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Inhibition of ROCK activity regulates the balance of Th1, Th17 and Treg cells in myasthenia gravis

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ABSTRACT

Aberrant ROCK activation has been found in patients with several autoimmune diseases, but the role of ROCK in myasthenia gravis (MG) has not yet been clearly investigated. Here, we demonstrated that ROCK activity was significantly higher in peripheral blood mononuclear cells (PBMCs) from MG patients. ROCK inhibitor Fasudil down-regulated the proportions of Th1 and Th17 cells in PBMCs of MG patients in vitro. Intraperitoneal injection of Fasudil ameliorated the severity of experimental autoimmune myasthenia gravis (EAMG) rats and restored the balance of Th1/Th2/Th17/Treg subsets. Furthermore, Fasudil inhibited the proliferation of antigen-specific Th1 and Th17 cells, and inhibited CD4 + T cells differentiated into Th1 and Th17 through decreasing phosphorylated Stat1 and Stat3, but promoted Treg cell differentiation through increasing phosphorylated Stat5. We conclude that dysregulated ROCK activity may be involved in the pathogenic immune response of MG and inhibition of ROCK activity might serve as a novel treatment strategy for MG.

1. Introduction

Myasthenia gravis (MG) is an antibody-mediated autoimmune disease of the neuromuscular junction and is clinically characterized by skeletal muscle weakness and fatigability [1]. Roughly 85% of MG patients have autoantibodies that bind to the nicotinic acetylcholine receptor (AChR) [2]. Antibodies against the muscle-specific receptor tyrosine kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4) are also defined as autoantibodies in non-AChR-MG patients as they are able to impair AChR clustering in neuromuscular junctions [3]. Although autoantibodies produced by B cells are directly responsible for the destruction of the muscle endplate, $CD4^+$ T helper (Th) cells are necessary in the pathogenesis of MG because antigen-experienced B cells rely on the action of Th cells to produce pathogenic antibodies with high affinity.

Among different subtypes of T helper cells, T helper type 1 (Th1) cells secrete IFN-γ, IL-2, TNF-α, TNF-β and promote cell-mediated immune response. Th2 cells mainly produce IL-4, IL-5, IL-6, IL-9, IL-10 and mediate the activation and maintenance of humoral immune response. Th17 cells produce IL-17, IL-21, IL-22 and have a pivotal role in the initiation and development of autoimmune diseases [4]. T regulatory cells (Treg) regulate Th and B-cell immune responses by suppressing the activation and proliferation of autoreactive cells and are thus essential for immune homeostasis. Imbalance of these four Th cell subsets is suggested to contribute to the pathogenesis of MG, and modulation of the differentiation of these Th cells would be an alternative to treatment of this autoimmune disorder [5].

Current treatment of MG utilizing corticosteroids and other immunosuppressants needs to be maintained for a long time and is associated with potential side effects arising from protracted immune suppression. Thence, there is an urgent need to explore new therapies for the treatment of MG.

Rho kinase (ROCK), originally identified as the key effector of the small GTPase RhoA, is one of the serine/threonine kinases. Two ROCK isoforms have been identified: ROCK1 and ROCK2 [6]. ROCK regulates a diverse range of key cellular functions including cytoskeletal reorganization, proliferation, and differentiation [7]. Although the RhoA-ROCK pathway has been extensively and widely investigated in the non-hematopoietic cells, a growing number of publications have shown that it is also critical for the recruitment and function of immune cells, including T and B cells [8,9]. ROCK2 has been shown to upregulate the secretion of IL-17 and IL-21 by CD4 + T cells through phosphorylation of IRF4 and Signal transducer and activator of transcription (Stat)3 in arthritis in mice and healthy human [10,11].

Furthermore, aberrant ROCK activation in peripheral blood mononuclear cells (PBMCs) has been found in a variety of autoimmune

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diseases, including systemic lupus erythematosus (SLE) [12,13], rheumatoid arthritis (RA) [14], and inflammatory bowel diseases (IBD) [15]. These findings suggest that elevated ROCK activity in PBMC may be a common feature of patients with autoimmune diseases, and targeting ROCK might be a promising therapeutic option. However, the involvement of ROCK in patients with MG has not been clearly investigated.

In this study, we detect the ROCK activity in PBMCs of MG patients, characterize the immune regulatory function of ROCK inhibitor on Th cells from MG patients and experimental autoimmune myasthenia gravis (EAMG) rats in vitro, and examine its therapeutic potential in the chronic stage of EAMG in vivo.

2. Materialas and methods

2.1. MG subjects

All MG patients were recruited at Huashan Hospital. Twenty-five patients with MG for ROCK activity test and twenty-two patients with MG for in vitro experiment were enrolled. Inclusion criteria for MG patients were as follows: age > 18 years and classification of MG Foundation of America (MGFA) was II~IV. Exclusion criteria were as follows: glucocorticoid or other immunosuppressant therapy within 6 months, active infection by history, known diagnosis of other autoimmune diseases or malignant tumor by history. Clinical data and blood samples were collected. MGFA quantitative MG scores (QMG) and manual muscle testing scores (MMT) were assessed at the time of blood sampling.

2.2. Healthy controls

Twenty-five sex- and age-matched (± 3 years) healthy controls were included in this study. Exclusion criteria were as follows: known diagnosis of any autoimmune disease, active infection or malignant tumors.

The institutional review board approved the study, and all blood donors provided written informed consent.

2.3. Animals

Female Lewis rats, body weighting 160-180 g (6–8 weeks old), were purchased from Vital River Laboratory Animal Co.Ltd. (Beijing, China) and kept at the local animal house under pathogen free conditions with standard rat chow and water ad libitum. The study was approved by the Ethics Committee of Fudan University Ethics Committee and was conducted in accordance with the guidelines of the International Council for Laboratory Animal Science.

2.4. Induction and clinical assessment of EAMG

EAMG was induced by 97–116 peptide of rat AChR α subunit (R97–116, DGDFAIVKFTKVLLDYTGHI, synthesized by Glbiochem, Shanghai, China) as described previously [16,17]. Rats in the experimental groups were anesthetized and immunized subcutaneously at the base of tail with 50 μg R97–116 peptide in complete Freund's adjuvant (CFA) (Sigma-Aldrich, USA) supplemented with 1 mg of *Mycobacterium tuberculosis* strain H37RA (Difco, Detroit, MI, USA) on day 0 and boosted on day 30 with the same peptide in incomplete Freund's adjuvant (IFA). The adjuvant control group (CFA group) was immunized with CFA and IFA emulsions without R97–116 peptide, respectively.

Rats in the Fasudil treatment group were given Fasudil daily (30 mg/kg, intraperitoneally, TianJin Hongri Pharmaceutical Co. Ltd., China) starting from day 29 after the first immunization. Animals in the three groups were weighted at the beginning of the experiment and every other day until sacrificed on day 62. Clinical muscle weakness was graded 0–4 based on the presence of tremor, hunched posture, muscle strength, and fatigability as described previously [18].

2.5. Isolation and culture of PBMCs

Blood samples were collected into tubes containing EDTA, and PBMCs were isolated by standard Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) gradient centrifugation. The cells were cultured in RPMI1640 (HyClone, Beijing, China) supplemented with 1% (*v*/v) penicillin-streptomycin (Gibco, NY, USA) and 10% (v/v) fetal bovine serum (Gibco, NY, USA) in the presence or absence of Fasudil (15 μg/ml) for 48 h in vitro with leukocyte activation cocktail (2 μl/ml, BD GolgiPlug, BD Pharmingen, San Jose, CA, USA) added during the final 5 h. Cells were harvested and analyzed by flow cytometry, and supernatants were collected to detect IFN-γ, IL-10, and IL-17 by ELISA.

