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Celastrol attenuates Guillain-Barré syndrome by inhibiting TLR4/NF-κB/STAT3 pathway-mediated Th1/Th17 cell differentiation

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Highlights

1. Celastrol dose-dependently restrained the pathological damage of GBS in EAN rats.
2. Celastrol suppressed Th1/Th17 cell differentiation in EAN rats.
3. Celastrol inhibited the TLR4/ NF-κB/STAT3 signaling pathway in EAN rats.

Abstract. Guillain-Barré syndrome (GBS) is an acute immune-mediated paralytic neuropathy with variable disease course and outcome. In this study, we aimed to investigate the therapeutic effects of celastrol on GBS and uncover its underlying mechanisms. Experimental autoimmune neuritis (EAN) is a typical animal model for GBS, and thus an EAN rat model was established with the injection of celastrol or/and LPS. We assessed the body weights and EAN clinical scores of rats. HE staining, flow cytometry, RT-qPCR, and Western blotting were respectively employed to measure pathological damage, proportions of cells (Th1, Th17, and Treg), Th1/Th17 cell differentiation-related mRNAs (IFN-γ, TBX21, IL-18, RORγT, IL-17, and IL-23) and TLR4/NF-κB/STAT3 pathway-related proteins (TLR4, NF-κB, p-NF-κB, STAT3, and p-STAT3). We found that celastrol attenuated clinical symptoms and pathological damage of GBS in EAN rats. Moreover, celastrol down-regulated Th1 and Th17 cell proportions, and the levels of IFN-γ, TBX21, IL-18, RORγT, IL-17, and IL-23 in EAN rats. Meanwhile, the levels of TLR4, p-NF-κB, and p-STAT3 were decreased by celastrol. Taken together, celastrol could restrain Th1/Th17 cell differentiation through inhibition of the TLR4/NF-κB/STAT3 pathway in EAN rats. Our findings suggest that celastrol may exert therapeutic effects on GBS by suppressing TLR4/NF-κB/STAT3 pathway-mediated Th1/Th17 cell differentiation.

Key words: Celastrol — Guillain-Barré syndrome — Th1 — Th17 — TLR4/NF-κB/STAT3 pathway

Introduction

Guillain-Barré syndrome (GBS) is an autoimmune disease associated with the peripheral nervous system, characterized by progressive limb weakness and a decrease in or loss of tendon reflexes (hyporeflexia and areflexia, respectively) (Rahimi 2020). Presently, intravenous immunoglobulin (IVIG), as well as plasma exchange (PE), is regarded as the standard treatment for GBS (Nguyen and Taylor 2022). However, effective supporting therapeutic strategies for GBS, such as traditional Chinese medicine (TCM) approaches, are still urgently required. Previous research has suggested that the integration of an empirical TCM strategy and the

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current treatment could have a better effect on GBS (Yang and Zhao 2020).

Celastrol, an active ingredient, was originally discovered and extracted from the root of *Tripterygium wilfordii* (a kind of Chinese medicine), with a wide range of pharmacological functions, such as anti-inflammation, antioxidation, and anti-tumor (Du et al. 2019). Previous studies have shown that celastrol can inhibit experimental autoimmune encephalomyelitis (Venkatesha and Moudgil 2019) and mitigate inflammatory diseases (Xu YY et al. 2020). Recently, a study has implied that celastrol has potentially favorable effects on alleviating neurodegeneration (Liu et al. 2022). These findings prompted us to hypothesize that celastrol might also have the potential to mitigate GBS. Therefore, we probed into the underlying benefits of celastrol to the treatment of GBS in this study.

CD4(+) T cells, known as crucial mediators of adaptive immune responses, have significant implications for the pathogenesis of various disorders including autoimmune diseases and chronic inflammation. Naive CD4(+) T cells can differentiate into a range of effector subsets (e.g. Th1, Th17, and Treg cells) that execute distinct immune functions. Therefore, the balance of Th1/Th17/Treg cells is vital for immunity homeostasis (Cai et al. 2021). Inhibition of the differentiation of Th1/Th17 cells may be a promising target for alleviating the severity of experimental autoimmune neuritis (EAN) and GBS, as evidenced by previous studies (Chen et al. 2018; Wang et al. 2019). Celastrol reportedly is capable of suppressing Th17 cell production and enhancing iTreg cell generation (Zhang et al. 2018). Otherwise, a study has demonstrated that celastrol enriched extract can block the Th17 pathway to mitigate psoriasis (Nguyen et al. 2020). Nevertheless, whether or how celastrol mediates the differentiation of Th1/Th17 cells in GBS has not been clarified.

Toll-like receptors (TLRs), known as a superfamily of single-pass transmembrane receptors, have important implications for the regulation of immune and inflammatory responses (Mokhtari et al. 2021). For instance, TLR4 silencing can mitigate inflammatory injury by decreasing the expression of pro-inflammatory cytokines (Wu et al. 2021). As a downstream effector of TLR4 signaling, nuclear factor-kappa B (NF-κB) acts as a key switch in inflammatory responses (Zhu et al. 2018). STAT3, a well-studied transcription factor, also has significant implications for immune and inflammatory responses (Lee et al. 2019; Wang et al. 2020). TLR4/NF-κB signaling pathway is implicated in the development of various autoimmune diseases, such as rheumatoid arthritis (Wang et al. 2018) and acute disseminated encephalomyelitis (ADEM) (Deng et al. 2020). Evidence has demonstrated that celastrol can inhibit the TLR4/NF-κB axis to relieve some disorders, such as neuropathic pain (Jin et al. 2022) and acute lung injury (He et al. 2021). Furthermore, a previous study has indicated the TLR4/NF-κB signaling pathway activation can contribute to onset of GBS (Li et al. 2021). Nonetheless, the regulatory role of the TLR4/NF-κB/STAT3 signaling pathway in GBS has not been illuminated.

