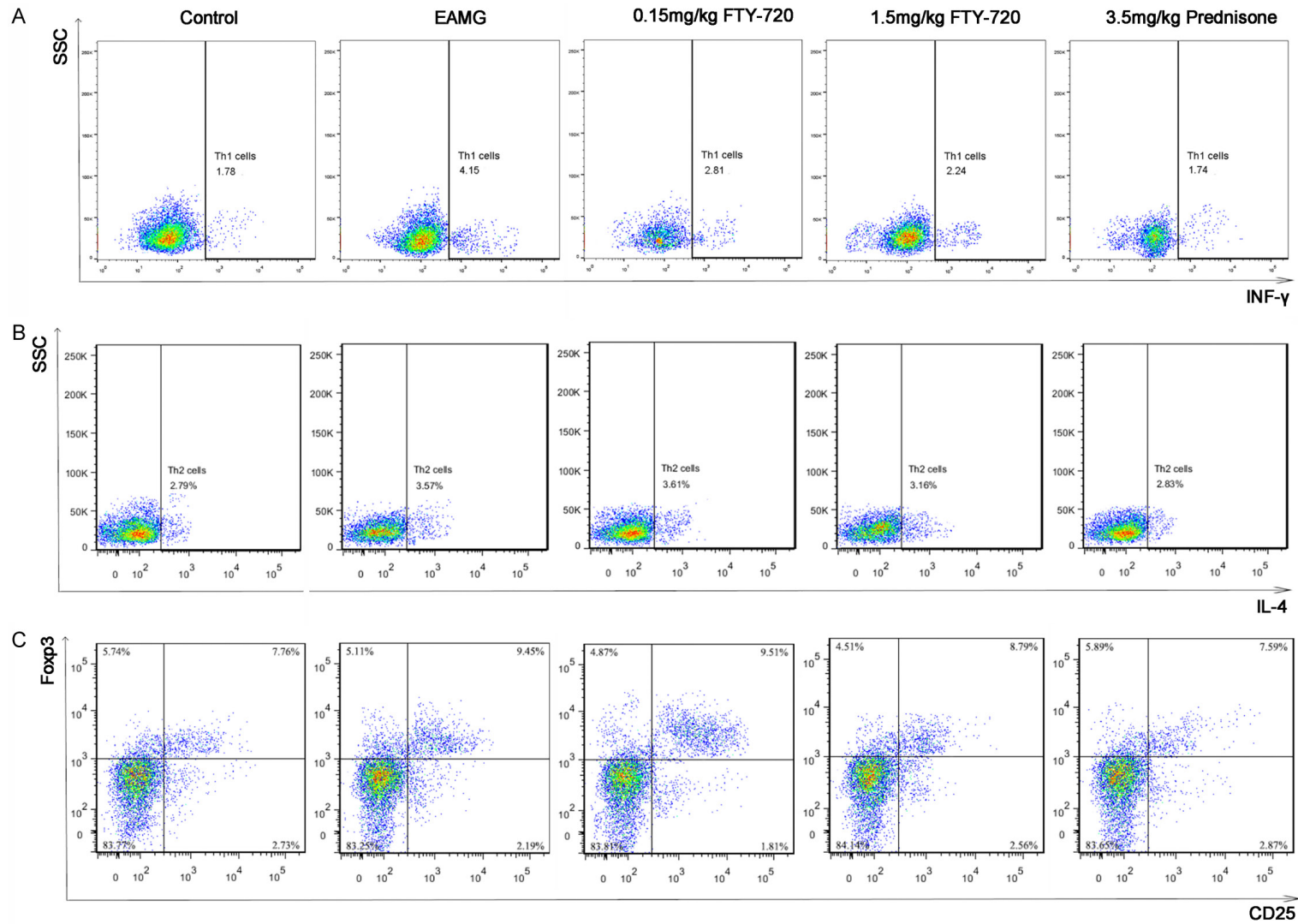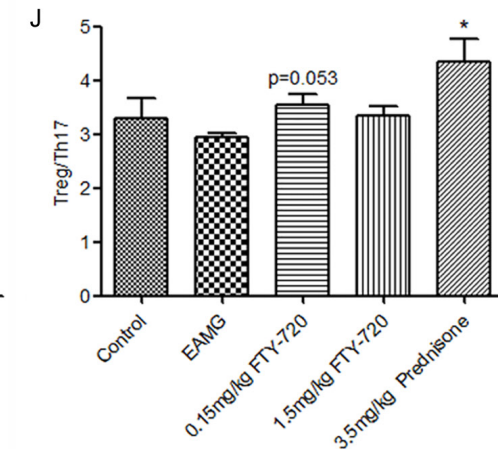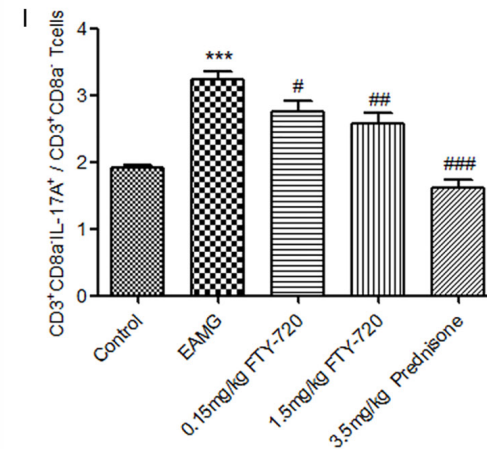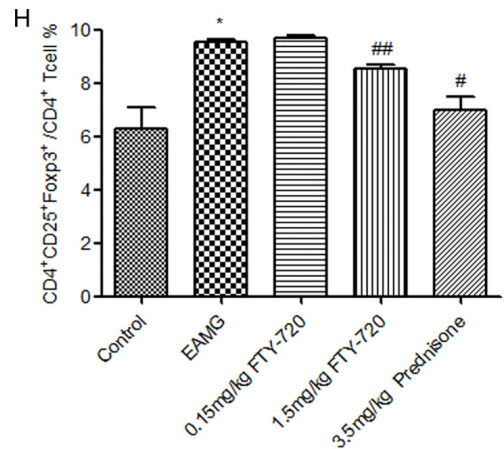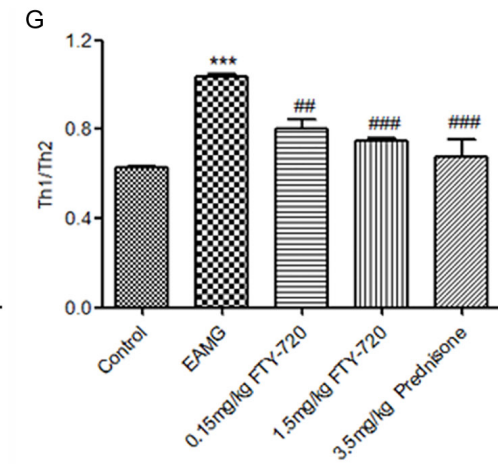