2.6. ROCK activity assay

ROCK activity in extracts of PBMCs was measured using the 96-well ROCK Activity Assay Kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. PBMCs lysates were prepared using 1% NP-40 lysis buffer. 120 μg of whole cell extracts were added to a 96-well plate coated with myosin phosphatase target subunit 1 (MYPT1), the ROCK substrate. The kinase reaction was initiated and kept for 60 min at 30 °C. After the reaction was terminated, anti-phospho-MYPT1 (Thr^{696}) antibody was added into each well. The plate was incubated at room temperature for 1 h and then washed. The HRP-conjugated secondary antibody was added for another hour. Substrate solution was then added and incubated for 20 min. The enzyme reaction was stopped and quantification performed on a spectrophotometer using 450 nm as the primary wavelength.

2.7. Preparation of mononuclear cells (MNCs) from spleen and lymph nodes

MNCs from spleens and inguinal lymph nodes of immunized rats were prepared by grinding tissues through a cell strainer (Becton Dickenson, Franklin Lakes, NJ, USA) in lymphocyte separation fluid (Dakewe, Shanghai, China). After gradient centrifugation, MNCs were isolated and washed three times and resuspended in RPMI 1640 (HyClone, Beijing, China) supplemented with 1% (*v*/v) penicillin-streptomycin (Gibco, NY, USA) and 10% (v/v) fetal bovine serum (Gibco, NY, USA) for the following experiments.

2.8. Flow cytometry analysis

The PBMCs from patients with MG were labelled with cell surface marker specific antibodies (anti-CD4 FITC, anti-CD25 PE, all from BD Biosciences), after surface staining, cells were then fixed and permeabilized using Intracellular Fixation & Permeabilization Buffer or Foxp3/Transcription Factor Buffer (all from eBioscience, San Diego, CA, USA), and labelled with anti-IFN-γ-PE, anti-IL-4-PE, anti-IL-17-PE, and anti-Foxp3-APC (BD Biosciences; San Diego, CA, USA) to detect the intracellular or intranuclear markers.

MNCs isolated from spleen and lymph nodes of EAMG rats on day 15 and day 45 were cultured in vitro with or without the presence of Fasudil (15 μg/ml) for 48 h and cells were incubated with leukocyte activation cocktail during the last 5 h. Then, cells were stained with FITC-conjugated anti-rat CD4 and PE-conjugated anti-rat CD25, after fixation and permeabilization, cells were labelled with PE-conjugated anti-rat IFN-γ , PE-conjugated anti-rat IL-4, PE-conjugated anti-rat IL-17, and APC conjugated anti-rat FOXP3.

Samples were analyzed performed on Attune NxT Flow Cytometer (Thermo Fisher, USA) and data were analyzed using FlowJo software.

2.9. Isolation and culture of $CD4^+$ T cells

$CD4^+$T cells were purified using the $CD4^+$ isolation kit from StemCell (Vancouver, BC, Canada). Cells were cultured in the presence or absence of Fasudil (15 μg/ml) at a final concentration of 1×10^6 cells/ml in 96-well plate with plate-bound anti-rat CD3 (5 μg/ml), anti-rat CD28 (2 μg/ml) and recombinant rat IL-2 (50 U/ml). After 72 h, cells harvested were used for quantitative real time-PCR and western blot analysis.

2.10. CFSE labeling

Isolated MNCs from EAMG rats were suspended in PBS at 2×10^7/ml and incubated with Carboxyfluorescein succinimidyl ester (CFSE; Selleck Chemicals, USA) at a final concentration of 1 μM for 5 min at 37 °C. Cells were washed for three times and resuspended in the medium at the indicated cell concentration.

2.11. Antigen-specific Th1 and Th17 cell proliferation assays

After CFSE staining, triplicate aliquots (200 μl) of MNCs suspensions containing 2×10^5 cells were cultured in 96-well, round-bottom microtiter plates and stimulated with R97–116 peptides (10 μg/ml), R97–116 peptides (10 μg/ml) plus Fasudil (15 μg/ml) or PBS. Five days later, cells were stimulated with leukocyte activation cocktail (2 μl/ml) for 5 h and incubated with PerCP-eFlour710-conjugated anti-rat-CD4, PE-conjugated anti-rat IFN-r, and PE-conjugated anti-rat IL-17 specific antibodies respectively, and analyzed by flow cytometry. Cell proliferation was determined in the FITC channel by the successive diminution of fluorescence intensities due to CFSE distribution to daughter cells. Gating was performed in order to observe $CD4^+$IFN-γ^+ and $CD4^+$IL-17^+ cells.

2.12. Cytokine ELISA

Cell culture supernatants and serum were collected and cytokine levels of IFN-γ, IL-10, IL-17 were determined using respective ELISA kits (eBioscience, San Diego, CA, USA) for human or rat according to the manufacturer's instructions. The analyses were performed in duplicate and the results are expressed as the mean cytokine concentration (pg/ml) ± SD.

2.13. Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen; Carlsbad, CA, USA), and cDNA was synthesized using an RT-PCR kit (TaKaRa; Dalian, China), and the mRNA expression levels were estimated by real-time quantitative PCR in the Roche LightCycler 480 System using an SYBR Green PCR kit (TaKaRa; Dalian, China). GAPDH was used as the endogenous reference gene, and relative Quantification normalized to GAPDH was carried out using the 2 ΔΔct algorithms.

Primers were listed as indicated: GAPDH sense: 5′-GGCAAGTTCA ACGGCACAGT-3′, antisense: 5′-TGGTGAAGACGCCAGTAGACTC-3′; IFN-γ sense: 5′- CACGCCGCGTCTTGGT-3′, antisense: 5′- TCTAGGCTT TCAATGAGTGTGCC-3′; T-bet sense: 5′-GTGAATGACGGTGAGCC AGA-3′, antisense: 5′-GGCGAGGGAACACTCGTATC-3′; IL-4 sense: 5′-CAAGTCTGGGGTTCTCGGTG-3′, antisense: 5′-AGTGTTGTGAGCGT GGACTC-3′; GATA3 sense: 5′- GGCGGCGAGATGGTACTG-3′, antisense: 5′- TCTGCCCATTCATTTTATGGTAGA-3′; IL-17 sense: 5′-GTTCAGTGT GTCCAAACGCC-3′, antisense: 5′-AGGGTGAAGTGGAACGGTTG-3′; RORC sense: 5′-TACACGGCCTTGGTTCTCAT-3′, antisense: 5′-GCAGAT GCTCCACTCTCCTC-3′.

2.14. Detection of anti-R97–116 IgG

Anti-R97–116 IgG was measured as described [19]. 96-well flat-bottomed plates (Corning, Corning, NY, USA) were coated with AChR R97–116 (5 μg/ml in 100 μl) overnight at 4 °C, washed with PBS-T (PBS 0.05% Tween 20) the following day and blocked with 10% fetal calf serum at room temperature (RT) for 2 h. Serum (1:500) were incubated at RT for 2 h in a volume of 100 μl. After five washes, HRP-conjugated rabbit anti-rat IgG (1:2000) was added and incubated at 37 °C for 1 h at RT. Finally, TMB substrate solution was added and the reaction allowed to develop at RT in the dark. Plates were read at an OD450 nm and the results expressed as OD value ± SD.

2.15. Detection of membrane attack complex (MAC) deposits at the NMJ by immunofluorescence microscopy

Sections (10 μm) in transverse direction were obtained from diaphragm samples of rats in the control and experimental groups. Slides were allowed to air-dry and then were fixed in cold acetone. After washing with PBS, the sections were blocked with 4% BSA for 90 min at room temperature and incubated for overnight at 4 °C with mouse anti-rat C5b-9 antibody(1:2000, Santa Cruz, Heidelberg, Germany). Sections were then washed and incubated with Alexa Fluor488 conjugated-anti-mouse IgG (Abcam, San Francisco, USA) and Alexa Fluor555 conjugated α-BTX for 60 min. The sections were washed and viewed in an Olympus fluorescence microscope.

2.16. Western blot

Protein of $CD4^+$T cells from EAMG rats was extracted with RIPA lysis buffer. Total protein concentration was determined using the BCA kit (Thermo Fisher, USA). Protein samples were electrophoretically separated on 10% SDS-PAGE gels and an equal amount of total protein (30 μg) was loaded into each lane. Proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk for 1 h and then incubated overnight with mouse anti-p-Stat1 (Santa Cruz, Dallas, TX, USA), rabbit anti-p-Stat3 (Abcam, Cambridge, MA, USA), rabbit anti-p-Stat5 (Invitrogen, Carlsbad, CA, USA). After several washes, membranes were incubated with anti-mouse or anti-rabbit Ig antibodies linked to HRP. Membranes were developed with ECL according to the manufacturer's instructions.

