

Reactive enteric glial cells participate in paralytic ileus by damaging nitrergic neurons during endotoxemia

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Research Article

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Abstract

Background: Paralytic ileus is common in patients with septic shock, which may cause high morbidity and mortality. Enteric neurons and enteric glial cells (EGCs) participate in the regulation of intestinal motility, but little is known about their interaction in endotoxemia. We aim to investigate whether reactive EGCs have harmful effects on enteric neurons and participate in intestinal motility disorder in mice during endotoxemia.

Methods: In this study, lipopolysaccharide (LPS) was used to induce endotoxemia in mice, and intraperitoneal injections of fluorocitrate (FC) before LPS injections to inhibit the reactive EGCs. The effects of reactive EGCs on intestinal motility were analyzed by motility assays *in vivo* and colonic migrating motor complexes (CMMCs) *ex-vivo*. The number of enteric neurons was evaluated by immunofluorescent staining HuCD, nNOS and CHAT *in vivo*. In addition, we stimulated EGCs with IL-1 β and TNF- α *in vitro* and cultured the primary enteric neurons in the conditioned medium, detecting the apoptosis and morphology of neurons through staining TUNEL, cleaved Caspase-3 protein and Anti- β -III tubulin.

Results: The expression of glial fibrillary acidic protein (GFAP) was significantly upregulated in LPS-injected animals, indicating EGCs were transformed into a reactive state. Inhibition of reactive EGCs improved intestinal motility and peristaltic reflex, and increased the density of the neuronal population in the colonic myenteric plexus significantly, especially the nitrergic neurons. *In vitro*, the apoptotic rate of enteric neurons significantly increased, and the dendritic complexity and the number of primary neurites were significantly reduced in the conditioned medium of reactive EGCs.

Conclusion: Reactive enteric glial cells participated in paralytic ileus by damaging nitrergic neurons during endotoxemia. It may provide a novel therapeutic strategy for intestinal motility disorders during endotoxemia or sepsis.

Introduction

Sepsis is a life-threatening organ dysfunction caused by the host's dysregulated response to infection[1]. The mortality rate of sepsis and septic shock has commonly been quoted as ranging from 25–60%[2]. Intestinal motility disorder, often manifests as paralytic ileus, is a common complication following sepsis and septic shock. It can cause bacteria accumulation in the intestine, and promote bacterial translocation. In turn, the occurrence of bacterial translocation will induce multiple organ dysfunction syndromes (MODS) and lead to high mortality[3]. Thus improving intestinal motility is critical during sepsis treatment[4]. Intestinal motility is regulated by the enteric nervous system (ENS), a complex network of neurons and glial cells, mainly resides in the submucosal and myenteric plexus[5]. There is evidence that dysfunction, degeneration, or loss of myenteric neurons will cause several intestinal motility disorders [6, 7]. Enteric glial cells (EGCs), as the most abundant cells in the ENS, provide nutritional

support for enteric neurons, promote the formation and function of synapses, and maintain neuronal survival[8].

EGCs may undergo a dramatic transformation called the “reactive glial phenotype” in response to intestinal infection with bacteria[9], viruses and inflammatory mediators[10, 11]. However, there is a controversy regarding the function of reactive EGCs. Previous studies have shown EGCs may have dual protective and harmful effects. For instance, the intestinal ischemia-reperfusion injury (IRI) leads to the significant upregulation of glial fibrillary acidic protein (GFAP), the EGC activation marker. That presents a beneficial effect on neurons through the increase of glial cell line-derived neurotrophic factor (GDNF) [12, 13]. On the contrary, a study from Gulbransen's group provided evidence that glial activation as a driver of enteric neurodegeneration caused the death of enteric neurons through purinergic pathways in colitis induced by dinitrobenzene sulfonic acid (DNBS) [14]. As for endotoxemia, it has been confirmed that lipopolysaccharide (LPS) increasing leads to proinflammatory cytokines release and myenteric neurons apoptosis *in vitro*[15]. In addition, systemic LPS administration also induces an increase in intestinal GFAP and S100b expression[16]. While the association between reactive EGCs and the loss of enteric neurons is still unknown. That is to say, it is unclear whether reactive EGCs are beneficial or harmful to the enteric neurons in endotoxemia mice.

In this study, we investigated the effect of reactive EGCs on intestinal motility and enteric neuron function in an endotoxemia model *in vivo*, and observed the effect of reactive EGCs on the growth and survival rate of enteric neurons under an inflammatory condition *in vitro*. Here we observed that LPS-induced reactive EGCs are harmful to the enteric neurons during endotoxemia and are associated with colonic transit delay, which might provide a novel strategy for prevention or treatment of LPS-induced intestinal paralysis and other relevant intestinal motility disorders.

Materials And Methods

1. Animals

C57BL/6 mice (male, 6~8 weeks old, weight 20~23 g) were obtained from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China) and housed in a temperature-controlled and humidity-controlled room with a 12:12 hour light: dark cycle, and provided with food and water. All procedures were approved by the Institutional Animal Care and Use Committees at Xi'an Jiaotong University (Xi'an, China). Animals were randomly divided into 3 groups: Con: vehicle control, normal saline (0.02 ml/g, IP), LPS: lipopolysaccharide (Sigma-Aldrich, E. coli O55:B55, USA, 20 mg/kg, IP), LPS + fluorocitrate (FC): LPS injection after intraperitoneal injections of FC (Sigma, St. Louis, MO, 20 μ mol/kg) twice per day (9 AM and 6 PM) for 7 days according to the previous research[17] (Supplementary Figure S1). Animals were observed and evaluated at least twice a day for post-treatment care until sacrifice, including breath, body temperature and activity.

2. Assessment of gastrointestinal motility

2.1 Stool frequency and fluid content

According to the previous method[18], each mouse was placed in a clear cage for 1hr. Fecal pellets were collected, counted, and weighed (wet weight) during this time. These were dried overnight at 60°C and weighed again (dry weight). Fluid content (%) = $100\% \times (\text{wet weight} - \text{dry weight}) / \text{wet weight}$.

2.2 Colon bead assay

Distal colon transit was assessed using glass beads (2 mm in diameter) as previously described[18], and executed 48 hrs after the LPS injection. After being overnight fasted, mice were anesthetized with inhalation of isoflurane and a single bead was inserted through the anus and pushed 2 cm towards the oral by a customized needle with a silicon cannula (1.9 mm in diameter) (Cadence Inc., Cat. No. 9921, Staunton, VA). Then, the needle was withdrawn lightly and the bead expulsion latency was obtained. The time required to eject the bead was measured as an estimate of colonic motility.

2.3 Whole-gut transit

Whole intestinal transit time was measured following the previous report with a little modification[19] and executed 48 hrs after the LPS injection. Male mice (8 weeks) have fasted only with access to water for 18 hrs before the experiment. 0.2 mL of a solution containing 6% (w/v) Carmine (Sigma-Aldrich, C1022, USA) and 0.5% (w/v) methylcellulose (Sigma-Aldrich, M0262, USA) dissolved in ultrapure water was orally administered to each mouse and left undisturbed in individual cages with food and water ad libitum. During 2 hrs after gavage, pellets coloration was checked regularly every 20 min. Time elapsed from gavage until the appearance of the first red pellet was obtained.