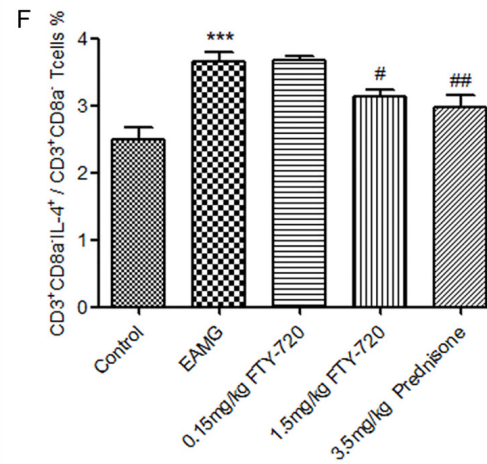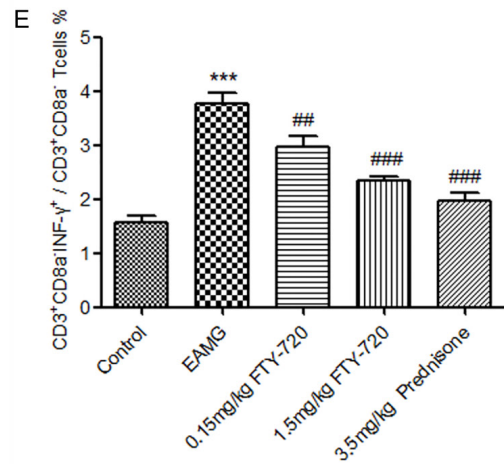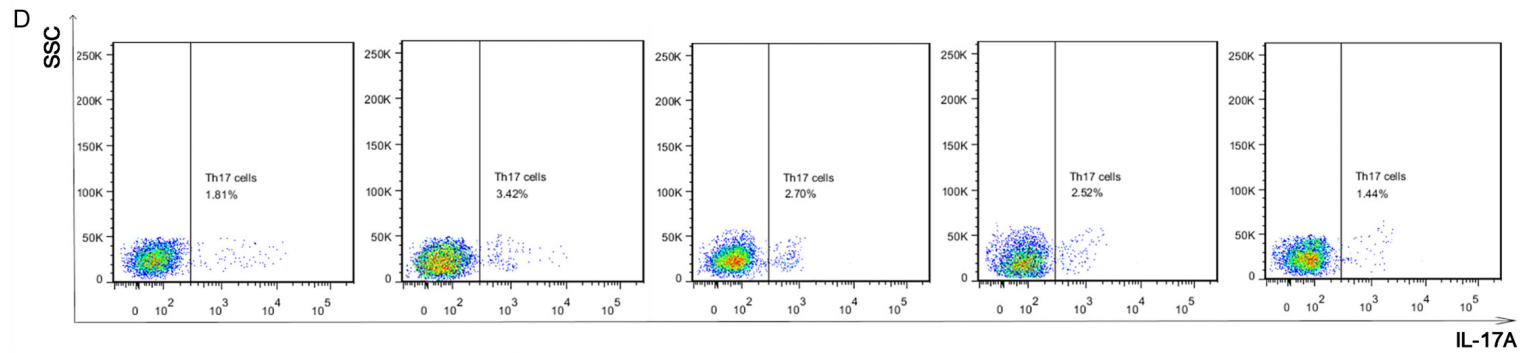
# Fingolimod ameliorates experimental autoimmune myasthenia gravis