2.17. Statistical analysis

Statistical analyses were performed using SPSS 22.0 and GraphPad Prism 7.

Data were expressed as mean ± SD. Statistical differences between 2 groups were determined using the Student's *t*-test or Mann–Whitney *U* test. Correlations were estimated with the Spearman correlation coefficient. All tests were two-tailed, and a *p*-value of < 0.05 was considered statistically significant.

3. Results

3.1. ROCK activity increased in PBMCs of patients with MG

First, we assessed whether ROCK activity changed in PBMCs of patients with generalized MG. We performed a cross-sectional analysis of ROCK activity in 25 MG patients and 25 age and sex-matched healthy controls (Table 1). We detected higher ROCK activity level in PBMCs from MG group than that from the control (Fig. 1A). There was no significant correlation between ROCK activity in PBMCs and clinical severity of MG patients measured by QMG or MMT scores.

3.2. Fasudil down-regulated Th1 and Th17 cells in PBMCs from MG patients in vitro

Twenty-two generalized MG patients were recruited for the study (Table 2). After cultured in the presence or absence of ROCK inhibitor,

Table 1
Demographic and clinical characteristics of MG patients and healthy controls for ROCK activity assay.

Parameter	Patients with MG	Healthy controls
	n = 25	n = 25
Age, y	44.20 ± 14.94	43.96 ± 14.69
Males, n (%)	12 (48)	11 (44)
Disease duration, m	8.68 ± 6.42	–
MGFA class, n %		
II a	7 (28)	–
II b	3 (12)	–
III a	6 (24)	–
III b	6 (24)	–
IV a	2 (8)	–
IV b	1 (4)	–
Thymic abnormalities		
Thymoma	7	–
Thymic hyperplasia	5	–
AChR Ab(+), n(%)	24	–
QMG score	13.04 ± 4.69	–
MMT score	11.04 ± 8.28	–

Fasudil (15 μg/ml) for 48 h in vitro, $CD4^+$ T cell subsets in PBMCs were measured by flow cytometry. The proportions of Th1($CD4^+IFN\text{-}\gamma^+$) and Th17($CD4^+IL\text{-}17^+$) cells decreased with Fasudil compared with the control cells (Fig. 1C, D). No significant differences in Th2($CD4^+IL\text{-}4^+$) and Treg($CD4^+CD25^+FOXP3^+$) subsets were observed.

To further clarify the Th cell profile after Fasudil treatment, cytokines secreted by Th cells including IFN-γ, IL-10, and IL-17 were examined in the culture medium by ELISA. IFN-γ($p < .001$)and IL-17 ($p < .01$) were significantly decreased in the Fasudil treatment group compared to the control group, while there is no significant difference in IL-10 ($p = .73$) between the two groups (Fig. 1B).

3.3. Administration of Fasudil ameliorated EAMG rats in vivo

To elucidate the therapeutic potential of ROCK inhibitor in vivo, we used a therapeutic approach of treatment in EAMG model [20]. The rat model of EAMG was induced by 97–116 peptide of the rat AChR α subunit emulsified in CFA. Fasudil (30 mg/kg) or PBS was administrated daily from day 29 to day 62. Lower clinical scores indicating less severe and less weight loss were demonstrated in the rats treated with Fasudil (Fig. 2A, B). Sera were collected from rats in the CFA, EAMG

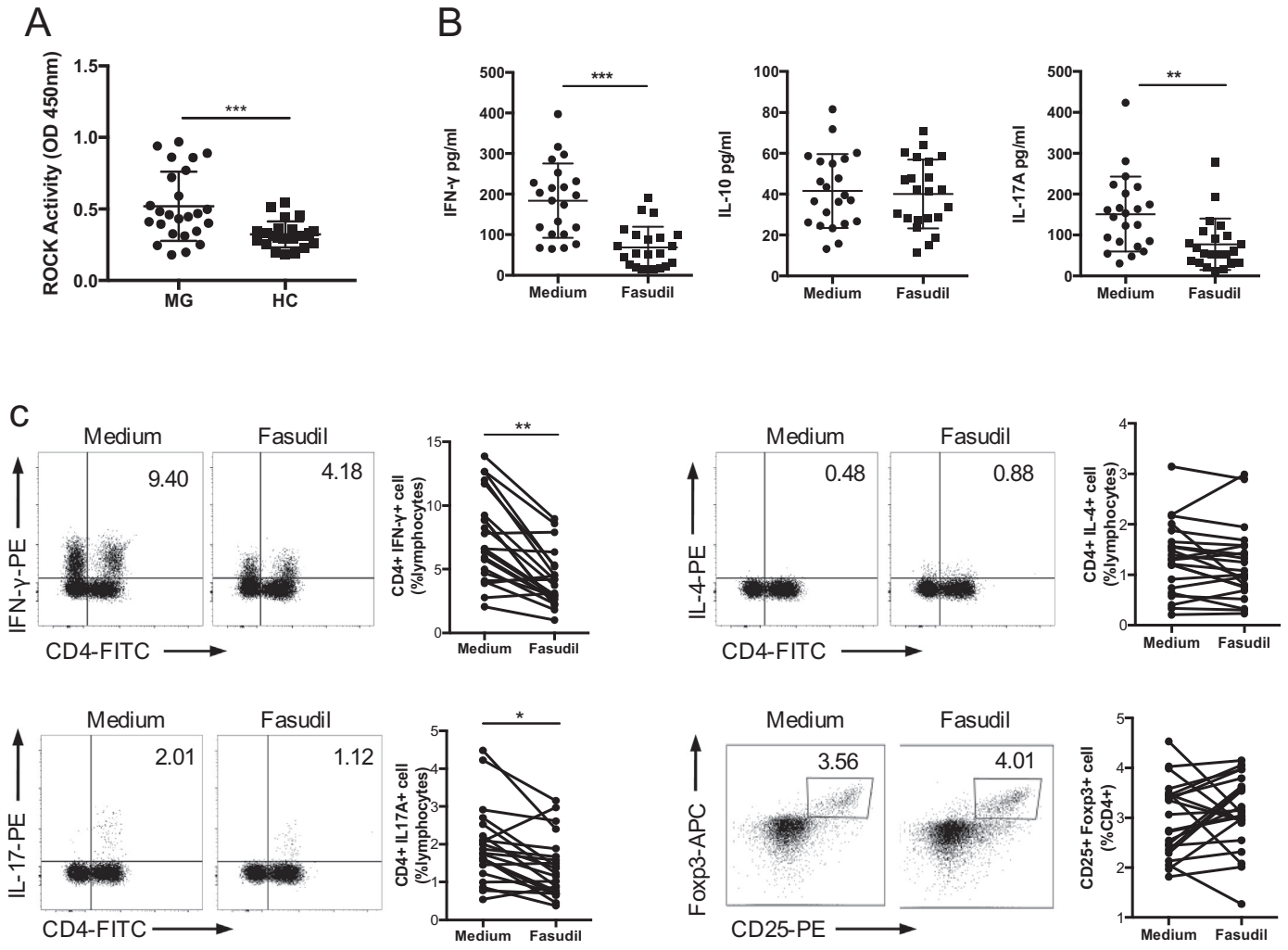

Fig. 1. ROCK activity in patients with MG and in vitro regulatory effect of Fasudil on T helper cell subsets in PBMCs of MG patients.
(A) Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of 25 patients with MG and sex, age-matched healthy controls. Whole cell extracts were prepared and ROCK activation was assessed by an ELISA-based ROCK activity assay (***$p < .01$). (B) PBMCs of MG patients were isolated and cultured in vitro with or without Fasudil for 48 h with leukocyte activation cocktail added during the final 5 h. Supernatants were collcected and analyzed for the level of IFN-γ, IL-10, and IL-17 by ELISA (**$p < .01$, ***$p < .001$). (C) PBMCs were harvested and $CD4^+IFN\text{-}\gamma^+$ cells, $CD4^+IL\text{-}4^+$ cells, $CD4^+IL\text{-}17^+$ cells and $CD4^+CD25^+$ $FOXP3^+$ cells were detected by flow cytometry ($n = 22$, *$p < .05$, **$p < .01$).