On the basis of the background, we aimed to explore the therapeutic functions of celastrol in GBS by evaluating the differentiation of Th1/Th17 cells. Besides, the signaling cascade mediating Th1/Th17 cell differentiation was investigated. Herein, we may uncover the mechanisms underlying celastrol ameliorating GBS and provide a novel drug therapy for GBS.

Materials and Methods

Experimental animals

All the female Lewis rats (aged 6–8 weeks, weighing 150–180 g) used in the experiments were purchased from Kaixue Biotechnology company (Shanghai, China). Rats were housed in separated cages under a 12-h light/dark cycle at room temperature (25°C) and fed *ad libitum*.

Experimental design

Experiment 1. A total of 30 female Lewis rats were randomly divided into 5 groups (n = 6): Control, Model, Celastrol (1 mg/kg), Celastrol (2 mg/kg), and Celastrol (4 mg/kg). Rats in Model and celastrol-treated groups were administered with a subcutaneous (s.c.) injection of 200 μl emulsion, which contained 1 mg of bovine peripheral myelin (BPM) in normal saline. An equivalent amount of complete Freund's adjuvant (containing 0.3 mg of *Mycobacterium tuberculosis*, H37RA) was also administered to these rats *via* s.c. injection. Meanwhile, the control rats were s.c. injected with the same amount of normal saline. After 5 days, 1 mg/kg, 2 mg/kg, and 4 mg/kg of celastrol were respectively administered to rats daily *via* intraperitoneal (i.p.) injection. The doses of celastrol applied in this study were in accordance with previous research (Yang et al. 2006; Wagh et al. 2021).

Experiment 2. A total of 30 female Lewis rats were randomly classified into 5 groups (n = 6): Control, Model, Celastrol (4 mg/kg), Celastrol (4 mg/kg)+PBS (phosphate buffer saline), and Celastrol (4 mg/kg)+LPS (lipopolysaccharide, a TLR4 agonist). Rats in Model and medicine-treated groups were s.c. injected with emulsion (200 μl, containing 1 mg BPM in normal saline) and the equivalent amount of complete Freund's adjuvant (containing 0.3 mg of *Mycobacterium tuberculosis*, H37RA). Meanwhile, control rats were treated with the same amount of normal saline *via* s.c. injection. Five days later, rats in Celastrol (4 mg/kg) group were i.p. injected with 4 mg/kg of celastrol daily. After 24 h of treatment with 4 mg/kg celastrol, rats in Celastrol

(4 mg/kg)+PBS and Celastrol (4 mg/kg)+LPS groups were respectively administered with PBS (50 μg/kg) and LPS (50 μg/kg) *via* i.p. injection.

After 28 days of treatment, all the rats were euthanized by i.p. injection with an overdose of pentobarbital (100 mg/kg). Sciatic nerves and whole blood were then collected. The experiments were conducted in accordance with previous studies (Wang et al. 2019; Gao et al. 2020). All animal protocols were approved by the Animal Experimental Ethics Committee of the Zhejiang Hospital Affiliated to Zhejiang University.

Detection of body weights and EAN clinical scores of rats

The rats in each group were weighed and scored daily for 28 days. EAN clinical scores (Mausberg et al. 2018; Zhao et al. 2020; Szepanowski et al. 2021) were graded as follows : 0 = normal; 1 = tail tension decrease; 2 = partial caudal paralysis; 3 = complete caudal palsy or without righting reflex; 4 = gait ataxia; 5 = mild hindlimb paralysis; 6 = moderate hindlimb paralysis; 7 = severe hindlimb paralysis; 8 = quadriplegia; 9 = dying; 10 = death.

Hematoxylin-eosin (HE) staining

The sciatic nerve tissues of the rats in each group were fixed in 4% paraformaldehyde for 24 h. Then, the tissues were dehydrated in graded concentrations of ethanol (50, 70, 85, 95, and 100%), subsequently embedded in paraffin, and sliced at 5 μm. Next, the sections were dewaxed, rehydrated in xylene, and fractionated with ethanol. Thereafter, the sections were stained with hematoxylin for 5 min and eosin for 2 min. Finally, the samples were obtained using a fluorescence microscope (DM3000, Leica, Germany).

Flow cytometry analysis

Flow cytometry was employed to assess the proportions of Th1, Th17, and Treg cells in the whole blood of rats in each group. Firstly, the whole blood of rats was collected in sterile 5 ml EDTA tubes, and PBS (Beyotime, Shanghai, China) was added to dilute the blood by 1:1. Then, lymphocyte separation solution was collected and stored in a sterile centrifuge tube for subsequent assessment. Mononuclear cells were absorbed after the blood samples were centrifuged for 20 min, and then Th1, Th17, and Treg cells were collected. To detect the surface markers, the cells were stained with FITC anti-CD4 and APC anti-CD25 antibodies (eBioscience, USA) and incubated at 4°C for 30 min. For intracellular cytokine staining, the cells were stained with PE anti-IFN-γ, APC anti-IL-17A, and PE anti-Foxp3 antibodies (eBioscience, USA) at 4°C for 30 min. Finally, the cells were suspended, and the proportions of Th1, Th17, and Treg positive cells were measured using a flow cytometer (CytoFLEX S, Beckman Coulter, Germany).