2.4 Colonic migrating motor complexes measured in *vitro*

According to the previous method[20], the entire colon (5~6 cm, n = 6) was dissected from the mouse and flushed to remove fecal content with Krebs solution. Then the empty colon was mounted in a horizontal organ bath with oxygenated Krebs solution at 35~36°C and maintained intraluminal pressure at 1~2 cmH₂O. Preparations were equilibrated for 30 min and four 15-minute videos of contractile activity were captured via a video camera (Logitech, Newark, CA) positioned 7~8 cm above the gut. Finally, these videos were converted to spatiotemporal maps via MATLAB (MathWorks, USA). The frequency of colonic migrating motor complexes (CMMCs) as well as the velocity and length of their propagation were analyzed by a researcher blinded to the test groups.

3. Cell cultures

3.1 Enteric glial cell line

The rat enteric glial cell line CRL2690 (American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, USA), 1mM glutamine (Gibco Life Technologies, USA), and penicillin/streptomycin (Gibco Life Technologies, USA).

3.2 Primary enteric glial cells

The isolation, identification, and culture of primary EGCs were performed as previously described[21]. In brief, the colonic tissue was collected from newborn mice (1~2 days old, C57BL/6 mice), and cells were cultured in DMEM-F-12 supplemented with 10% FBS, 1mM glutamine and 100IU/ml penicillin/streptomycin. More details are in the supplementary information. They were passaged to new plates after 12~14 days for purity assay and the following experiments. Culture consisted of approximately 90% of enteric glia, as judged by immunolabelling with a chicken antibody specific for GFAP (1:500, GeneTex, GTX85454, USA) and rabbit antibody for S100b protein (1:100, GeneTex, GTX57757, USA)(Supplementary Figure S2).

3.3 Primary enteric neurons

The isolation and culture of enteric neurons are similar to that of primary EGCs. The difference is that the colonic tissue was collected from embryonic day 14~15 (E14~15) rats, and cells were cultured in a neurobasal medium containing 2 mM glutamine, 1 mM (100 IU/ml) penicillin/streptomycin, and 1 mM B-27 supplement. The purity of neurons is also more than 90% after 12~14 days, staining with mouse anti-HuC/D primary antibody (1:100; Invitrogen) (Supplementary Figure S3).

4. Conditioned medium experiments

IL-1 β (80 ng/ml, Peprotech, AF-211-11B, USA) and TNF α (60 pg/ml, Peprotech, AF-315-01A, USA) were used to induce EGCs into reactive for 24 hrs according to the previous study[21]. Briefly, the enteric glial cell line was culture about 48 hrs in DMEM supplemented with 10% FBS, 1mM glutamine, and penicillin/streptomycin until the cell attachment rate was up to 70~80%. Then, we changed the media into the serum-free one supplemented with 80 ng/ml IL-1 β and 60 pg/ml TNF α for 24 hrs. Next, the medium containing cytokines was removed and replaced with a fresh neurobasal medium for another 24 hrs. At this time, the reactive EGCs conditioned medium (ECM) was centrifuged at 1000 rpm for 5 min, filtered through a 0.22 μ m syringe filter (0.22 μ M filter, Thermo, USA), and conserved at -80°C until use. The conditioned medium of control (resting) EGCs was collected as above process except supplementary with IL-1 β and TNF α .

4.1 Detection of apoptosis

To detect apoptosis, TUNEL staining was carried out using the fluorescein tagged In Situ Cell Death kit (Promega, G3250, USA) according to the procedures of specification. **Cells suspension** was obtained after isolating enteric neurons from colons of fetal mice, this was **counted** using a classic hemocytometer. Primary enteric neurons were plated about 4×10^4 cells/cm² in 24-well plates and grown for 12~14 days after maturing in the neurobasal medium. They were treated with the conditioned medium of control (resting) and reactive EGCs respectively. 48 hrs after the treatment, neurons were fixed in 4% PFA for 20 min at 25°C and then cleared with PBS. The next steps were according to the procedures of the specification.

4.2 Neurite formation assay

Primary enteric neurons were plated about 1,000 cells/cm² in 24-well plates when **cell suspension** was obtained. The neurobasal medium was replaced with the conditioned medium of control and reactive EGCs until enteric neurons were grown for 8~9 days. Then, enteric neurons were fixed and stained with antibodies against the neuron-specific beta III Tubulin (TuJ-1, Abcam, ab78078, USA) 72 hrs after incubation. For analysis of neurite formation, Sholl analysis was used to assess the complexity of neurites. Pictures of TuJ-1-labeled cells were taken under the confocal microscope (Olympus, FV1000, Japan) with 40× objective and a template of concentric circles distant from 10 to 500 μm (10 μm interval) from the ganglion center was overlaid on the ganglion using Image J software. The number of primary neurites and branching points was also counted. For each group (3 biological replications), 5 ganglia were analyzed.

5. Immunofluorescent staining

Whole-mount longitudinal muscle/myenteric plexus (LMMP) preparations of colonic myenteric plexus were prepared according to a published method in Tricia H. Smith et al[22]. An entire colon was divided into two parts, and half part of each colon was used as LMMP preparations, and the other part was for Western blot. After washing in phosphate-buffered saline, colon LMMP preparations that separated from 1-1.5-cm colonic fragments were fixed in 4% paraformaldehyde (PFA) 1 hr and then permeabilized with 0.3% Triton-X for 40 min before immunostaining. Preparations were blocked with 10% goat serum for 1 hr and exposed to primary antibodies: chicken anti-GFAP (1:500, GeneTex, GTX85454, USA), mouse anti-HuC/D (1:100, Invitrogen, 16A11, USA) for labeling the general population of myenteric neurons, goat anti-choline acetyltransferase (ChAT, 1:200, Millipore, AB144P, USA) for labeling cholinergic neurons, and rabbit anti-neuronal nitric oxide synthase (nNOS, 1:200, GeneTex, GTX133403, USA) for labeling nitrergic neurons. Preparations were incubated with primary antibodies for 16-24 hrs, visualized with species-specific secondary antibodies (Alexa Fluor 488 or 594, 1:500) and mounted.

6. Confocal Image Acquisition and Analysis

Images of the myenteric ganglia were taken under the FluoView 1000 confocal microscope (Olympus, Tokyo, Japan). A computer-controlled motorized stage was used to scan images (20× or 40× objective) without projections of Z-stacks, and the exposure brightness, contrast, and time were maintained for each photomicrograph. At least, 3 images that were captured with a 20× or 40× objective were counted from each animal (n = 3 biological replications). ImageJ (version 2.0) was used to analyze the fluorescent intensity and neuronal counts. For quantification of enteric neurons, 3 ganglia were randomly selected from each LMMP preparation, 3 preparations for each animal, and 3 animals in each treatment group. In each ganglion, the number of HuC/D-immunoreactive neuronal bodies and nNOS-immunoreactive neurons was manually counted in a blind fashion. In addition, the intensity fluorescence of ChAT was detected by ImageJ, along with several adjacent background readings. Then, the CTCF (corrected total cellular fluorescence) [= integrated density - (area of selected cell × mean fluorescence of background readings)] was calculated[23]. All the studies were performed in a double-blind fashion.

7. Western blot analysis

Protein samples were extracted from colonic tissue in RIPA buffer containing complete protease and phosphatase inhibitor cocktail. After assessing the protein content of each sample by BCA Protein Assay kit (Thermo Fisher Scientific, 23227, USA), they were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. After blocking, the blots were incubated with anti-GFAP (1:10000, GeneTex, GTX100850, USA) primary antibody overnight at 4°C. Subsequently, the membrane was incubated with a secondary antibody (anti-rabbit IgG-horseradish peroxidase, 1:2000, GeneTex, GTX213110-01, USA) followed by visualized with enhanced chemiluminescence. The signals on the blots were detected with Tanon 5200 Multi (Minhang District, Shanghai Municipality, China). β -actin was used as an internal standard. Immunoblots were quantified by Image J software (version 2.0).