Table 2
Patient demographics and clinical characteristics for in vitro study.

Subject	Sex	Age(y)	Disease duration (m)	MGFA class	AChR Ab titer (nmol/L)	QMG score	MMT score	Thymectomy
MG-01	F	59	3	IIb	14.71	6	11	n
MG-02	F	39	2	IIIa	2.47	16	27	y
MG-03	F	28	36	IIa	11.09	5	4	y
MG-04	F	39	8	IIb	5.23	10	6	n
MG-05	M	55	1	IIa	14.76	7	5	n
MG-06	M	42	1	IIIa	9.01	16	23	n
MG-07	M	29	6	IIa	3.33	9	9	n
MG-08	M	34	3	IIa	14.90	8	3	n
MG-09	M	30	5	IIa	15.36	7	7	n
MG-10	F	21	12	IIa	3.84	12	4	n
MG-11	F	48	9	IIa	14.31	11	7	n
MG-12	M	62	1	IIa	9.74	10	11	n
MG-13	F	24	28	IIa	15.76	7	5	y
MG-14	F	49	2	IIa	7.09	13	6	n
MG-15	F	48	11	IIb	7.20	13	16	n
MG-16	M	45	24	IIa	6.39	8	8	n
MG-17	F	23	11	IIa	8.37	9	13	n
MG-18	F	26	12	IIa	9.40	6	8	y
MG-19	M	27	18	IIa	12.16	11	6	n
MG-20	F	57	2	IIa	1.34	12	9	n
MG-21	F	31	7	IVa	8.72	26	39	n
MG-22	M	33	9	IIIb	1.65	19	22	n

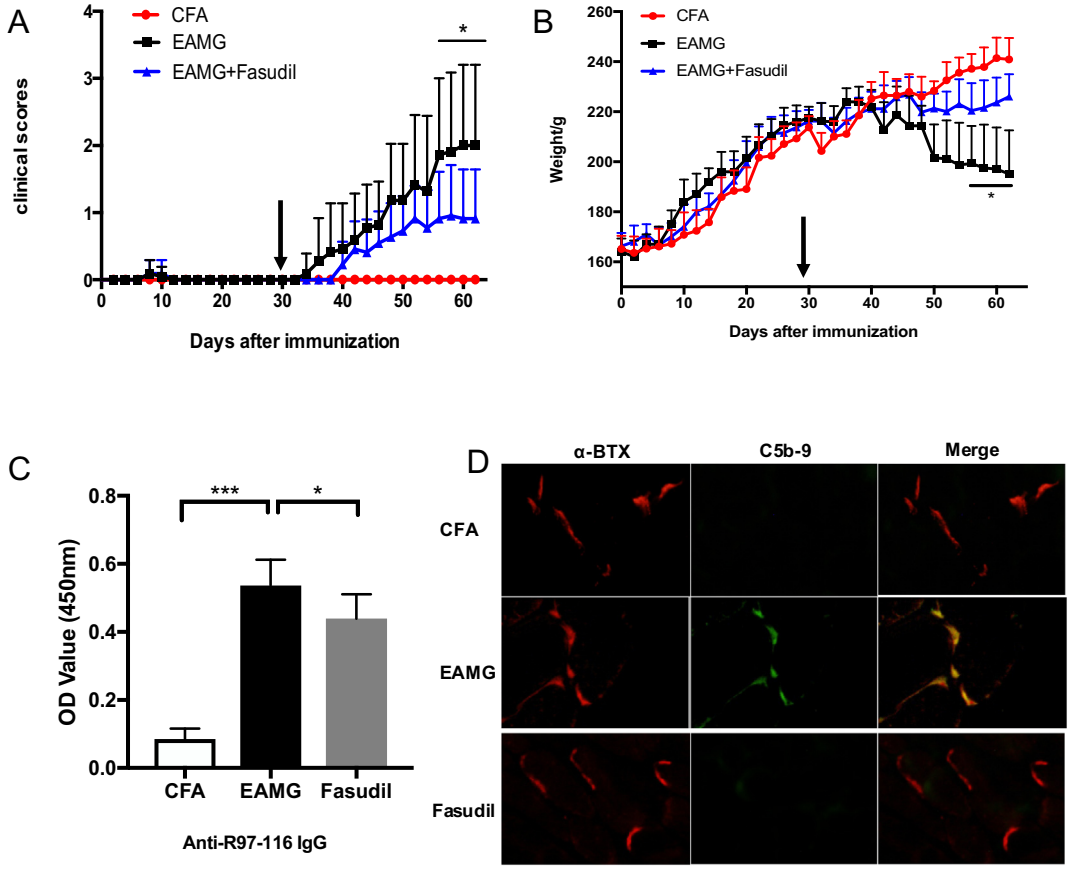

Fig. 2. Fasudil ameliorated experimental autoimmune myasthenia gravis (EAMG) symptoms.
(A,B) Fasudil (30 mg/kg) was intraperitoneally injected to EAMG rats daily from day 29 to day 62, $n = 8$ rats/group. Clinical scores and body weight were measured in rats from the complete Freund's adjuvant (CFA) group, EAMG group, and Fasudil treatment groups. Arrows indicate the day that Fasudil were started. Data are expressed as the mean + SD, $^{*}p < .05$, $^{***}p < .001$. (C) EAMG versus Fasudil treated groupAnti-R97–116 IgG titer in sera from rats in EAMG and Fasudil groups were determined by ELISA. (D) Sections from rats in the CFA, EAMG, Fasudil treatment groups were examined for the presence of AChR (red fluorescence) and C5b-9 diposition (green fluorescence) to assess changes of the neuromuscular junctions. Data are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