RT-qPCR analysis

Total RNA was extracted from the sciatic nerve tissues of the rats in each group using TRIZOL reagent (15596018, Invitrogen, Carlsbad, US) according to the manufacturer's instructions. A reverse transcription reaction was performed using a FastKing OneStep Probe RT-qPCR MasterMix (KR118-02, TIANGEN, Beijing, China). RT-qPCR was conducted in a Real-Time PCR System (MX3000P, Stratagene, CA, USA) under the following conditions: denaturation at 95°C for 3 min, 40 circles at 95°C for 12 s, and 62°C for 40 s. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences involved in this experiment are listed in Table 1.

Table 1. The primers for RT-qPCR

Genes	Primer sequences (5' to 3')
IFN-γ	F: GGCAAAAGGACGGTAACACG
	R: GTGCTGGATCTGTGGGTTGT
TBX21	F: GTCGTCCTATTCTGGGAGCG
	R: TTGGAAGCCCCCTTGTTGTT
IL-18	F: TGGCTGCCATACCAGAAGAA
	R: ATAGGGTCACAGCCAGTCCT
RORγT	F: ACCAACCTCTTC TCACGGG
	R: CTTCCATTGCTCCTGCTTTC
IL-17	F: TGAAGGCAGCGGTACTCATC
	R: GGGTGAAGTGGAACGGTTGA
IL-23	F: GCTGTGCCTAGGAGTAGCAG
	R: TGGAACGGAGAAGAGAACGC
GAPDH	F: TGTGGGCATCAATGGATTTGG
	R: ACACCATGTATTCCGGGTCAAT

F, forward; R, reverse.

Western blotting

Total proteins were extracted from the sciatic nerve tissues of the rats in each group by lysing in RIPA buffer (P0013B, Beyotime, Shanghai, China), and quantified using a BCA kit (P0010S, Beyotime, Shanghai, China). Then, the proteins obtained were transferred onto several polyvinylidene difluoride (PVDF) membranes (FFP24, Beyotime, Shanghai, China). The membranes were blocked with 5% non-fat milk (P0216-300g, Beyotime, Shanghai, China) at room temperature for 1 h and then incubated with anti-TLR4 (1:100, 48-2300, Invitrogen, Shanghai, China), anti-NF-κB (1:1,000, 8242S, Cell Signal Technology, US), anti-p-NF-κB (1:1,000, 3033S, Cell Signal Technology, USA), anti-STAT3 (1:1,000,

ab68153, Abcam, UK), and anti-p-STAT3 (1:1,000, ab32143, Abcam, UK) primary antibodies at 4°C overnight. After washing, the membranes were incubated with the Goat anti-Rabbit IgG H&L (HRP) (1:2,000, ab205718, Abcam, UK) secondary antibody at room temperature for 1 h. An anti-GAPDH antibody (1:10,000, ab8245, Abcam, UK) served as an internal control. Protein bands were observed using NanoDrop™ 8000 (ND-8000-GL, Thermo Scientific, USA).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The one-way ANOVA test was used to compare the differences between groups. All statistical analyses were performed on GraphPad 7.0 software. $p < 0.05$ was considered statistically significant.

Results

Celastrol mitigated the severity of GBS in EAN rats

Effects of celastrol on ameliorating the clinical symptoms of EAN rats

To determine the effects of celastrol on reliving GBS, EAN rats were weighed and scored. The body weights of EAN rats decreased notably compared with the control rats ($p < 0.01$). Celastrol markedly increased the body weights of EAN model rats in a dose-dependent manner ($p < 0.01$). Moreover, the daily EAN clinical scores of control rats were normal (EAN clinical score = 0); however, the scores of the EAN rats were much higher than those of the control rats ($p < 0.01$). The clinical scores were significantly down-regulated by celastrol in a dose-dependent manner ($p < 0.05$) (Fig. 1A). Our data showed that 4 mg/kg of celastrol exerted optimal effects on mitigating the pathological symptoms in EAN rats and thus this dose was chosen for the subsequent experiments.

Effects of celastrol on amelioration of pathological damage of GBS in EAN rats

To explore the functions of celastrol in mitigating pathological damage of GBS, HE staining was applied and the fiber arrangement was observed. HE staining showed that the fiber arrangement was disordered in the EAN rats compared with that in control rats. Celastrol dose-dependently improved the fiber arrangement in EAN rats, indicating that celastrol alleviated pathological damage of GBS (Fig. 1B).

Celastrol suppressed Th1/Th17 cell differentiation in EAN rats

Effects of celastrol on reducing the proportions of Th1/Th17 positive cells in EAN rats

Th1/Th17 cell differentiation plays a crucial part in GBS. To determine whether celastrol has regulatory impacts on