8. Statistical Analysis

Results are presented as mean \pm Standard Error of Mean (SEM). All statistical analysis was conducted using Prism 7.0 (GraphPad, San Diego, CA, USA). The Student's *t*-test was used to determine statistical differences between each experimental group and the control group data. One-way ANOVA with Sidak's multiple comparisons test was used for the data with group numbers over two. $P < 0.05$ was considered significant and denoted by *.

Results

1. Reactive EGCs are induced whereas are reversed by FC in LPS-injected animals

We assayed GFAP expression of colonic tissue in LPS-injected animals, as it is a typical identification marker of enteric glia and astrocytes to indicate their activation. As expected, results of Western blot showed that GFAP expression was significantly upregulated after the LPS injection compared with the control group (Fig. 1a and b, control vs. LPS, $P < 0.01$). FC is a metabolic compound that makes EGCs metabolism static [17]. To testify whether EGCs are no longer reactive in the mice treated with FC, we injected LPS and assessed the protein expression of GFAP 48hrs later. Western blot analysis showed that FC treatment significantly reduced the expression of GFAP in colon tissue compared with the LPS group, while it has no significant difference with the control one (Fig. 1a and b, mean FC + LPS vs. LPS, $P < 0.001$; vs. control, $P > 0.05$). In addition, we isolated the LMMPs from colon tissue and detected the intensity of GFAP expression in the myenteric nerve plexus by immunostaining. Compared with the control group, the intensity of GFAP increased in the LPS group (Fig. 1c-f, mean LPS vs. Control, $P < 0.05$). However, it decreased in the LPS plus FC group relative to the LPS group (Fig. 1c-f, mean FC + LPS vs. LPS, $P < 0.05$). Thus, it indicated that EGCs convert into a reactive state after LPS injection in mice, while, FC treatment impeded this produce.

2. Inhibition of reactive EGCs improves intestinal motility and peristaltic reflex in LPS-injected animals

To determine whether the reactive EGCs are involved in the regulation of GI motility, we measured fecal pellet output, propulsive colorectal motility, total gastrointestinal transit time (TGIT), fluid content *in vivo*. As shown in Figure 2, LPS significantly reduced gastrointestinal motility, which includes stool frequency (mean control 8.83 ± 0.83 , vs. LPS 2.50 ± 0.43 , Fig. 2a, $P < 0.01$), colon transit time (mean control 49.83 ± 9.63 s, vs. LPS 1044 ± 235.10 s, Fig. 2b, $P < 0.001$) and TGIT (mean control 72.50 ± 4.61 min, vs. LPS 541.20 ± 63.00 min, Fig. 2c, $P < 0.0001$). Stool frequency (mean FC + LPS 5.33 ± 0.56 vs. LPS group, Fig. 3a, $P < 0.05$), colon transit time (mean FC + LPS 188.80 ± 60.35 , vs. LPS group, Fig. 3b, $P < 0.01$) and TGIT (mean FC + LPS 201.30 ± 44.18 min vs. LPS group, Fig. 3c, $P < 0.01$) were significantly alleviated by FC treatment, comparing with the group only with LPS injection. Besides, there is no significant difference in stool water content between the control and the LPS mice, and between the LPS mice and the LPS mice treated with FC (mean control 0.63 ± 0.037 , vs. LPS 0.61 ± 0.04 ; LPS vs. FC + LPS 0.48 ± 0.07 , Fig. 2d, $P > 0.05$).

CMMCs, which are dependent on ENS [24], were investigated in the isolated preparations of colons to determine whether the observed changes in motility are due to an intrinsic ENS defect. Spatiotemporal maps of CMMCs were constructed and analyzed (Fig. 2e-g), showing that frequency (mean control 1.15 ± 0.19 CMMCs/min, vs. LPS 0.36 ± 0.06 CMMCs/min, Fig. 2h, $P < 0.001$) and velocity (mean control 22.95 ± 3.90 mm/min, vs. LPS 5.30 ± 0.36 mm/min, Fig. 2i, $P < 0.001$) were significantly decreased in the LPS group compared with the control group. However, both of them were back to normal when treated with FC (frequency, mean FC + LPS 0.78 ± 0.04 CMMCs/min vs. LPS, $P < 0.05$; velocity, mean FC + LPS $17.27 \pm$

1.15 mm/min, vs. LPS, $P < 0.05$). To sum up, the reactive EGCs slow down intestinal transit and disrupt the peristaltic reflex, while FC can mitigate the deficiency induced by LPS.

3. Inhibiting reactive EGCs reduces the loss of nitrergic neurons in the myenteric plexus of LPS-injected animals

To study how reactive EGCs affect enteric neurons in LPS-injected animals, the density of the general neuronal population (HuC/D-immunoreactive) in the colonic myenteric plexus was detected among the control, LPS, and LPS plus FC groups. LPS reduced the number of general neurons by 28% compared with the control mice (mean control $662.85 \pm 55.71/\text{mm}^2$, vs. LPS $476.19 \pm 62.22/\text{mm}^2$, Fig. 3a and b, $P < 0.05$). However, the neuronal population did not decrease in the FC plus LPS group, but increased significantly compared with the LPS group (mean FC + LPS $719.05 \pm 50.16/\text{mm}^2$, vs. LPS group, $P < 0.05$). To define whether the reactive EGCs affect the subpopulation of cholinergic and nitrergic neurons, we measured ChAT and nNOS expression in the colonic myenteric plexus by immunofluorescence staining. LPS reduced the population of nNOS neurons per square millimeter of colonic LMMPs (mean control $292.40 \pm 27.82/\text{mm}^2$, vs. LPS $156.40 \pm 24.62/\text{mm}^2$, $P < 0.05$), which reversed to baseline after FC treatment (mean FC + LPS $251.40 \pm 23.39/\text{mm}^2$, vs. LPS group, Fig. 3c, $P < 0.05$). No statistically significant difference was found in ChAT fluorescence intensity between the LPS mice and the LPS mice treated with FC (Fig. 3d, $P > 0.05$). These observations demonstrated that the reactive EGCs cause enteric neuron loss, especially nitrergic neurons in LPS-injected animals.

4. Reactive EGCs induce primary enteric neurons into apoptosis in vitro

It has been testified that reactive EGCs lead to a decrease of enteric neurons in the myenteric plexus *in vivo*. We studied how reactive EGCs influence the state of enteric neurons *in vitro*. Firstly, we stimulated the enteric glial cell line with IL-1 β and TNF α to develop a serum-free culture model for the reactive EGCs *in vitro*, mimicking the pathological process in LPS-injected animals (Fig. 4a-d). Meanwhile, we identified that IL-1 β and TNF α made primary EGCs reactive (Supplementary Figure S4). Next, purified enteric neurons were incubated with Neurobasal medium, the conditioned medium of resting or reactive EGCs. Then the cell apoptosis was assessed using TUNEL tests and cleaved Caspase-3 protein staining. The TUNEL assay showed that primary enteric neurons revealed $27.54 \pm 5.28\%$ positive apoptotic neurons at 48 hrs after cultured in the conditioned medium of reactive EGCs compared with $3.06\% \pm 0.34$ for cultures in the conditioned medium of resting EGCs (Fig. 5a and b, $P < 0.01$). Immunofluorescence of cleaved Caspase-3 protein displayed that $6.84 \pm 0.35\%$ positive apoptotic neurons cultured in the conditioned medium of reactive EGCs compared with $3.46 \pm 1.16\%$ for neurons incubated with the conditioned medium of the resting EGCs (Fig. 5c and d, $P < 0.01$).