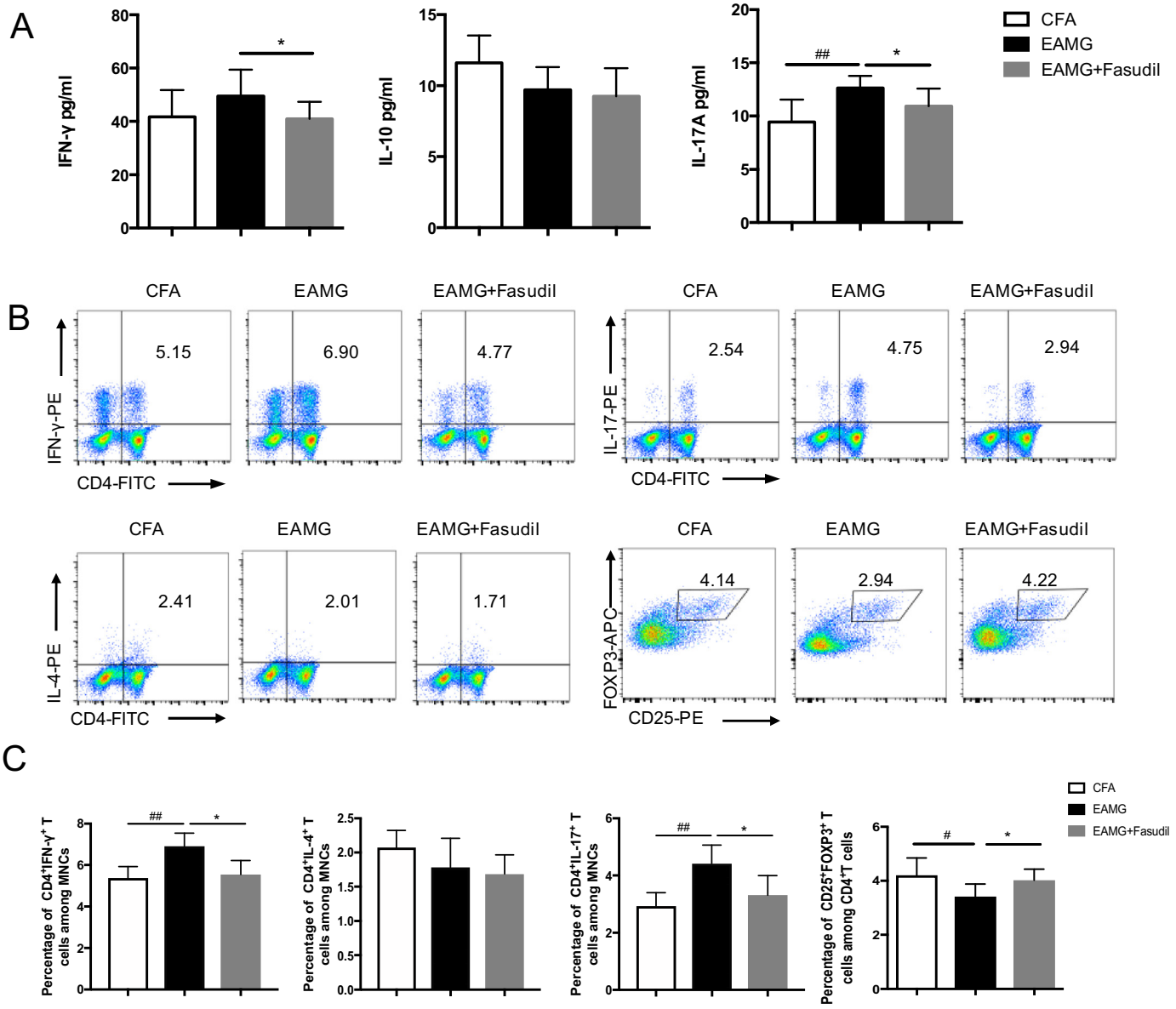

Fig. 3. Fasudil treatment altered the Th cell subsets distribution and cytokines production in vivo.
(A) Sera were obtained from rats of the three groups on day 62, and cytokines levels of IFN-γ , IL-10 and IL-17 were determined by ELISA. (B) Mononuclear cells (MNCs) of the spleens and lymph nodes were isolated from rats in three groups on day 62. (C) $CD4^+IFN\text{-}\gamma^+$ cells, $CD4^+IL\text{-}4^+$ cells, $CD4^+IL\text{-}17^+$ cells and $CD4^+CD25^+$ $FOXP3^+$ cells were detected by flow cytometry. The percentages of $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$ and $CD4^+CD25^+$ $FOXP3^+$ cells in MNCs were calculated, $n = 8$ rats/group, $^{\#}/^{*}p < .05$, $^{\#\#}p < .01$. Data are representative of three independent experiments.

and Fasudil groups on day 62 and the levels of anti-R97–116 IgG were determined by ELISA. The rats in Fasudil group had a lower level of anti-R97–116 IgG compared with that in EAMG group (Fig. 2C). A typical pathological change during EAMG development is the deposition of complement complex. To further assess this, we performed a histological examination of the diaphragm from rats in the three groups on day 62. Rats in the EAMG group showed more deposition of C5b-9 at NMJ compared with the CFA group, while deposition of C5b-9 significantly reduced in the Fasudil treatment group (Fig. 2D).

Besides, we found that ROCK inhibitor down-regulated the Th1 and Th17 cells but up-regulated Treg subsets in MNCs obtained from rats on day 62, and there was no significant effect on the percentage of Th2 cells. Levels of IFN-γ and IL-17 in serum were significantly lower in rats of Fasudil group compared with untreated EAMG rats (Fig. 3).

3.4. Fasudil down-regulated Th1, Th17 and up-regulated Treg proportions in MNCs from EAMG rats in vitro

We used MNCs of EAMG rats to explore the immune regulatory mechanism of Fasudil. The in vitro experiment was repeated with MNCs collected from EAMG rats sacrificed on day 15 (early phase) and day 45 (late phase) [20]. After Fasudil treatment for 48 h in vitro, the Th1 and Th17 subsets in MNCs decreased both on day15 and day45 (Figs. 4 and 5). However, Treg cells increased on day 45 after Fasudil administration (Fig. 5B). No difference in Th2 subsets was observed on day15 or day45. The levels of IFN-γ and IL-17 in cell supernatants reduced both on day15 and day45, while the level of IL-10 remained unchanged (Figs. 4A and 5A). The changes observed on day 45 were more pronounced and closer to the results of in vivo experiments.

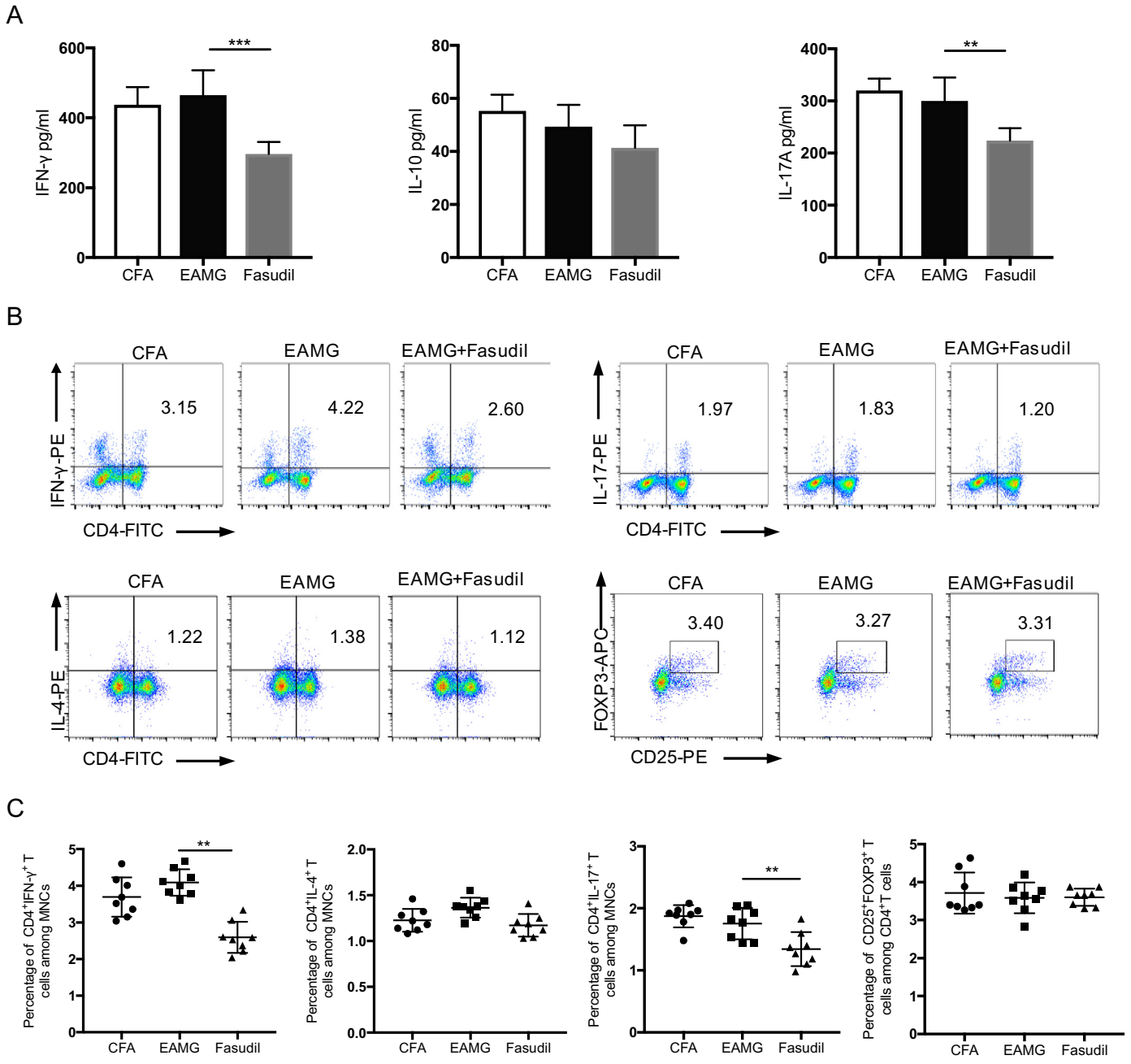

Fig. 4. Fasudil treatment altered the Th cell subsets distribution and cytokine production in vitro on day 15.
(A) MNCs were isolated from EAMG rats on day 15 and cultured in vitro for 48 h with or without Fasudil. Supernatants were collcected and analyzed for the level of IFN-γ, IL-10, and IL-17 by ELISA. (B) $CD4^+IFN\text{-}\gamma^+$ cells, $CD4^+IL\text{-}4^+$ cells, $CD4^+IL\text{-}17^+$ cells and $CD4^+CD25^+$ $FOXP3^+$ cells were detected by flow cytometry. (C) Graphic representation of the data shown in (B), data are expressed as mean ± SD of n = 8 rats/group representative of three independent experiments, $^{**}p < .01$, $^{***}p < .001$.