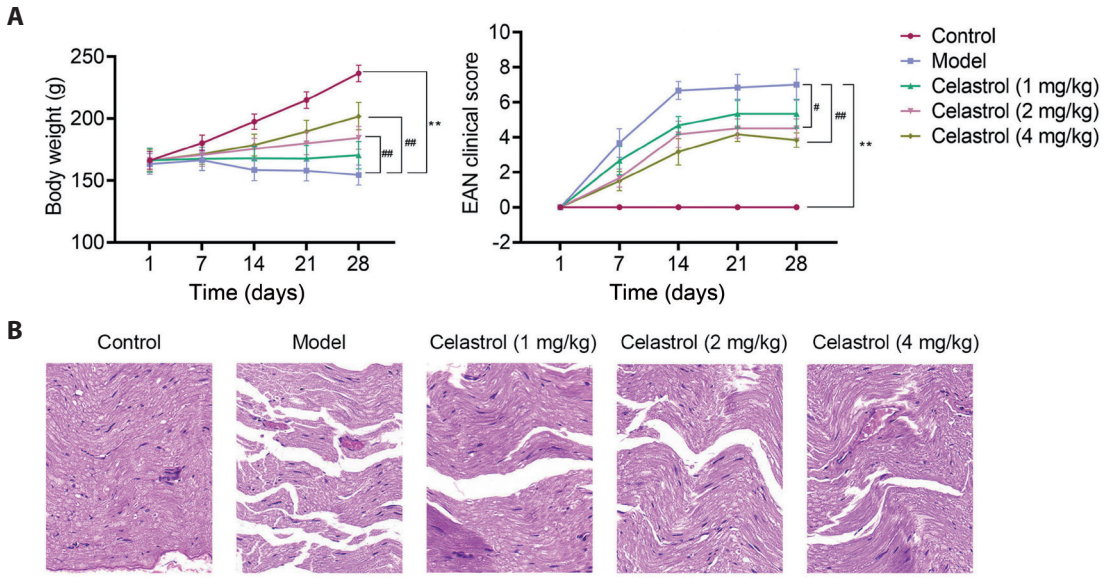


Figure 1. Effects of celastrol on remission of clinical symptoms and pathology of GBS in EAN rats. **A.** Determination of clinical symptoms by assessing the body weights and clinical scores of rats. **B.** Detection of sciatic nerve damage of EAN rats with HE staining (scale bar = 20 μm). ** $p < 0.01$ *vs.* Control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* Model group. GBS, Guillain-Barré syndrome; EAN, experimental autoimmune neuritis; HE, hematoxylin-eosin.

Th1/Th17 cell differentiation, the proportions of Th1, Th17, and Treg positive cells were measured. The proportions of Th1 and Th17 positive cells in EAN rats were higher than those in control rats ($p < 0.01$). However, 4 mg/kg of celastrol induced a notable decrease in the proportions of Th1 and Th17 positive cells in EAN rats ($p < 0.01$) (Fig. 2A, B). Moreover, Tregs proportion in the EAN rats decreased observably compared with control rats ($p < 0.01$), whereas celastrol exhibited no marked impact in this proportion (Fig. 2C).

Effects of celastrol on lowering the levels of Th1/Th17 cell differentiation-related factors

To further explore the regulatory effects of celastrol on Th1 and Th17 cell differentiation, the expression levels of IFN-γ, TBX21, IL-18, RORγT, IL-17, and IL-23 were measured. IFN-γ, TBX21, and IL-18 are associated with Th1 cell differentiation, while RORγT, IL-17, and IL-23 are correlated with Th17 cell differentiation. The levels of IFN-γ, TBX21, and IL-18 in the EAN rats were markedly higher than those in the control rats ($p < 0.01$). Celastrol notably reduced the levels of IFN-γ, TBX21, and IL-18 in the EAN rats in a dose-dependent manner ($p < 0.01$), except that 1 mg/kg of celastrol exhibited no marked effect on the down-regulation of TBX21. In addition, the levels of RORγT, IL-17, and IL-23 in the EAN rats were markedly elevated compared with those in control rats ($p < 0.01$). Importantly, we found that celastrol dose-dependently lowered the levels of RORγT, IL-17, and IL-23 (Fig. 2D).

Celastrol suppressed the TLR4/NF-κB/STAT3 signaling pathway in EAN rats

There is crosstalk between TLR4/NF-κB and STAT3 in the regulation of immune and inflammatory responses (Wu et al. 2019). To determine whether celastrol mitigates the pathological damage of GBS *via* targeting the TLR4/NF-κB/STAT3 signaling pathway, the expression of relevant proteins (TLR4, NF-κB, p-NF-κB, STAT3, and p-STAT3) was assessed. The levels of TLR4, p-NF-κB, and p-STAT3 in the EAN rats were significantly higher than those in the control rats ($p < 0.01$). Interestingly, our data showed that celastrol dose-dependently reduced the levels of TLR4, p-NF-κB, and p-STAT3 ($p < 0.01$) (Fig. 3).

Celastrol alleviated GBS in EAN rats by inhibiting the TLR4/NF-κB/ STAT3 pathway-mediated Th1/Th17 cell differentiation

Effects of celastrol on ameliorating the clinical symptoms of EAN rats

To further address whether celastrol has effects on reliving GBS *via* TLR4/NF-κB/STAT3 axis, the EAN rats were weighed and scored after LPS (a TLR4 agonist) treatment. Our results showed that LPS weakened the effects of celastrol on amelioration of GBS symptoms, as the body weights of celastrol-treated rats were down-regulated and the clinical scores were up-regulated when administered with LPS (Fig. 4A).

Effects of celastrol on alleviating pathological damage in EAN rats

To further investigate whether celastrol can alleviate the pathological damage of GBS *via* the TLR4/NF-κB/STAT3 signaling pathway, the fiber arrangement was observed after LPS administration. HE staining showed the fiber arrangement was disorder in the EAN rats compared with control rats. The fiber arrangement was more orderly than those in the EAN rats. Furthermore, the results demonstrated that LPS attenuated the effects of celastrol on easing the pathological damage of GBS (Fig. 4B).