5. Reactive EGCs disrupt neurites formation of primary enteric neurons in vitro

To test if reactive EGCs affect neurites formation of enteric neurons *in vitro*, primary enteric neurons were incubated with the conditioned medium of resting or reactive EGCs and observed the morphology through TuJ-1 immunostaining (Fig. 6a). The dendritic complexity of neurons cultured in reactive EGCs

conditioned medium significantly declined compared with those grown in the resting EGCs conditioned medium (Fig. 6b, $P < 0.05$). In addition, the primary neurites number of neurons cultured in reactive EGCs conditioned medium decreased by 54.17% (+resting EGCs 6.00 ± 0.58 , vs. +reactive EGCs 2.75 ± 0.85 , Fig. 6c, $P < 0.05$) and the number of branches was also reduced by 77.14%, comparing with neurons grown in the resting EGCs conditioned medium (+resting EGCs 19.25 ± 1.03 , vs. +reactive EGCs 4.40 ± 0.87 , Fig. 6d, $P < 0.0001$). Neurons cultured in reactive EGCs conditioned medium tended to show shorter neurites compared to those grown in the resting EGCs conditioned medium (+resting EGCs 141.80 ± 30.22 , vs. +reactive EGCs 72.03 ± 20.21 , Fig. 6e, $P > 0.05$), However, there was no significant difference between them (Supplementary Figure S5).

Discussion

In this study, we demonstrated that reactive EGCs caused a significant decrease in intestinal motility via increasing the apoptosis of nitrergic enteric neurons in LPS-injected animals. However, this tendency was reversed, when the function of reactive EGCs was inhibited by FC. In addition, reactive EGCs also disrupted the neurites formation of primary enteric neurons *in vitro*. Together, our results indicate that the reactive EGCs may cause damage to the enteric neuron during endotoxemia, which deteriorates the intestinal motility disorder, suggesting that inhibition of the reactive EGCs may be a therapeutic strategy for the treatment of LPS-induced intestinal paralysis and other intestinal motility disorders.

Early research showed that EGCs play an important role in regulating and protecting enteric neurons, and dysfunction of EGCs will break the intestinal homeostasis, leading to a series of gastrointestinal disorders[25]. Furthermore, previous studies demonstrate that enteric glia could be activated by bacteria, which will respond to an immune reaction in the gastrointestinal tract and release ATP[14], inflammatory cytokines[26], and prostaglandin, etc[27]. However, whether the reactive EGCs participate in neurodegenerative processes and whether the interaction between EGCs and neurons influences intestinal motility has not been clarified yet. To elucidate this question, we tested the intestinal motility of mice injected LPS and FC plus LPS, as well as the number of colonic LMMP neurons. Compared with the control, there was a significant delay of intestinal transition accompanied by a significant loss of neurons after the LPS injection. The result revealed that inhibition of EGC activation could improve intestinal motility and prevent neurons from losing. Thus, we inferred that the reactive EGCs were associated with the decrease of enteric neurons. Meanwhile, it has been proved that ENS plays a significant role in motility regulation[28] and the loss of enteric neurons causes intestinal dysmotility[7]. Therefore, if the reactive EGCs are inhibited, the loss of neurons would be alleviated as well as the intestinal dysmotility.

GFAP, a glial fibrillary acidic protein, is the typical identification marker of enteric glia and astrocytes, whose expression in mature EGCs is modulated by cell differentiation, inflammation, and injury[29]. Increased GFAP has been observed in ulcerative colitis, Crohn's disease[25], and Parkinson's disease[30]. Astrocytes and EGCs can be induced into reactive by cytokines and LPS[31, 32]. To study the function of reactive EGCs in the endotoxemia model, we choose LPS *in vivo*. There was an upregulation of the GFAP expression in the LMMP of colon tissue in line with another research[16]. However, GFAP is dynamic and

its observed expression varies depending on glial state[33], subtype[34], and genetic targeting strategy[35]. Changes in morphology or GFAP expression are not a definitive indication of a reactive glial phenotype, since the functional change is more crucial for a different cell phenotype. Additionally, LPS triggers immune responses in endotoxemia, leading to a massive release of cytokines such as IL-1 β and TNF- α [4, 36]. In our study, we stimulated the enteric glial cell line and primary EGCs with IL-1 β and TNF- α *in vitro* to mimic the inflammatory response induced by LPS *in vivo*. As expected, the GFAP expression levels increased significantly. To make a definitive indication of a reactive glial phenotype, we collected the conditioned medium of the reactive EGCs and incubated it with the primary enteric neurons, detecting effects of reactive EGCs on neurons survival and neurites' formation.

EGCs were primarily considered as a component to provide structural support for the enteric neural net. Currently, it has been confirmed that EGCs could release nerve growth factor (NGF), neurotrophin 3 (NT-3), and GDNF to maintenance and contribute to the survival of enteric neurons[37, 38]. Interestingly, glia transforms into a reactive phenotype in response to intestinal infection with bacteria or viruses, inflammatory mediators[39]. The function of reactive EGCs, though unlike the central nervous system (CNS), is still poorly understood. As shown for the CNS, 2 different types of reactive astrocytes termed "A1" and "A2" were induced by neuroinflammation and ischemia respectively[40]. In addition, A1 reactive astrocytes become neurotoxic, leading to the neuron's death[41]. As for ENS, the intestinal ischemia-reperfusion injury (IRI) leads to the significant upregulation of GFAP, which may present a beneficial effect on neurons, because GDNF increased under this condition [12, 13]. On the contrary, a study from Gulbransen's group provided evidence that glial activation is a driver of enteric neurodegeneration in colitis[14]. Besides, Andromeda's group used human EGCs from GI-surgical specimens and treated them with LPS to study the gene expression and relevant functions. Their results showed that the "reactive human EGC phenotype" leads to alterations of important molecular and functional signaling pathways that could disrupt GI motility[42]. After all, there is still a lack of study about the influence of reactive EGCs on enteric neurons. In our study, we used a conditioned medium of reactive EGCs to interfere with primary enteric neurons, and the results indicated that the reactive EGCs damaged enteric neurons through increasing neurons' apoptosis and disrupting neurites' formation. On the other hand, it suggested that EGCs might release some specific substances to be harmful to enteric neurons. Thus, in the following study, we can try to find out such potential substances or targets that reactive EGCs damage enteric neurons and block them, which may be a potential new therapeutic target to modulate the intestinal dysmotility of patients suffering from sepsis or endotoxemia, or other diseases associated with motility disorders.

Fluoroacetate (FAC) and its metabolite FC were important tools that have been used to define the role of astrocytes in central[43, 44] and peripheral neural networks previously[45, 46]. Also, FC can be selectively taken up by enteric glia in the intestine [47] and cause a reversible and selective disruption of glial function owing to a fall in glia ATP levels through inhibiting the tricarboxylic acid TCA cycle. However, it does not influence glial and neuronal morphology in both the myenteric and submucosal plexuses of the ileum and colon in mice treated with 20 μ mol/kg FC[17]. In our research, EGCs were not induced into reactive by LPS after FC administration, meanwhile, gastrointestinal motility and the number of enteric

neurons were not decreased anymore, comparing with the LPS group. Hence, it suggested that the reactive EGCs appear to an adverse effect on intestinal motility in LPS-injected animals.