3.5. Fasudil suppressed antigen-induced proliferation of Th1 and Th17 cells

To determine if ROCK inhibitors could affect AChR-specific T helper cell proliferation, spleen and lymph node MNCs harvested from EAMG rats on day 45 were stimulated with R97–116 peptide and cultured in the presence or absence of Fasudil in vitro. Single stimulation with R97–116 promoted Th1 and Th17 cell proliferation in MNCs, while treatment with Fasudil suppressed antigen-specific Th1 and Th17 proliferation significantly (Fig. 6).

3.6. Fasudil affected $CD4^+$ T cells differentiation through regulating phosphorylation of Stat1, Stat3, and Stat5

To further explore the underlying mechanisms of ROCK inhibitor on Th cell subsets, we purified $CD4^+$ T cells from EAMG rats on day 45 and stimulated with anti-CD3, anti-CD28, and recombinant rat IL-2. Cells were cultured in the presence or absence of Fasudil for 72 h and then harvested for analysis of mRNA expression and protein levels. The mRNA expression of IFN-γ and IL-17 were markedly decreased after Fasudil treatment, but no significant difference was detected in IL-4 (Fig. 7A). Consistently, mRNA levels of T-bet and RORC were significantly reduced, whereas the level of GATA3 was found not to

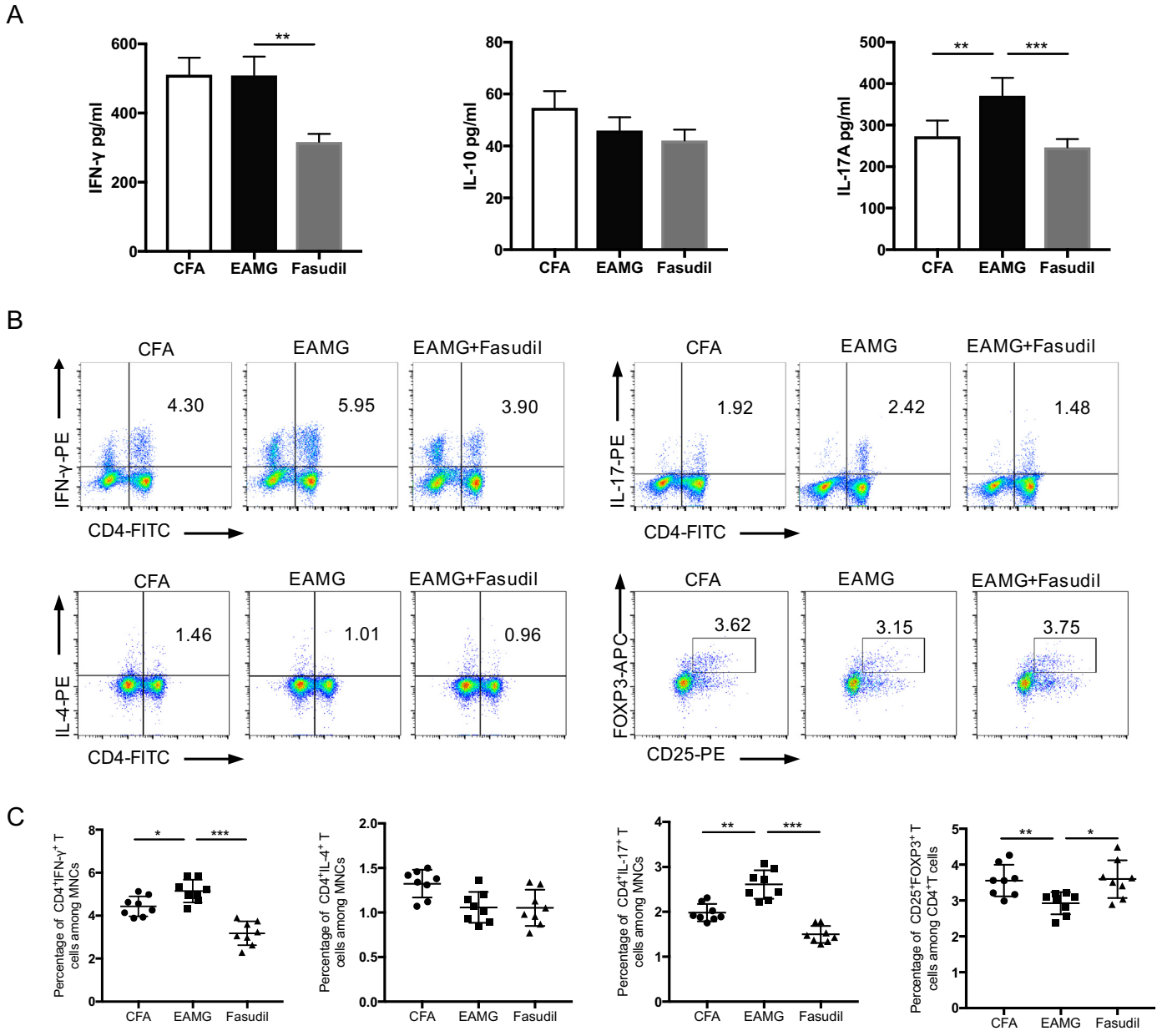

Fig. 5. Fasudil treatment altered the Th cell subsets distribution and cytokine production in vitro on day 45.
(A) MNCs were isolated from EAMG rats on day 45 and cultured in vitro for 48 h with or without Fasudil. Supernatants were collcected and analyzed for the level of IFN-γ, IL-10, and IL-17 by ELISA. (B) $CD4^+IFN\text{-}\gamma^+$ cells, $CD4^+IL\text{-}4^+$ cells, $CD4^+IL\text{-}17^+$ cells and $CD4^+CD25^+$ $FOXP3^+$ cells were detected by flow cytometry. (C) Graphic representation of the data shown in (B), data are expressed as mean ± SD of n = 8 rats/group representative of 3 independent experiments , $*p < .05$, $**p < .01$, $***p < .001$.

change significantly (Fig. 7A). Levels of phosphorylated Stat1, Stat3 and Stat5 were also measured. Fasudil treatment significantly down-regulated phosphorylated Stat1 and Stat3 but up-regulated phosphorylated Stat5 in $CD4^+$T cells of EAMG rats (Fig. 7B). Collectively, these results indicated that inhibition of ROCK activity suppressed the differentiation of EAMG $CD4^+$T cells into Th1 and Th17 cells but facilitated Treg cells differentiation.

4. Discussion

In this study, we have shown that ROCK activity was aberrantly upregulated in PBMCs from patients with generalized MG. ROCK inhibitor, Fasudil, could regulate Th cell subsets of MG and EAMG in vitro by affecting its proliferation and differentiation, and administration of Fasudil could suppress ongoing EAMG in vivo.