Figure 5. Effect of FTY-720 on Th1/Th2 and Treg/Th17 cells. The representative scatterplots of the content of CD3$^+$CD8a$^-$INF-γ$^+$ Th1 cells, CD3$^+$CD8a$^-$IL-4$^+$ Th2 cells, CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, and CD3$^+$CD8a$^-$IL-17A$^+$ Th17 cells from the rats in different groups are shown in (A-D). The percent of Treg cells among CD4$^+$ T cells is shown in (H); Th1, Th2, and Th17 cells among splenic CD3$^+$CD8a$^-$ T cells are shown in (E, F, I), respectively. Graphs of the Th1/Th2 ratio and the Treg/Th17 ratio are shown in (G and J), respectively. Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. EAMG).
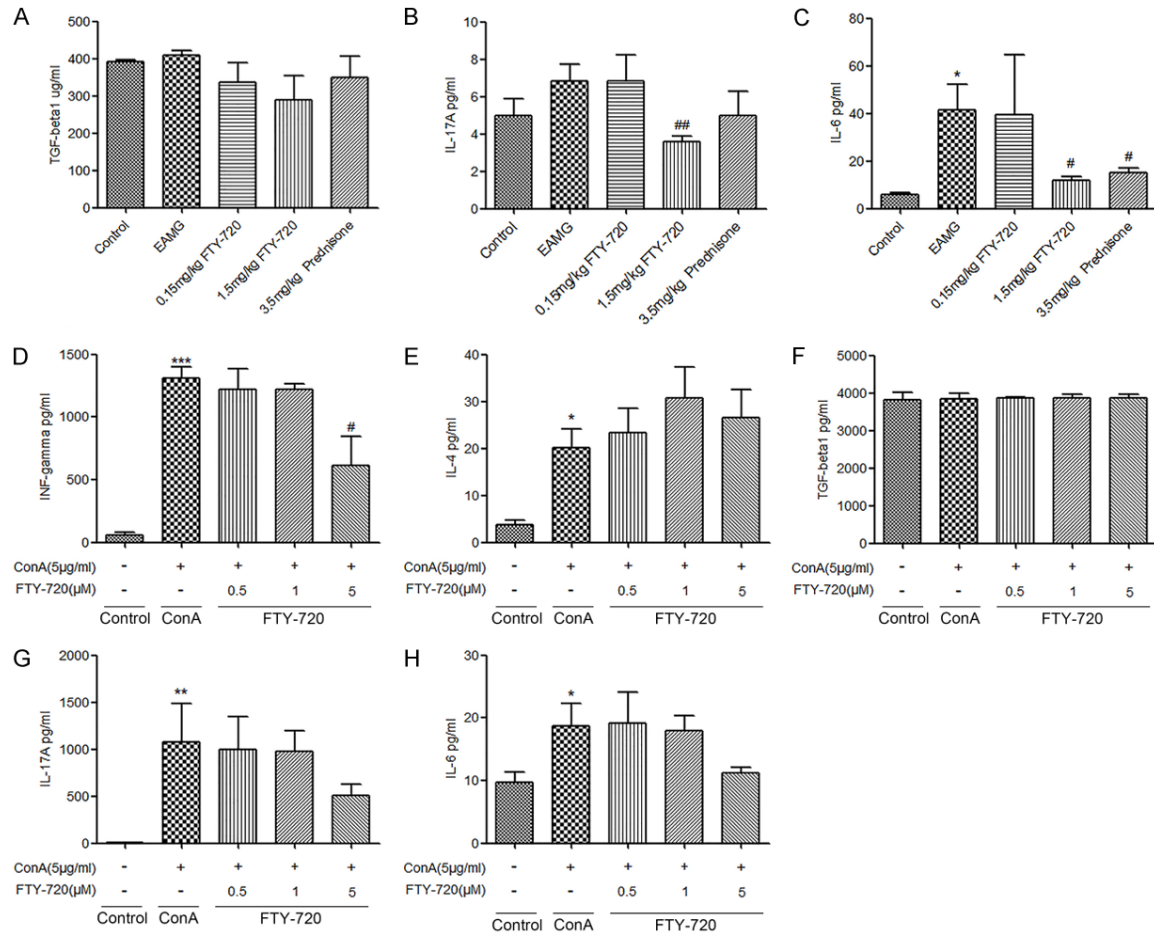
Figure 6. Effect of FTY-720 on cytokine secretion. Sera from different groups were harvested to detect TGF-β1, IL-17A, and IL-6. Splenic lymphocytes from SD rats were pretreated with FTY-720 (0.5, 1, 5 µM), and cell culture supernatants were harvested to detect INF-γ, IL-4, TGF-β1, IL-17A, and IL-6 after stimulation with ConA for 48 h. The serum levels of TGF-β1, IL-17A, and IL-6 are shown in (A-C), respectively; and the levels of INF-γ, IL-4, TGF-β1, IL-17A, and IL-6 from culture supernatants are shown in (D-H), respectively. Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. ConA or EAMG).