Effects of celastrol on regulating Th1/Th17 cell differentiation in EAN rats

To further validate whether celastrol exerts inhibitory effects on Th1 and Th17 cell differentiation *via* the TLR4/NF-κB/STAT3 signaling pathway, the proportions of Th1, Th17, and Treg positive cells in the whole blood of EAN rats were detected after LPS treatment. We found that the proportions of Th1 and Th17 positive cells in the EAN rats increased significantly compared with those in control rats ($p < 0.01$). However, these proportions dropped notably in medicine-treated rats. Nevertheless, LPS abolished the effects of celastrol on inhibiting Th1 and Th17 cell differentiation ($p < 0.01$) (Fig. 4C, D). Similarly, the Treg cell proportion declined significantly in the EAN rats ($p < 0.01$), but there was no significant change in this proportion in medicine-treated rats (Fig. 4E).

Effects of celastrol on inhibiting the TLR4/NF-κB/ STAT3 signaling pathway in EAN rats

To further verify the inhibitory effects of celastrol on the TLR4/NF-κB/STAT3 signaling pathway, the expression of pathway-related proteins was detected after LPS treatment. The levels of TLR4, p-NF-κB, and p-STAT3 in the EAN rats were significantly higher than those in the control rats ($p < 0.01$). Nonetheless, the levels of TLR4, p-NF-κB, and p-STAT3 in the medicine-treated rats decreased markedly compared with the EAN rats ($p < 0.01$). In addition, LPS eliminated celastrol-induced suppression of the TLR4/NF-κB/STAT3 signaling pathway ($p < 0.01$) (Fig. 4F).

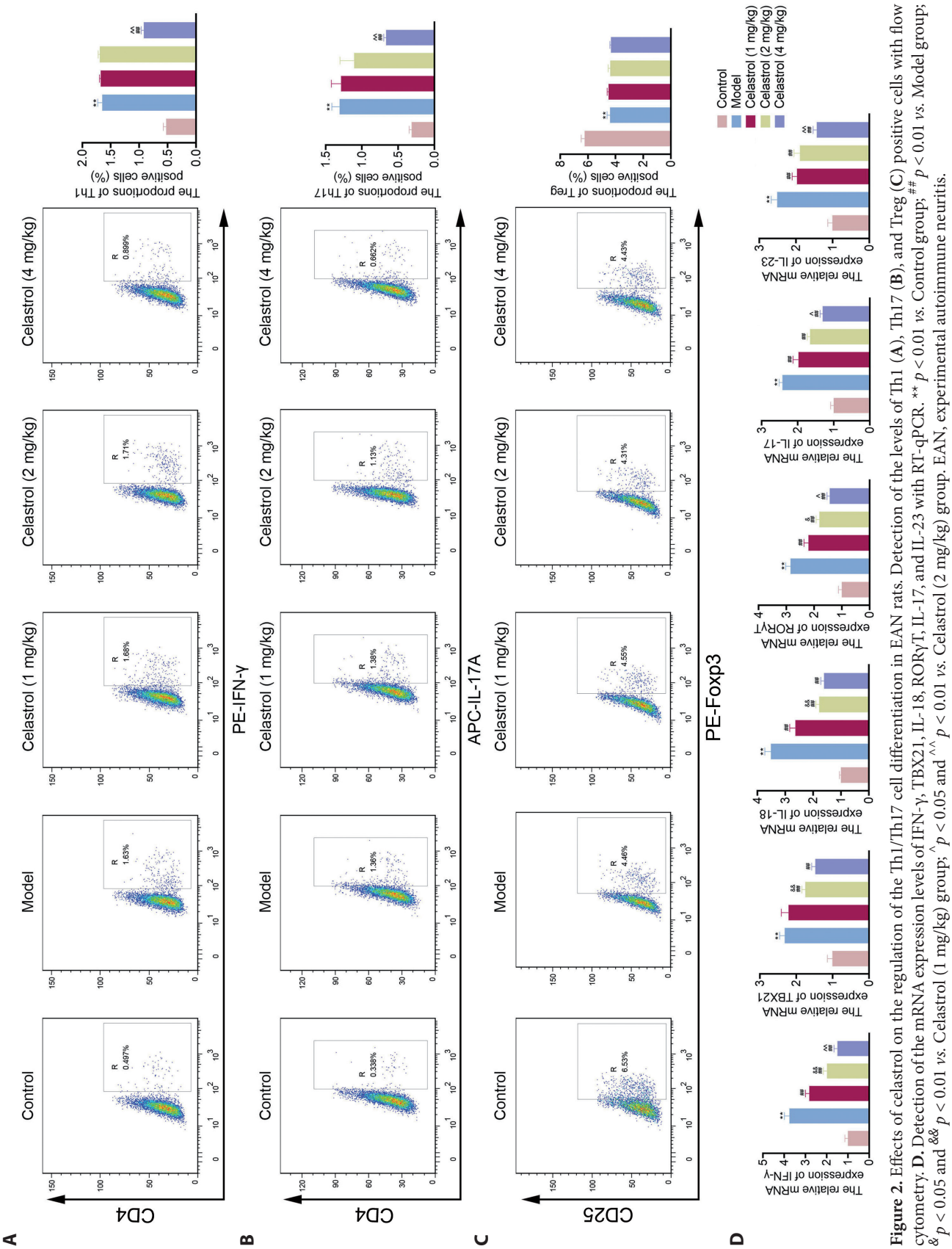


Figure 2. Effects of celastrol on the regulation of the Th1/Th17 cell differentiation in EAN rats. Detection of the levels of Th1 (**A**), Th17 (**B**), and Treg (**C**) positive cells with flow cytometry. **D.** Detection of the mRNA expression levels of IFN-γ, TBX21, IL-18, RORγT, IL-17, and IL-23 with RT-qPCR. $^{**}p < 0.01$ *vs.* Control group; $^{\#\#}p < 0.01$ *vs.* Model group; $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ *vs.* Celastrol (1 mg/kg) group; $^{\wedge}p < 0.05$ and $^{\wedge\wedge}p < 0.01$ *vs.* Celastrol (2 mg/kg) group. EAN, experimental autoimmune neuritis.