There is still a phenomenon that cannot be elucidated. We found that the amount of total and nitrergic neurons in the myenteric nerve clusters was decreased, except for cholinergic neurons, which is similar to a previous study[6]. The ENS contains many different types of neurons[48]. Given other research, cholinergic neurons and nitrergic neurons are mainly involved in intestinal motility regulation[49]. At least 70% of all myenteric neurons are cholinergic, which excite smooth muscle[50]. While most enteric neurons that inhibit smooth muscle contain nitric oxide synthase 1 (NOS1) [50]. This might seem contradictory. On the one hand, it may be relevant to the interaction and coordination among different kinds of enteric neurons, and the broken balance of intestinal neural networks might cause intestinal disorders. The other hand, this study only focused on two kinds of neurons, changes in other kinds of neurons during endotoxemia are still unclear. All these need further research.

Conclusions

In conclusion, the present data indicated that reactive enteric glial cells are involved in paralytic ileus through promoting nitrergic enteric neuronal apoptosis and impeding the formation of neurites during endotoxemia. These findings may provide a novel therapeutic strategy for intestinal motility disorders, including LPS-induced intestinal paralysis. In addition, the specific mechanism about how the reactive EGCs damage enteric neurons needs further study.

Abbreviations

EGC	Enteric glial cell
LPS	Lipopolysaccharide
FC	Fluorocitrate
CMMC	Colonic migrating motor complex
GFAP	Glial fibrillary acidic protein
MODS	Multiple organ dysfunction syndromes
ENS	Enteric nervous system
IRI	Ischemia-reperfusion injury
GDNF	Glial-derived neurotrophic factor
DNBS	Dinitrobenzene sulfonic acid
LMMP	Longitudinal muscle/myenteric plexus
CTCF	Corrected total cellular fluorescence
FBS	Fetal bovine serum
TuJ-1	Anti- β -III tubulin
TNF- α	Tumor necrosis factor- α
IL-1 β	Interleukin 1 β
TGIT	total gastrointestinal transit time
HBSS	Hank's balanced salt solution
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
PBS	Phosphate buffer saline
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulfate
PFA	paraformaldehyde

Declarations

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Competing Interests The authors declare that they have no conflict of interest.

Author Contributions Qiang Wang and Shuang Li designed experiments and developed methodologies; Na Li, Hui Gao and Jing Xu performed the research and wrote the manuscript; Yuxin Zhang and Haiqing Chang analyzed and interpreted data; Qiang Wang, Jing Xu and Shuwen Tan revised the manuscript.

Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval All procedures performed in studies involving animals were approved by the ethical standards of Institutional Animal Care and Use Committees of Xi'an Jiaotong University, Xi'an, China. Mice received humane care following the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

Consent to participate Not applicable.

Consent for publication Not applicable

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Figures

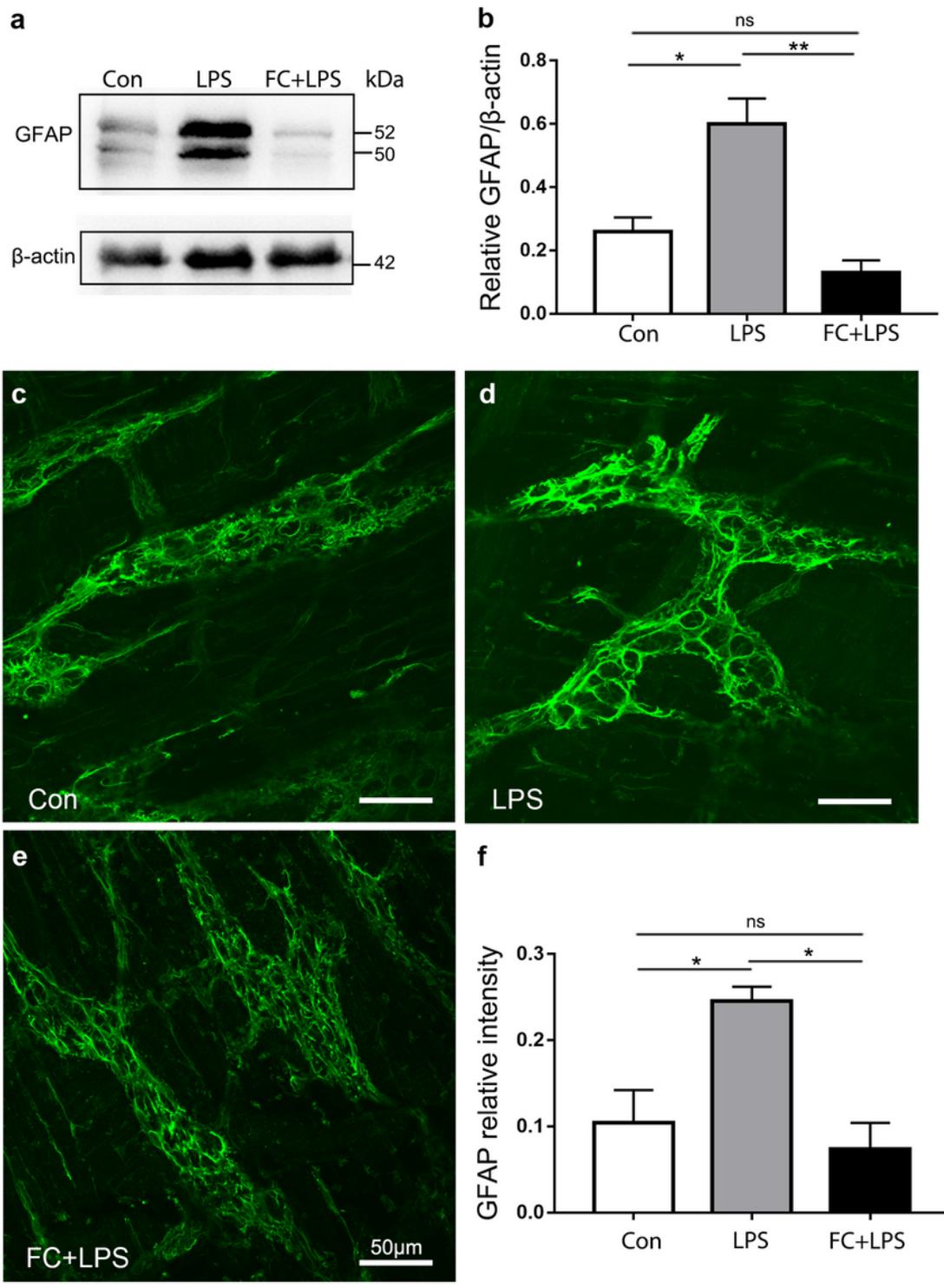


Figure 1

Effects of FC on EGCs activation in septic mice. (a-b) Western blot showed that the protein expression of GFAP in colon tissue increased after the LPS injection whereas it was reversed by FC treatment. (c-f) Immunostaining in the colon myenteric plexus showed that the GFAP fluorescence intensity increased in the LPS group relative to the control group, however, it was reduced in the LPS plus FC group compared

with the LPS group. One-way ANOVA, $n = 3$ mice per group, * $P < 0.05$, ** $P < 0.01$, ns, no significant difference.