which promoted the pathological development of EAMG. FTY-720 treatment shifted CD4$^+$ T cells from Th1 and Th17 cells to Th2 and Treg cells. The new balance of Th cells indicates, in part, a modified T cell migration, which might be regulated by FTY-720 via the S1P-S1PR1 signaling pathway. However, the effect of FTY-720 on specific Th subsets requires further investigation.

Moreover, IL-6 and TGF-β regulate the differentiation of Treg cells and Th17 cells. TGF-β induc-

es naïve T cells into Treg cells, although the synergistic effect of TGF-β and IL-6 induces naïve T cells into Th17 cells, which means that IL-6 suppresses the differentiation of naïve T cells into Treg cells [38]. Our data revealed that FTY-720 suppressed the secretion of IL-17A, IL-6, and INF-γ, but had no obvious effect on the secretion of TGF-β1 and IL-4. Based on the obtained data and related literature, we speculated that FTY-720 reduced pro-inflammatory or inflammatory cytokines such as IFN-γ, thereby decreasing antigen-presenting activity.

Furthermore, FTY-720 decreased IL-6, thereby facilitating the differentiation of naïve CD4$^+$ T cells into immunosuppressive Treg cells. In addition, FTY-720 downregulated Th1/Th17 cells, thereby reducing the levels of complement-fixing IgG subclasses. Meanwhile, FTY-720 had no marked effect on Th2 and Treg cells, whose abilities could suppress the activation of APC and Th1 cells and kill antigen-presenting and autoantibody-producing B cells, respectively. Finally, by regulating Th cell balance and cytokine secretion, FTY-720 decreased the anti-AChR Abs titer, increased AChR content in the NMJ, and enhanced neuromuscular transmission (Figure S1).