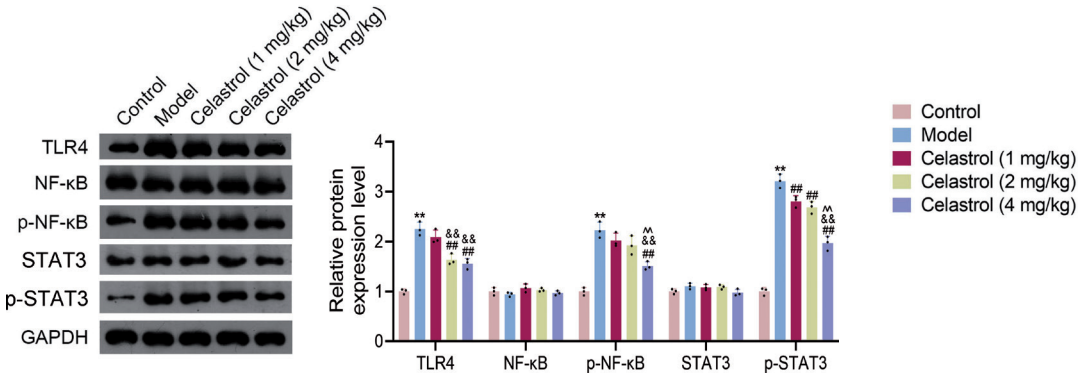


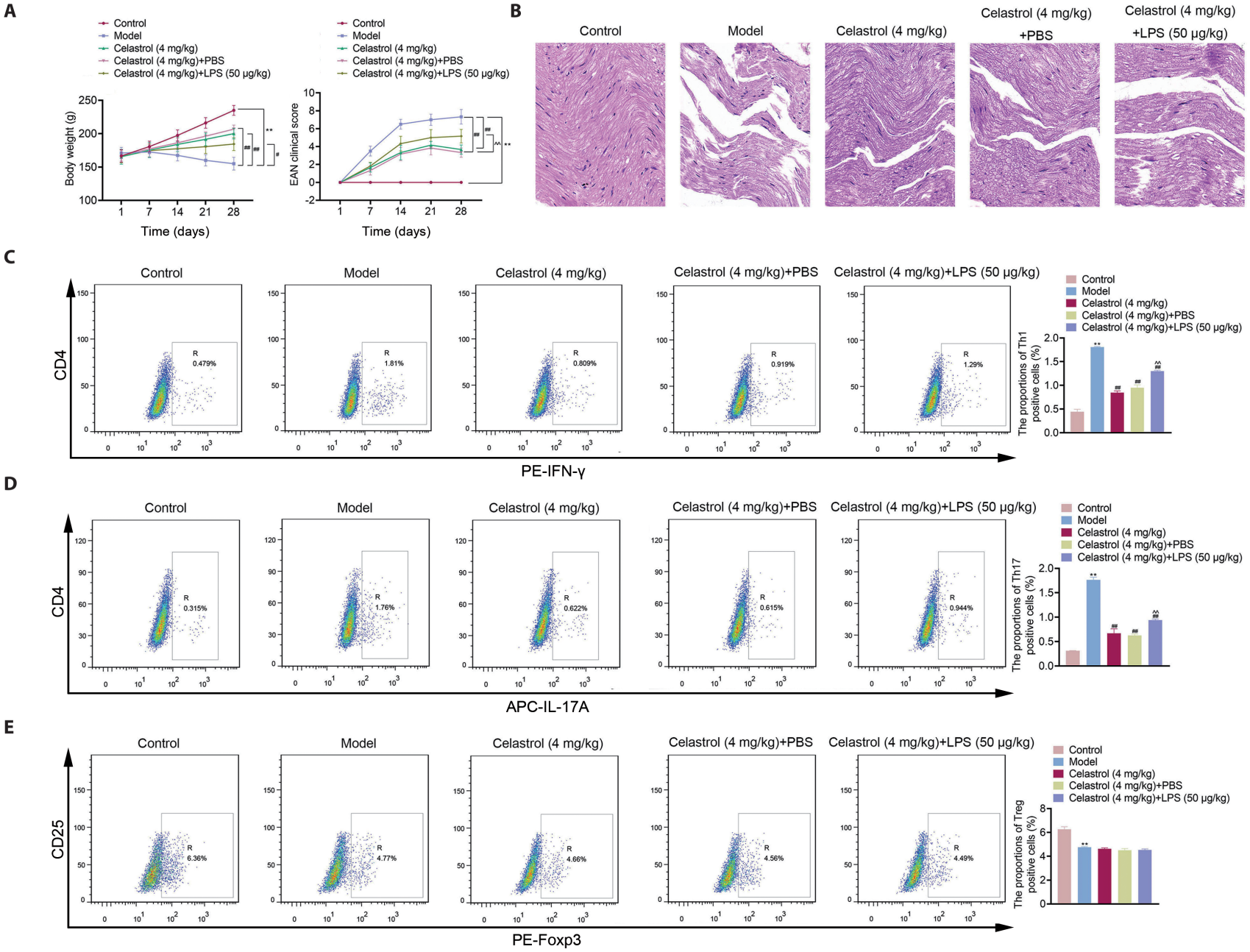
Figure 3. Effects of celastrol on the inhibition of the TLR4/NF-κB/STAT3 signaling pathway in EAN rats. Detection of the protein expression levels of TLR4, NF-κB, p-NF-κB, STAT3, and p-STAT3 with Western blotting. ** $p < 0.01$ *vs.* Control group; $^{\#\#}$ $p < 0.01$ *vs.* Model group; $^{\&\&}$ $p < 0.01$ *vs.* Celastrol (1 mg/kg) group; $^{\wedge\wedge}$ $p < 0.01$ *vs.* Celastrol (2 mg/kg) group. EAN, experimental autoimmune neuritis.