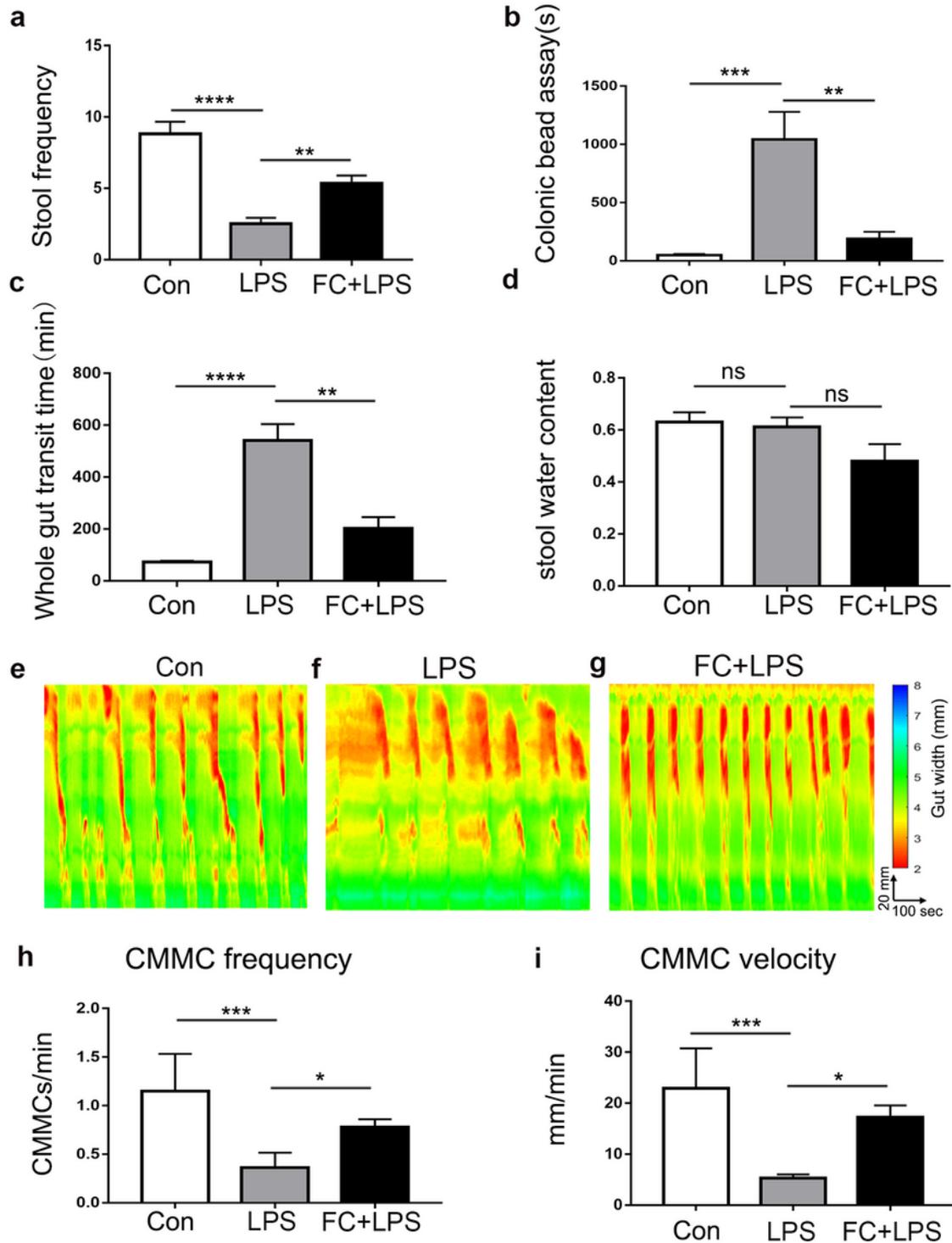


Figure 2

Effects of inhibiting reactive EGCs on intestinal motility in septic mice. The intestinal motility decreased at 48 hrs after the LPS injection and partly reversed back to normal under the condition of EGC ablation.

(a) Stool frequency, (b) Colonic bead assay(s), (c) Whole gut transit time, (d) Stool water content. (e-g) Spatiotemporal maps illustrating CMMCs recorded for control, LPS injection, and FC treatment. (h-j) LPS reduced CMMC frequency and velocity in the colon, while they were significantly improved by FC treatment. One-way ANOVA, $n = 6$ mice per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, no significant difference.