Accumulating evidence shows that ROCK plays a crucial role in the pathogenesis of autoimmune diseases [21]. Consistent with previous studies showing that ROCK activity is elevated in several autoimmune disorders such as SLE, RA, and IBD, our data first demonstrated that PBMCs of generalized MG patients exhibited enhanced ROCK activity compared to healthy controls, which suggested that hyperactive ROCK might contribute to the immune response in MG. However, we did not find a significant correlation between the levels of ROCK activity and disease severity measured by QMG or MMT scores. This result might be attributed to the small number of subjects or the fact that peripheral blood does not always represent ongoing inflammation in neuromuscular junction in MG; additional ROCK activity target, such as myosin light chain (MLC), cofilin and etc. might be a better representative of ROCK activity in MG. Further studies are required to verify the correlation between ROCK activity and disease severity in MG patients with

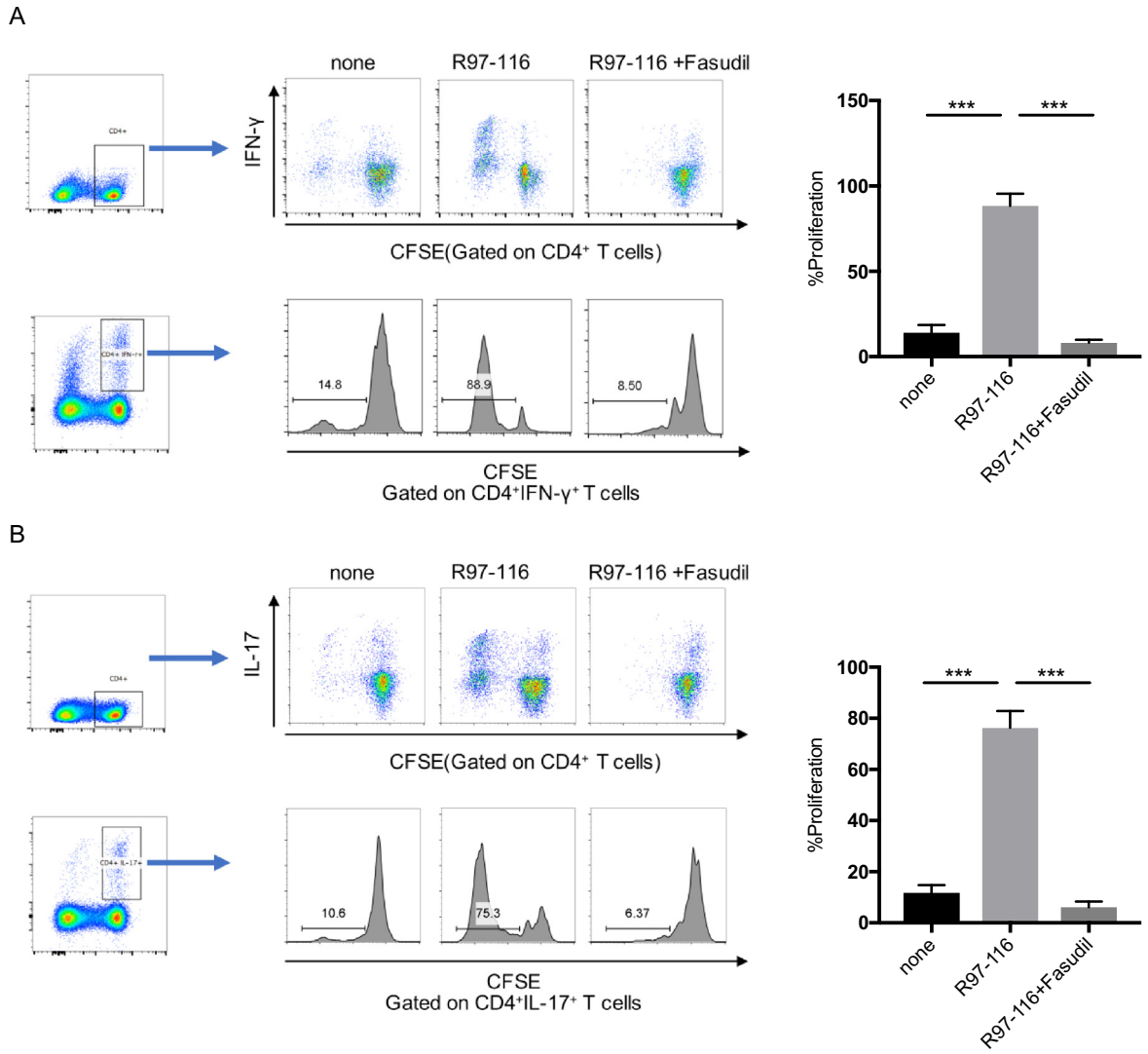

Fig. 6. Fasudil inhibited the proliferation of AChR-specific Th1 and Th17 cells in vitro. MNCs isolated from EAMG rats on day 45 after first immunization were stained with CFSE and stimulated with AChR R97–116 peptide in the presence or absence of Fasudil (15 μg/ml) for 5 days. Cells were harvested and analyzed by flow cytometry for staining with anti-CD4, anti-IFN-γ, anti-IL-17 antibodies and with CFSE. Gating was performed to analyze $CD4^+$, $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ cells. The bars in each panel represent the location of the dividing cells and the numbers indicate the percent of proliferating antigen-specific Th1(A) and Th17 (B) cells. The results of flow cytometry analyses are of one representative experiment, out of 5 individual experiments.

different clinical subtypes using larger samples.

The changes in ROCK activity in MG patients provided the rationale for our attempts to detect the immunomodulatory effect of ROCK inhibitor. Currently, a wide range of ROCK inhibitors has been employed in research. Fasudil is one of the non-isoform-selective ROCK inhibitors which has been used clinically with good tolerability. In vivo administration of Fasudil to SLE and RA mice attenuated disease severity associated with decreased levels of IL-17 and IL-21 and diminished autoantibody production [10,21]. In our study, Fasudil was used to test the immunomodulatory effect of ROCK inhibition in MG and EAMG, especially its regulatory effect on T helper cells.

Abundant data indicates that Th1, Th2, Th17, and Treg cells are involved in the development of MG and EAMG, via a complex web of interactions among the cells and their cytokines. Th1 cells secrete cytokines that could promote B cells to synthesize immunoglobulin isotypes and IgG subtypes capable of activating complement [22,23]. Mice deficient for the IFN-γ receptor or T-bet, a Th1 transcription factor, are less susceptible to EAMG [24–26]. Th17 cells also play a critical role in the pathogenesis of MG [27]. IL-17−/− mice exhibited significantly milder EAMG [28]. Studies in MG patients found significantly increased concentrations of IL-17 in serum and plasma, and elevated plasma IL-17A levels were associated with QMG scores [29]. Moreover, circulating autoreactive $CD4^+$ T cells of MG patients produce high levels of IFN-γ and IL-17 in response to AChR peptide stimulation, suggesting a mixed phenotype of Th1/Th17 cells [30–32]. Therefore, novel therapies suppressing auto-aggressive Th1, Th17 cells and reinstating tolerance in the immune system might be useful in the management of MG [33].

A recent study utilizing naive murine T cells has shown that ROCK2 is selectively activated under Th17 conditions, and T cells from heterozygous ROCK2-deficient mice exhibit impaired Th17 differentiation [10]. Furthermore, the use of silencing approaches and/or pharmacological inhibitors has also implicated the ROCK, and ROCK2 in particular, in the production of IL-17 and IL-21 by human T cells [11,13]. In our study, we found treatment with ROCK inhibitor Fasudil could down-regulated the proportions of Th1 and Th17 cells as well as the secretion of their related cytokines, IFN-γ and IL-17, in PBMCs of MG patients in vitro, and in MNCs of EAMG rats both in the early phase and late phase in vitro. Administration of Fasudil suppressed the proliferation of AChR-specific Th1 and Th17 cells stimulated by the R97–116