Discussion

GBS is an immune-mediated inflammatory disease affecting the peripheral nervous system, which is presently the most frequent cause of acute flaccid paralysis globally (Du et al. 2020). GBS typically manifests as a sudden occurrence of rapidly progressive and symmetrical weakness of the limbs, with or without peripheral sensory disturbance, decline in or loss of tendon reflexes, and increased protein concentrations in the cerebrospinal fluid with a normal white cell count (Liu et al. 2018). In addition, IVIG, PE, and corticosteroids have been commonly applied to treat GBS (Liu et al. 2018). Moreover, previous studies have shown that some traditional medicines, such as berberine (Li H et al. 2014) and *Yisui Tongjing* (Zhang et al. 2016), have potential therapeutic properties for GBS. Despite the development of targeted molecular-based strategies for GBS, more effective and safer drugs for GBS treatment are still needed.

Tripterygium wilfordii, also called Lei Gong Teng, is traditional Chinese medicine, which has been extensively used in the treatment of autoimmune and inflammatory diseases, such as rheumatoid arthritis (Li and Hao 2019). As the most active ingredient of *Tripterygium wilfordii*, celastrol has exhibited the favorable potential to treat inflammation and autoimmune diseases including systemic lupus erythematosus (SLE) (Xinqiang et al. 2020). In the present study, the clinical symptoms of GBS in EAN rats were observably improved by celastrol. GBS primarily damages the peripheral nervous system (Alarcón-Narváez et al. 2021). This study showed that celastrol mitigated the pathological damage of GBS in EAN rats. Taken together, these results imply that celastrol could alleviate the severity of GBS.

Suppression of the proportions of Th1/Th17 cells and their cytokines was previously demonstrated to ameliorate the symptoms of GBS/EAN and thus it might be a therapeutic approach for GBS (Li S et al. 2014; Wu et al. 2016). There are various inflammatory cytokines such as IFN-γ, and IL-17 involved in the pathogenesis of EAN (Zhang et al. 2013; Wang et al. 2014). Recent data has indicated that celastrol can ameliorate the pathological injury of ulcerative colitis by mediating the balance of Th1/Th17/Treg cells (Li et al. 2022). Furthermore, a recent study has demonstrated that down-regulation of IL-6 expression can alleviate the pathological damage of GBS (Mao et al. 2022). Hence, we assumed that celastrol might also mediate the differentiation of Th1/Th17 cells and secretion of inflammatory factors in GBS. Pro-inflammatory cytokine IFN-γ functions as a key regulator in the Th1 response, which is also produced by Th1 cells (Katsuyama et al. 2021). A study has indicated an increase in the IFN-γ level in the serum of patients with GBS (Li et al. 2020). TBX21 (T-bet) is a transcriptional factor that controls the Th1 genetic program and activates IFN-γ gene transcription (Jablonka-Shariff et al. 2021). As signaling downstream of IFN-γ, TBX21 activation triggers pro-inflammatory effects of Th1 cells to connect innate and adaptive immune responses (Ullrich et al. 2020). IL-18 is also a pro-inflammatory cytokine involved in the innate immune response and acts as a decent inducer of IFN-γ (Yasuda et al. 2019). RORγt is a transcription factor that regulates Th17 cell differentiation (Qiu et al. 2021). Also, overexpression of RORγt was found during the progression of GBS in an *in vivo* study (Liu et al. 2019). IL-17 is a critical pro-inflammatory cytokine, mainly produced by Th17 cells, and IL-17 inhibitors have been authorized for the treatment of some immune and inflammatory diseases (Miossec 2021). IL-23 is vitally implicated in promoting the production of IL-17 by activating the Th17 cells (Liu et al. 2020) . Evidence has suggested that IL-23/IL-17 immune axis might contribute to the onset of GBS (Debnath et al. 2018). Otherwise, previous research has indicated that down-regulation of the pro-inflammatory


A
Control
Model
Celastrol (4 mg/kg)
Celastrol (4 mg/kg)+PBS
Celastrol (4 mg/kg)+LPS (50 μg/kg)
Body weight (g)
Time (days)
EAN clinical score
Time (days)
B
Control
Model
Celastrol (4 mg/kg)
Celastrol (4 mg/kg) +PBS
Celastrol (4 mg/kg) +LPS (50 μg/kg)
C
CD4
PE-IFN-γ
R 0.479%
R 1.81%
R 0.809%
R 0.919%
R 1.29%
The proportions of Th1 positive cells (%)
D
CD4
APC-IL-17A
R 0.315%
R 1.76%
R 0.622%
R 0.615%
R 0.944%
The proportions of Th17 positive cells (%)
E
CD25
PE-Foxp3
R 6.36%
R 4.77%
R 4.66%
R 4.56%
R 4.49%
The proportions of Treg positive cells (%)