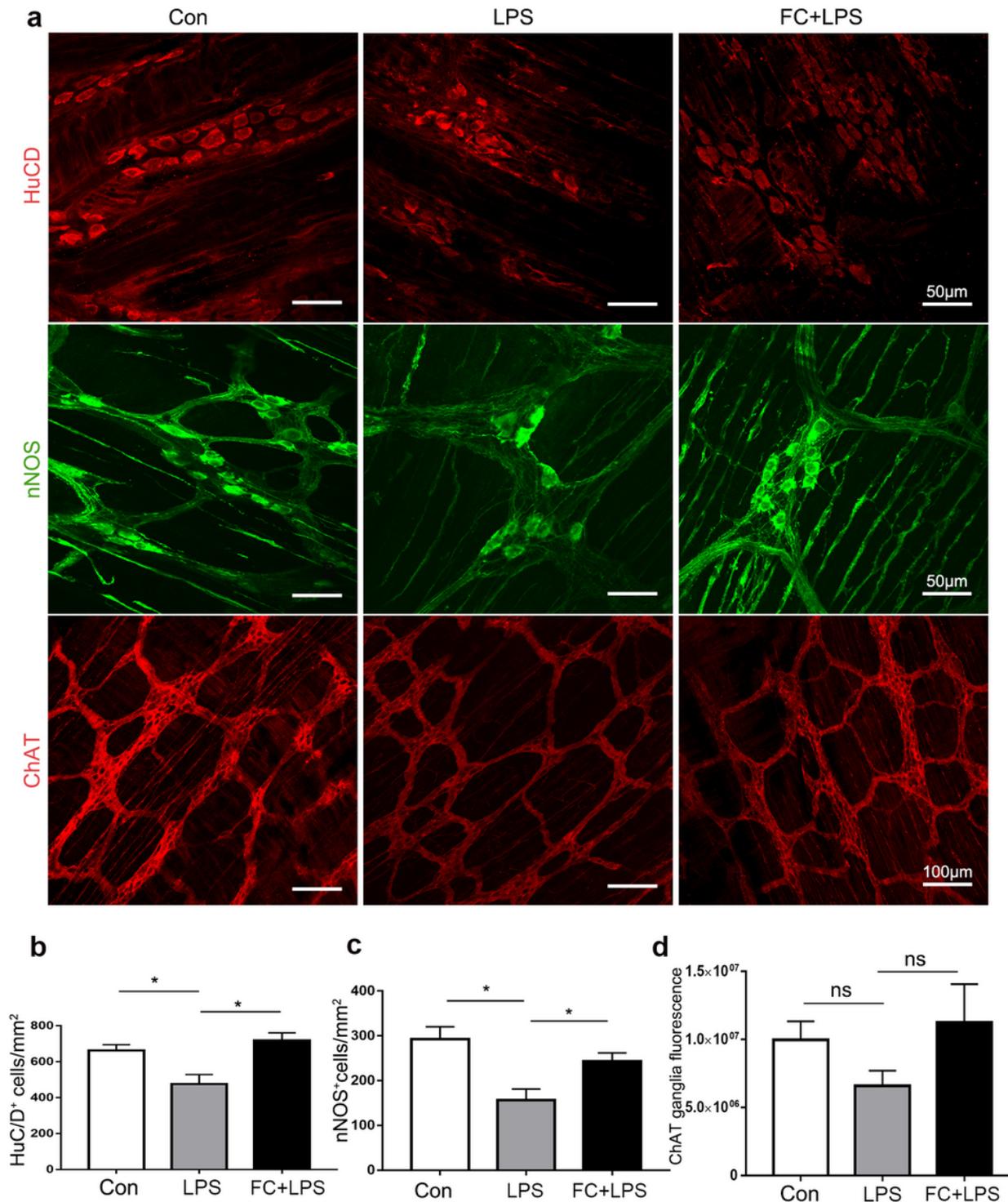


Figure 3

Effects of inhibiting reactive EGCs on the number of neurons in septic mice. (a) Representative images of immunofluorescence staining in the colonic myenteric plexus under different treatments. The number of the general neuronal population (HuC/D-immunoreactive) (b) and nitrenergic neurons (nNOS-immunoreactive) (c) decreased at 48 hrs after the LPS injection, and was restored to normal after FC treatment. Myenteric ganglia fluorescence intensity (CTCF) of ChAT (d) showed no significant difference among these groups. One-way ANOVA, $n = 3$ mice per group, * $P < 0.05$, ns, no significant difference.

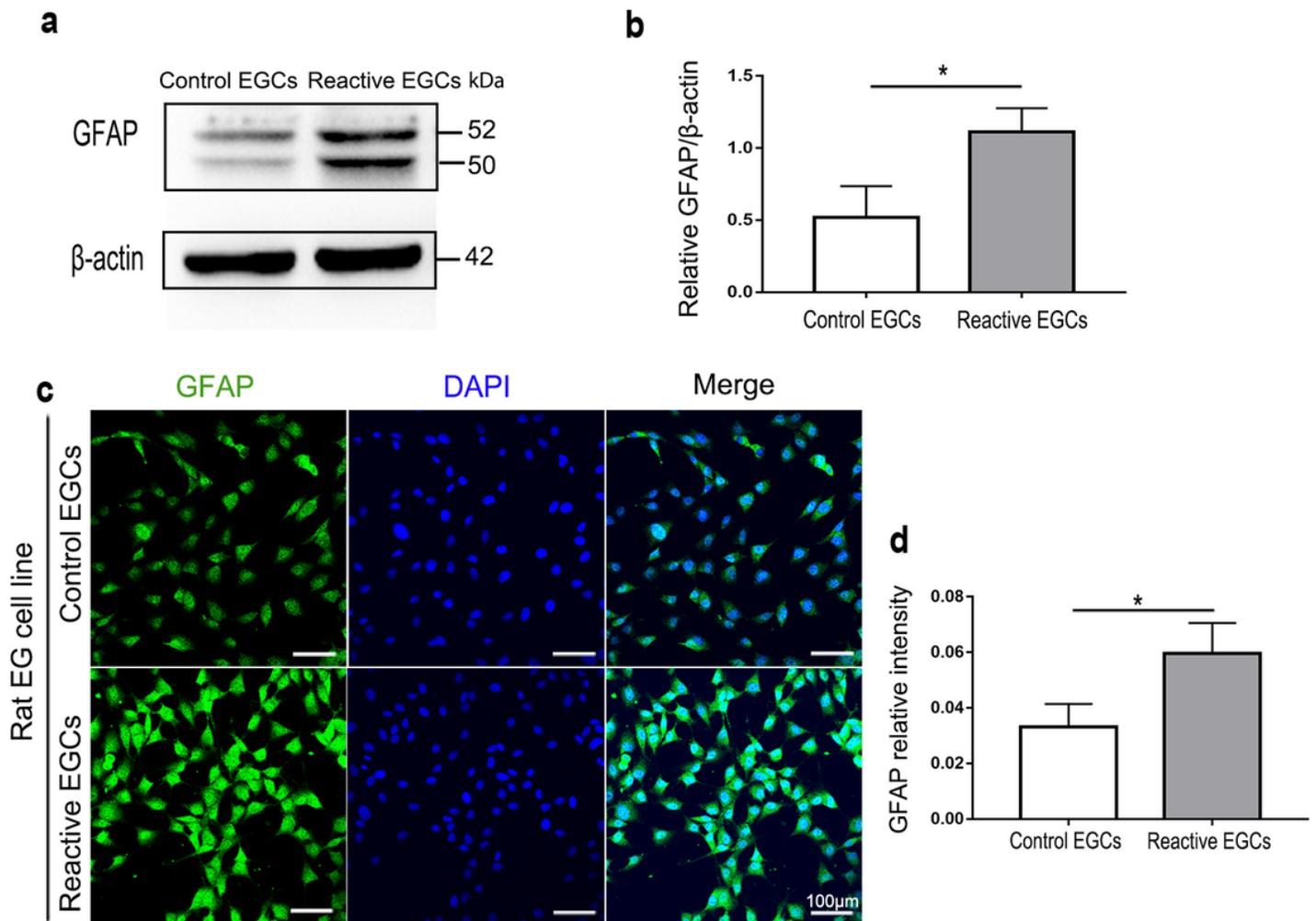


Figure 4

Reactive EGCs were induced by IL-1 β and TNF- α in vitro. (a-b) Western blot showed that GFAP expression of enteric glial cell line increased under IL-1 β /TNF- α treatment. (c-d) Immunostaining showed higher GFAP fluorescence intensity of EGCs with L-1 β /TNF- α treatment relative to the control group. Student's t -test, $n = 3$ biological replications, * $P < 0.05$.