In conclusion, this study demonstrated that FTY-720 exhibits a preventive effect on EAMG by inhibiting lymphocyte proliferation, regulating the balance of Th1/Th2 cells and Treg/Th17 cells, and altering the secretion of cytokines. Although more in-depth research is still needed, the present data may lay the foundation for the development of new drugs to treat MG.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Rui Wang and Hong-Lin Li, Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai, PR China. Tel: +86-021-64250823; Fax: +86-021-64250823; E-mail: ruiwang@ecust.edu.cn (RW); hlli@ecust.edu.cn (HLL)

References

[1] Meriggioli MN and Sanders DB. Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity. Lancet Neurol 2009; 8: 475-490.

[2] Chamberlain JL, Huda S, Whittam DH, Matiello M, Morgan BP and Jacob A. Role of complement and potential of complement inhibitors in myasthenia gravis and neuromyelitis optica spectrum disorders: a brief review. J Neurol 2019; [Epub ahead of print].

[3] Moiola L, Protti MP, Manfredi AA, Yuen MH, Howard JF Jr and Conti-Tronconi BM. T-helper epitopes on human nicotinic acetylcholine receptor in myasthenia gravis. Ann N Y Acad Sci 1993; 681: 198-218.

[4] Lefeuvre CM, Payet CA, Fayet OM, Maillard S, Truffault F, Bondet V, Duffy D, de Montpreville V, Ghigna MR, Fadel E, Mansuet-Lupo A, Alifano M, Validire P, Gossot D, Behin A, Eymard B, Berrih-Aknin S and Le Panse R. Risk factors associated with myasthenia gravis in thymoma patients: the potential role of thymic germinal centers. J Autoimmun 2020; 106: 102337.

[5] Truffault F, de Montpreville V, Eymard B, Sharshar T, Le Panse R and 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: 108-124.

[6] Villegas JA, Bayer AC, Ider K, Bismuth J, Truffault F, Roussin R, Santelmo N, Le Panse R, Berrih-Aknin S and Dragin N. Il-23/Th17 cell pathway: a promising target to alleviate thymic inflammation maintenance in myasthenia gravis. J Autoimmun 2019; 98: 59-73.

[7] Fattorossi A, Battaglia A, Buzzonetti A, Ciaraffa F, Scambia G and Evoli A. Circulating and thymic CD4 CD25 T regulatory cells in myasthenia gravis: effect of immunosuppressive treatment. Immunology 2005; 116: 134-141.

[8] Luther C, Poeschel S, Varga M, Melms A and Tolosa E. Decreased frequency of intrathymic regulatory T cells in patients with myasthenia-associated thymoma. J Neuroimmunol 2005; 164: 124-128.

[9] Gertel-Lapter S, Mizrachi K, Berrih-Aknin S, Fuchs S and Souroujon MC. Impairment of regulatory T cells in myasthenia gravis: studies in an experimental model. Autoimmun Rev 2013; 12: 894-903.

[10] Koneczny I and Herbst R. Myasthenia gravis: pathogenic effects of autoantibodies on neuromuscular architecture. Cells 2019; 8: 671.

[11] Gilhus NE and Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. Lancet Neurol 2015; 14: 1023-1036.

[12] Skeie GO, Apostolski S, Evoli A, Gilhus NE, Illa I, Harms L, Hilton-Jones D, Melms A, Verschuuren J and Horge HW; European Federation of Neurological Societies. Guidelines for

treatment of autoimmune neuromuscular transmission disorders. Eur J Neurol 2010; 17: 893-902.

[13] Dalakas MC. Immunotherapy in myasthenia gravis in the era of biologics. Nat Rev Neurol 2019; 15: 113-124.

[14] Huwiler A and Zangemeister-Wittke U. The sphingosine 1-phosphate receptor modulator fingolimod as a therapeutic agent: recent findings and new perspectives. Pharmacol Ther 2018; 185: 34-49.