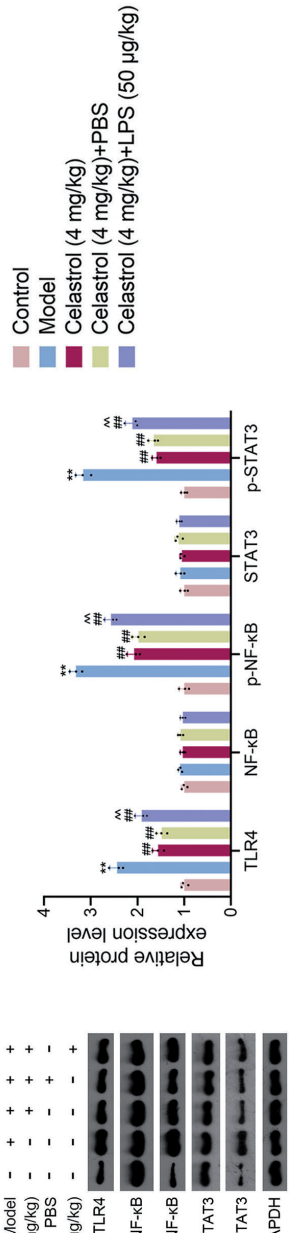


Figure 4. Effects of celastrol on amelioration of GBS in EAN rats *via* blockade of the TLR4/NF-κB/STAT3 pathway-mediated Th1 and Th17 cell differentiation. **A.** Detection of clinical symptoms by assessing the body weights and clinical scores of rats. **B.** Detection of sciatic nerve damage of EAN rats with HE staining (Scale bar = 20 μm). Detection of the levels of Th1 (**C**), Th17 (**D**), and Treg (**E**) positive cells with flow cytometry. **F.** Detection of the protein expression levels of TLR4, NF-κB, p-NF-κB, STAT3, and p-STAT3 with Western blotting. ** $p < 0.01$ *vs.* Control group; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ *vs.* Model group; $^{\wedge\wedge}$ $p < 0.01$ *vs.* Celastrol (4 mg/kg)+PBS group. GBS, Guillain-Barré syndrome; EAN, experimental autoimmune neuritis; HE, hematoxylin-eosin; PBS, phosphate buffer saline.

cytokines IFN-γ and IL-17 might alleviate GBS (Fagone et al. 2018). Herein, the proportions of Th1 and Th17 positive cells dropped observably after celastrol treatment. Meanwhile, celastrol down-regulated the expression levels of IFN-γ, TBX21, IL-18, RORγT, IL-17, and IL-23. Accordingly, we supposed that celastrol might suppress GBS in EAN rats by inhibiting the differentiation of Th1/Th17 cells.

TLR4, known as a member of the TLR family of receptors, triggers pro-inflammatory immune responses *via* recognizing pathogens and endogenous ligands, which is also a well-featured receptor for LPS (Xu et al. 2019). The NF-κB family of transcriptional factors mediates a great number of genes linked with different cellular processes, such as cell proliferation, differentiation, genome stability, and innate/adaptive immune responses (Peng et al. 2020). STAT3, a cytoplasmic transcription factor participates in multiple biological activities, including cell differentiation, immune and inflammatory responses (Lee et al. 2019). Accumulating evidence has demonstrated that the TLR4/NF-κB signaling pathway plays a crucial role in inflammatory and immune diseases *via* regulating Th1/Th17 cell responses (Lou et al. 2018; Liu et al. 2021; Ma et al. 2021). Moreover, a previous study declared that activation of STAT3 could facilitate Th17 cell differentiation and autoimmune inflammation (Damasceno et al. 2020). Specifically, inhibition of TLR4/NF-κB can down-regulate the proportions of Th1/Th17 cells, thus attenuating the pathological injury of autoimmune hepatitis (Liu et al. 2021) and collagen-induced-arthritis (Xu N et al. 2020) . A previous investigation suggested crosstalk between endoplasmic reticulum (ER) stress and NF-κB activation in neuroinflammation, which also demonstrated that inhibition of ER stress and NF-κB activation could alleviate neuroinflammation (Logsdon et al. 2016). Furthermore, a recent *in vivo* study has indicated that suppression of the TLR4/NF-κB/STAT3 axis-mediated neuroinflammation can ameliorate sciatic nerve damage in rats (Yardim et al. 2022). These facts pushed us to speculate on an interaction between TLR4/NF-κB axis and STAT3 signaling in GBS. Interestingly, we found that celastrol significantly decreased the proportions of Th1/Th17 cells, accompanied by the falling levels of TLR4, p-NF-κB, and p-STAT3. LPS eliminated the effects of celastrol on attenuating the pathological damage of GBS and down-regulating the proportions of Th1/Th17 cells in EAN rats. This suggests that celastrol may mitigate GBS by inhibiting TLR4/NF-κB/STAT3 signaling pathway-mediated Th1/Th17 cell differentiation.

Conclusions

In summary, our study demonstrates that celastrol can alleviate GBS by inhibiting the TLR4/NF-κB/STAT3-mediated Th1/Th17 cell differentiation. Nevertheless, our work still has

some limitations. There are multiple pathological and signaling mechanisms implicated in the development of GBS; however, we only focused on Th1/Th17 cell differentiation and the TLR4/NF-κB/STAT3 axis in this research. Besides, the effects of celastrol on reliving GBS have not been confirmed in clinical trials, and the pathway mechanism by which celastrol attenuates GBS needs to be further elucidated.

Ethics approval. All animal protocols were approved by the Animal Experimental Ethics Committee of the Zhejiang Hospital Affiliated to Zhejiang University and were carried out in accordance with the NIH guidelines.

Data availability. The data that support the findings of this study are available from the corresponding author on special request.

Conflict of interests. The authors confirm that there is no conflict of interest related to the manuscript.

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