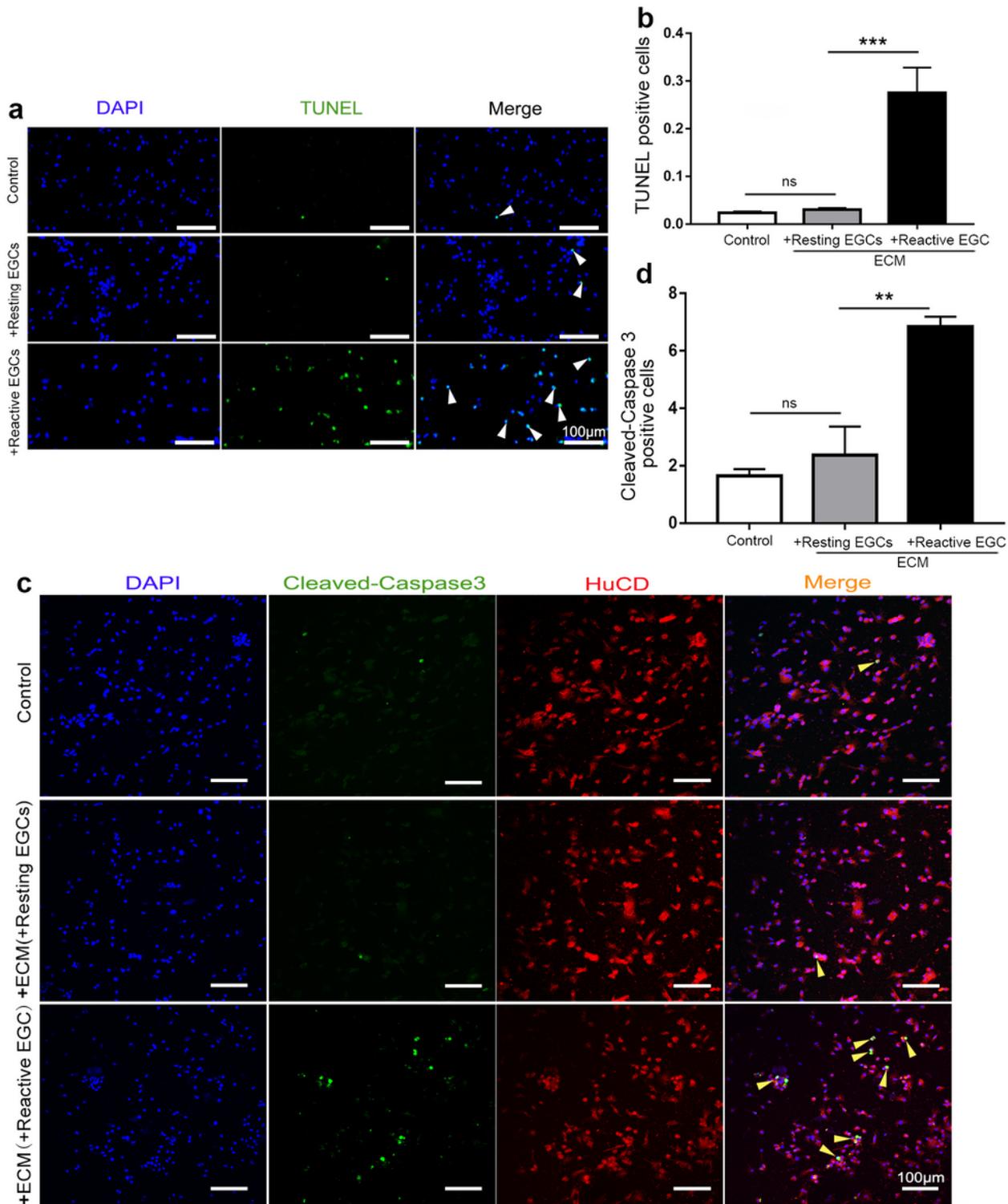


Figure 5

Effects of reactive EGCs on the apoptosis of primary enteric neurons. (a) The representative TUNEL staining images (the white arrows in a) showed that apoptotic cells in cultured enteric neurons with blank control (Neurobasal medium), the conditioned medium of resting EGCs, and the conditioned medium of reactive EGCs. (b) The TUNEL positive cells percentage increased at 48 hrs after cultured with the conditioned medium of reactive EGCs, compared with neurons incubated with the conditioned medium of

the resting EGCs. (c-d) Fluorescence staining for the cleaved form of caspase-3 in primary enteric neurons (the yellow arrows in c) showed that more positive apoptotic neurons were observed in the conditioned medium of the reactive EGCs group than in the conditioned medium of the resting EGCs. One-way ANOVA, $n = 3$ biological replications, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, no significant difference. Control, blank control (Neurobasal medium); +ECM(+resting EGCs), the conditioned medium of resting EGCs; +ECM(+reactive EGCs), the conditioned medium of reactive EGCs.

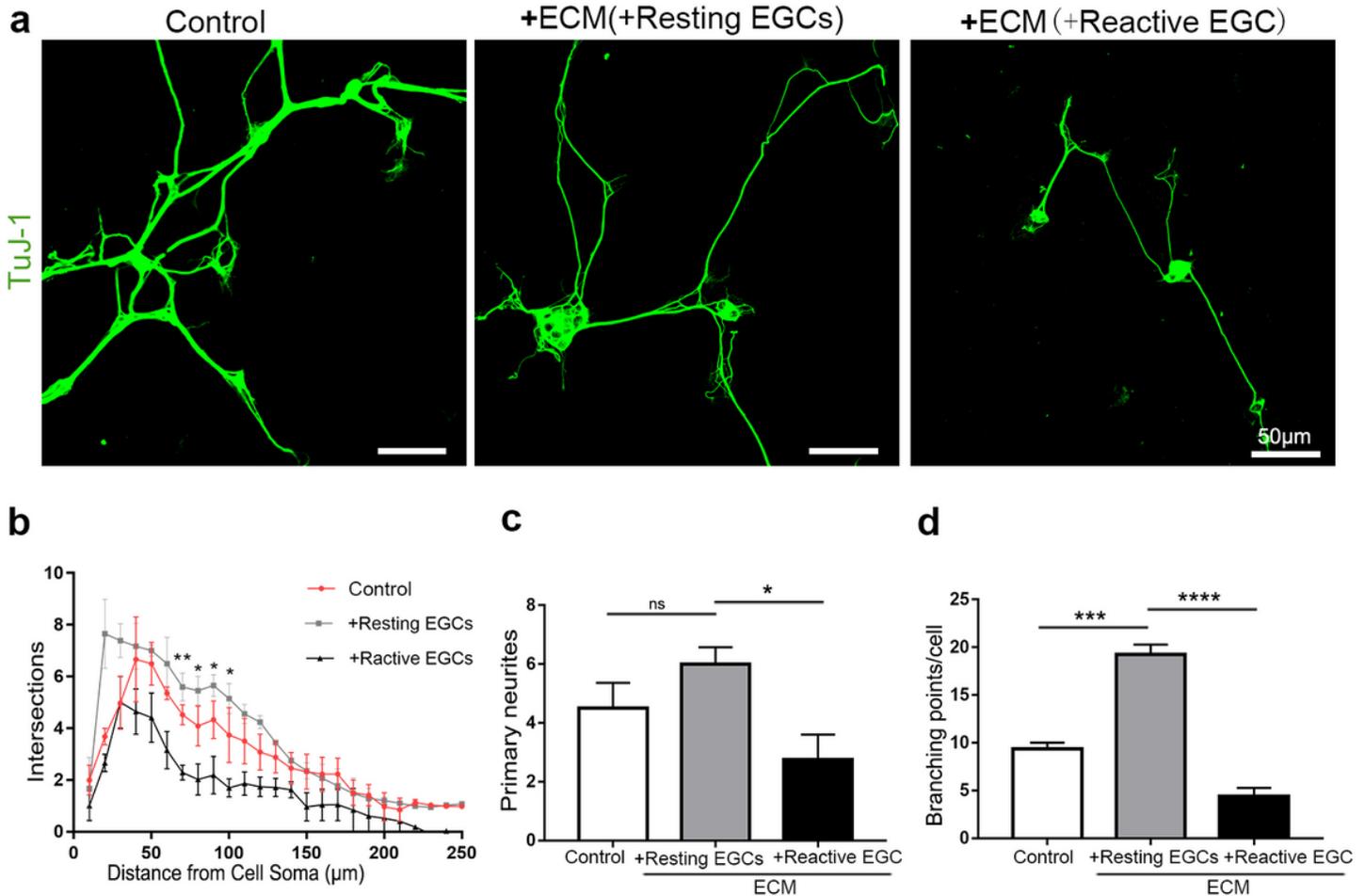


Figure 6

Effects of reactive EGCs on neurites formation in primary enteric neurons. (a) The representative immunofluorescence images of TuJ-1 to present the morphology of cultured enteric neurons under different treatments. (b) Sholl analysis showed that the dendritic complexity of primary enteric neurons was reduced in cultured with the conditioned medium of reactive EGCs, compared with neurons incubated with the conditioned medium of the resting EGCs. The number of primary neurites (c) and branching points (d) decreased in the medium of the reactive EGCs group relative to the group adding the conditioned medium of the resting EGCs. One-way ANOVA, $n = 3$ biological replications, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, ns, no significant difference. Control, blank control (Neurobasal medium); +ECM(+resting EGCs), the conditioned medium of resting EGCs; +ECM(+reactive EGCs), the conditioned medium of reactive EGCs.

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