[15] Chiba K, Yanagawa Y, Masubuchi Y, Kataoka H, Kawaguchi T, Ohtsuki M and Hoshino Y. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J Immunol 1998; 160: 5037-5044.

[16] Brinkmann V. Sphingosine 1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology. Pharmacol Ther 2007; 115: 84-105.

[17] Mizushima T, Ito T, Kishi D, Kai Y, Tamagawa H, Nezu R, Kiyono H and Matsuda H. Therapeutic effects of a new lymphocyte homing reagent FTY720 in interleukin-10 gene-deficient mice with colitis. Inflamm Bowel Dis 2004; 10: 182-192.

[18] Nofer JR, Bot M, Brodde M, Taylor PJ, Salm P, Brinkmann V, van Berkel T, Assmann G and Biessen EA. FTY720, a synthetic sphingosine 1 phosphate analogue, inhibits development of atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation 2007; 115: 501-508.

[19] Tsunemi S, Iwasaki T, Kitano S, Imado T, Miyazawa K and Sano H. Effects of the novel immunosuppressant FTY720 in a murine rheumatoid arthritis model. Clin Immunol 2010; 136: 197-204.

[20] Okazaki H, Hirata D, Kamimura T, Sato H, Iwamoto M, Yoshio T, Masuyama J, Fujimura A, Kobayashi E, Kano S and Minota S. Effects of FTY720 in MRL-lpr/lpr mice: therapeutic potential in systemic lupus erythematosus. J Rheumatol 2002; 29: 707-716.

[21] Huang J, Zhang T, Wang H and Zhao Y. Treatment of experimental autoimmune myasthenia gravis rats with FTY720 and its effect on Th1/Th2 cells. Mol Med Rep 2018; 17: 7409-7414.

[22] Kohno T, Tsuji T, Hirayama K, Iwatsuki R, Hirose M, Watabe K, Yoshikawa H, Kohno T, Matsumoto A, Fujita T and Hayashi M. A novel immunomodulator, FTY720, prevents development of experimental autoimmune myasthenia gravis in C57BL/6 mice. Biol Pharm Bull 2005; 28: 736-739.

[23] Meacci E and Garcia-Gil M. S1P/S1P receptor signaling in neuromuscolar disorders. Int J Mol Sci 2019; 20: 1-23.

[24] Huang GJ and Huang QW. Animal model in medical experiment: development and application. Beijing: Chemical Industry Press; 2008.

[25] Xie X, Mu L, Yao X, Li N, Sun B, Li Y, Zhan X, Wang X, Kang X, Wang J, Liu Y, Zhang Y, Wang G, Wang D, Liu X, Kong Q and Li H. ATRA alters humoral responses associated with amelioration of EAMG symptoms by balancing Tfh/Tfr helper cell profiles. Clin Immunol 2013; 148: 162-176.

[26] Conti-Fine BM, Milani M and Kaminski HJ. Myasthenia gravis: past, present, and future. J Clin Invest 2006; 116: 2843-2854.

[27] Wang ZY, Karachunski PI, Howard JF Jr and Conti-Fine BM. Myasthenia in SCID mice grafted with myasthenic patient lymphocytes: role of CD4+ and CD8+ cells. Neurology 1999; 52: 484-497.

[28] Hohlfeld R and Engel AG. Induction of HLA-DR expression on human myoblasts with interferon-gamma. Am J Pathol 1990; 136: 503-508.

[29] Conti-Fine BM, Milani M and Wang W. CD4+ T cells and cytokines in the pathogenesis of acquired myasthenia gravis. Ann N Y Acad Sci 2008; 1132: 193-209.

[30] Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, Le TV, Lorenz RG, Xu H, Kolls JK, Carter RH, Chaplin DD, Williams RW and Mountz JD. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. Nat Immunol 2008; 9: 166-175.

[31] Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K and Iwakura Y. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol 2006; 177: 566-573.

[32] Peters A, Pitcher LA, Sullivan JM, Mitsdoerffer M, Acton SE, Franz B, Wucherpfennig K, Turley S, Carroll MC, Sobel RA, Bettelli E and Kuchroo VK. Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. Immunity 2011; 35: 986-996.