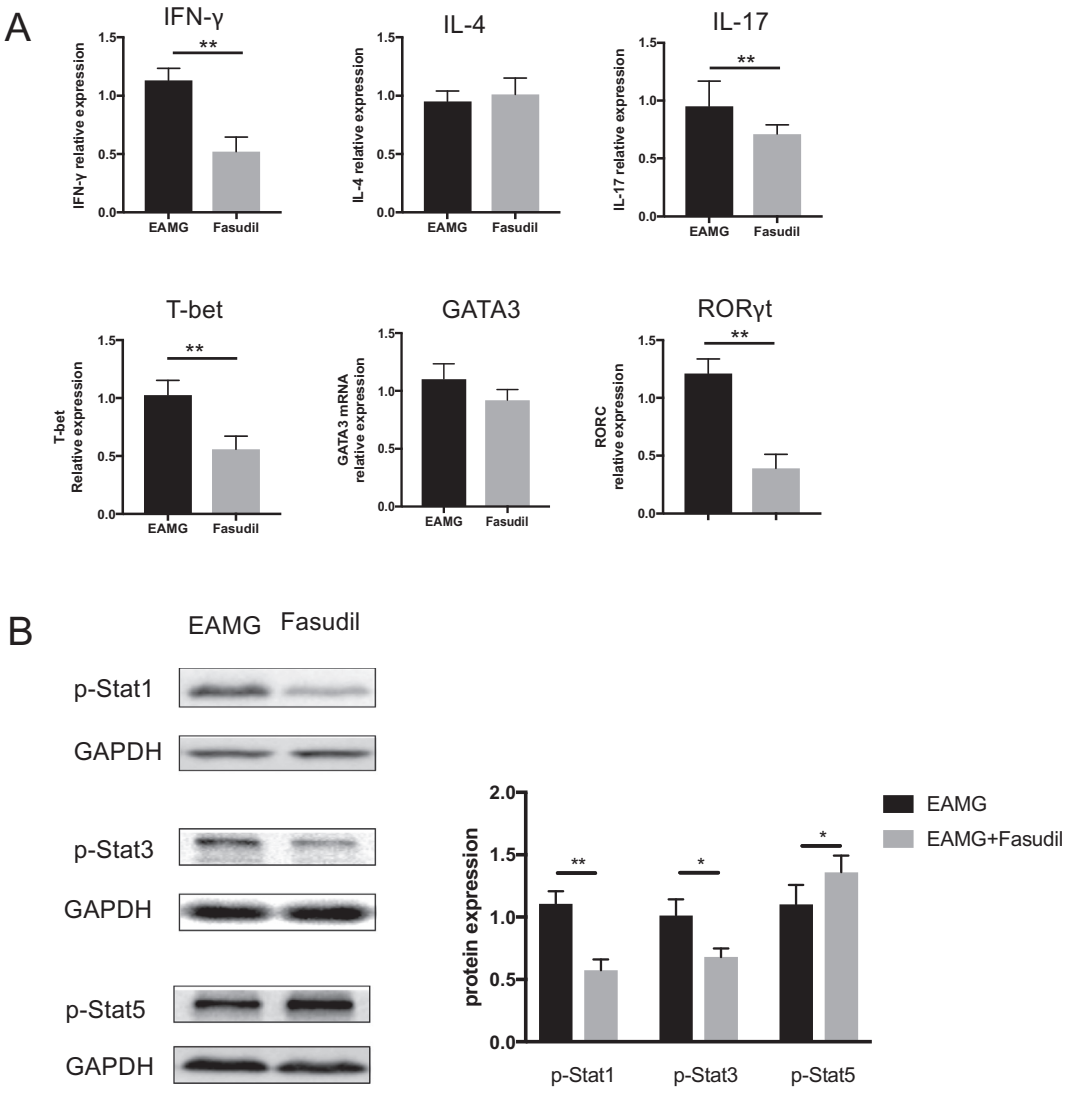

Fig. 7. Fasudil suppressed Th1 and Th17 differentiation but promoted Treg differentiation in vitro.
(A) $CD4^+$T cells isolated from EAMG rats were cultured in vitro and stimulated with anti-CD3, anti-CD28 and IL-2 in the presence or absence of Fasudil for 72 h. The mRNA expression of IFN-γ, T-bet, IL-4, GATA3, IL17, and RORC was analyzed by qRT-PCR, the results are expressed as mean ± SD, $^{**}p < .01$, n = 8 rats/group. (B) $CD4^+$T cells were collected and phosphorylation of Signal transducer and activator of transcription (Stat) 1, Stat3, Stat5 were analyzed using western blots, the results are expressed as mean ± SD $^{*}p < .05$, $^{**}p < .01$.
Each result is a representative of 3 independent experiments.

peptide. All these observations suggested that ROCK inhibition might be a way forward for treatment of MG.

Treg cells are important for maintaining self-tolerance and a decreased frequency or an impaired function of Treg cells are involved in the development of autoimmune diseases [34]. In MG patients, the thymic and peripheral blood Treg cells are defective in numbers and/or in functions, which are related to disease severity [35,36]. Approaches to correct the defects of these Treg cells may be a novel way in the treatment of MG. It has been reported that selective ROCK2 inhibitor could promote human Treg function [11]. Data in our report demonstrated that in vitro ROCK inhibition in MNCs purified from EAMG rats on day45 resulted in an increase in the percentage of $CD4^+CD25^+FOXP3^+$ cells. However, no significant change was found in the frequency of Treg cells on the day15, it was possible that defects in Treg cells became more evident with EAMG progression as demonstrated in a previous study [37].

Considering the potential of the ROCK as therapeutic targets for MG, we test the therapeutic effect of Fasudil in vivo. It was observed that Fasudil treatment administered at the chronic stage effectively suppressed EAMG induced by R97–116 peptide immunization in rats. Besides, the improvement of symptoms was accompanied by a decrease in autoantibody production and complement deposition in NMJ as well as the down-modulation of Th1 and Th17 cells, and expansion of regulatory T cells. Heng Li et al. reported that intraperitoneal injection of Fasudil from day 6 after the first immunization could attenuate the development of ongoing EAMG [38], but they found no difference in Th1/Th2/Th17 cytokines after Fasudil treatment. The differences in our study may due to the fact that we used a different approach to induce EAMG, and we started the administration of Fasudil at a later time. Collectively, in this study, we found that after treatment with Fasudil, the changes of Th subsets in PBMCs from MG patients were similar to those observed in EAMG rats.

Stat family proteins have essential roles in Th cell differentiation [39]. Stat1, Stat3, and Stat5 are the key transduction factors for the differentiation of Th1, Th17 and Treg cells, respectively [39]. Previous studies have shown that selective inhibition of ROCK2 suppresses phosphorylation of Stat3 but promotes Stat5 phosphorylation [11]. In our study, purified $CD4^+$T cells of EAMG rats were treated with

Fasudil, the phosphorylation levels of Stat1 and Stat3 increased, while the phosphorylation of Stat5 decreased. Consistently, the mRNA levels of IFN-r, T-bet, IL-17, and RORC reduced after treatment with Fasudil. These data indicated that inhibition of ROCK activity could suppress the differentiation of Th1 and Th17 cells but promote the differentiation of Treg cells in EAMG rats.

Our study contained some limitations. Only one kind of ROCK inhibitors, Fasudil, was used in our research, other ROCK inhibitors could be used to verify that the immunomodulatory effect is achieved by inhibiting ROCK activity. Further studies with larger cohorts of clinically heterogeneous MG subjects, including longitudinally acquired samples, will be necessary to assess the association of ROCK activity with clinical status and response to treatment.

In summary, our study suggested that dysregulated ROCK activity might be involved in the pathogenic immune response of MG. After treatment with ROCK inhibitor in vitro, changes in Th cell subsets and cytokines production in PBMCs of MG patients were similar to those were observed in MNCs of EAMG rats. Furthermore, administration of ROCK inhibitor inhibited the progression of EAMG in vivo. These data thus indicate that inhibition of ROCK activity may provide a novel way to treat MG, but more studies would be needed.

Authors' contributions

JS, JYX, WBY, CY, SSL, LZ performed the research, analyzed the data, and wrote the manuscript. WHZ, JHL, BGX, QD contributed to the conception and design of the study. CBZ participated in the design of the study, helped in funding the project, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study involving human subjects, according to the Declaration of Helsinki, was approved by the local Ethics Committee. All patients gave their written informed consent to the study. All of the experimental protocols with rats have been approved by the Ethics Committee of Fudan University.

Conflict of interests

The authors declare that they have no competing interests.

Acknowledgments

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