[33] Patera AC, Pesnicak L, Bertin J and Cohen JI. Interleukin 17 modulates the immune response to vaccinia virus infection. Virology 2002; 299: 56-63.

[34] Abbas AK, Murphy KM and Sher A. Functional diversity of helper T lymphocytes. Nature 1996; 383: 787-793.

[35] Zhao DM, Thornton AM, DiPaolo RJ and Shevach EM. Activated CD4+CD25+ T cells selectively kill B lymphocytes. Blood 2006; 107: 3925-3932.

[36] Taylor A, Verhagen J, Blaser K, Akdis M and Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth fac-

tor-beta: the role of T regulatory cells. Immunology 2006; 117: 433-442.

[37] Mu L, Sun B, Kong Q, Wang J, Wang G, Zhang S, Wang D, Liu Y, Liu Y, An H and Li H. Disequilibrium of T helper type 1, 2 and 17 cells and regulatory T cells during the development of experimental autoimmune myasthenia gravis. Immunology 2009; 128: e826-836.

[38] Kimura A and Kishimoto T. IL-6: regulator of Treg/Th17 balance. Eur J Immunol 2010; 40: 1830-1835.
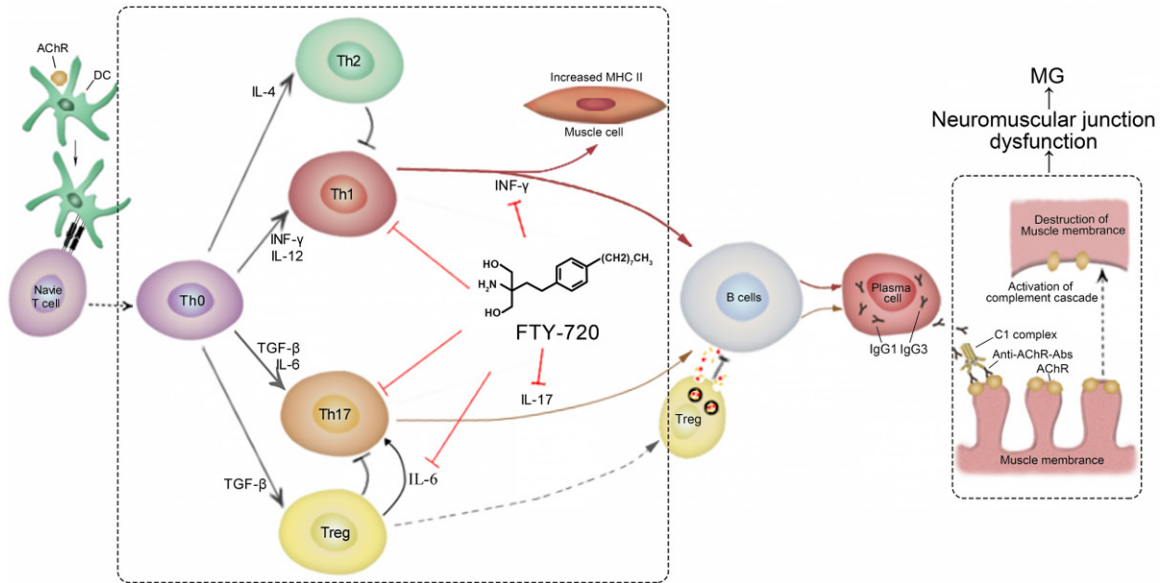
Figure S1. Role of Th cells and cytokines in the pathogenesis of MG [29]. Th1 cells and cytokines induce the production of complement-fixed IgG subclasses. Th1 cytokine IFN-γ facilitates the expression of MHC II molecules, which induce antigen presentation and activate anti-AChR CD4$^+$ T cells. Th17 cells and IL-17A also increase in the IgG1 and IgG3 subclasses, which activates the complement cascade. Th2 cells, however, suppress Th1 cells. In addition, Treg cells could directly kill antigen-presenting and autoantibody-producing B cells in a cell contact-dependent manner by secreting perforin and granzyme. Moreover, Treg cells suppress Th17 cells. In MG, anti-AChR Abs belong to the high-affinity IgG1 and IgG3 subclasses. The Fc region of Abs binds to C1q and triggers the complement cascade, which destroys the muscle membrane and decreases AChR, leading to impeded neuromuscular transmission.