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Title: Resveratrol suppressed microglia activation and promoted functional recovery of traumatic spinal cord via improving intestinal microbiota

Running Title: Resveratrol improves intestinal dysbiosis in SCI

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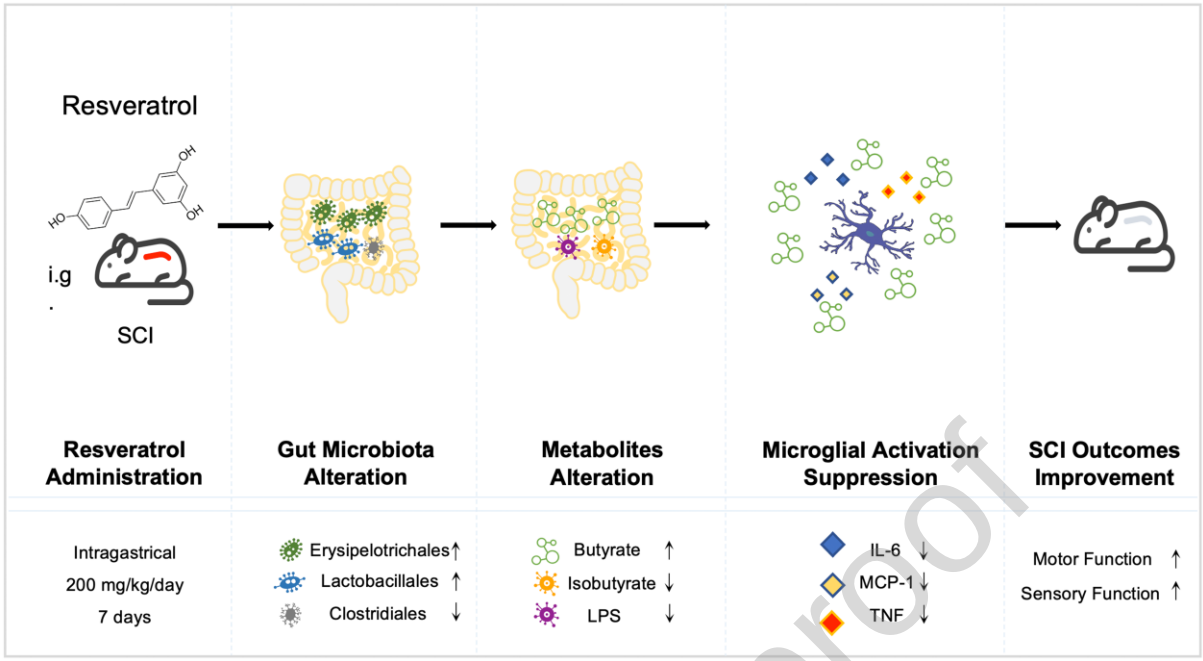
Resveratrol suppressed microglia activation and promoted functional recovery of traumatic spinal cord via improving intestinal microbiota

ABSTRACT:

Spinal cord injury (SCI) can change the intestinal microbiota pattern and modify corresponding metabolites, which in turn affect the prognosis of SCI. Among many metabolites, short-chain fatty acids (SCFAs) are critical for neurological recovery after SCI. Recent research showed that resveratrol exerts anti-inflammatory properties. But it is unknown if the anti-inflammatory properties of resveratrol are associated with intestinal microbiota and metabolites. Here, we thus investigated the alteration in gut microbiota and determined the consequent change of SCFAs following resveratrol treatment. The SCI mouse models with retention of gut microbiota (donor) and depletion of gut microbiota (recipient) were established. Fecal microbiota transplantation from donors to recipients was performed with intragastrical administration. Spinal cord tissues of mice were examined by H&E, Nissl, and immunofluorescence staining. The expressions of the inflammatory profile were examined by qPCR and cytometric bead array. Fecal samples of mice were collected and sent to 16S rRNA sequencing. The results showed that resveratrol inhibited the microglial activation and promoted the functional recovery of SCI. The analysis of intestinal microbiota and metabolite indicated that SCI caused dysbiosis and the decrease in butyrate, while resveratrol restored microbiota pattern, reversed intestinal dysbiosis, and increased the concentration of butyrate. Both fecal supernatants from resveratrol-treated donors and separate butyrate suppressed the expression of proinflammation genes in BV2 microglia. Our result demonstrated that fecal microbiota transplantation from resveratrol-treated donors had beneficial effects on the functional recovery of SCI. One mechanism of resveratrol effects was to restore the disrupted gut microbiota and butyrate to normal patterns.

Graphical abstract



Keywords:

Resveratrol; microbiota; fecal supernatant; fecal microbiota transplantation; SCFAs; spinal cord injury

Chemical compounds studied in the article

resveratrol (PubChem CID: 445154)

butyric acid (PubChem CID: 264)

isobutyric acid (PubChem CID: 6590)

lipopolysaccharide (PubChem CID: 11970143)

sodium butyrate (PubChem CID: 5222465)

ampicillin (PubChem CID: 6249)

neomycin (PubChem CID: 8378)

metronidazole (PubChem CID: 4173)

Abbreviations:

SCI: Spinal cord injury; SCFAs: Short-chain fatty acids; BCFAs: Branched-chain fatty acids; Res: Resveratrol; H&E: Hematoxylin-eosin; qPCR: Quantitative real-time polymerase chain reaction; CBA: Cytometric bead array; IBA: Ionized calcium binding adapter molecule; iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; ELISA: Enzyme linked immunosorbent assay; UPLC: Ultra-performance liquid chromatography; GC: Gas chromatography; FMT: Fecal microbiota transplantation; BBB: Blood-brain barrier; BMS: Basso mouse scale; PWMT: Paw withdrawal mechanical threshold; OTUs: Operational taxonomic units; FBS: Fetal bovine serum; TNF: Tumor necrosis factor; IL: Interleukin; MCP: Monocyte chemotactic protein; LBP: LPS-binding protein; GFAP: Glial fibrillary acidic protein; RT: Room temperature; DAPI: 4', 6-diamidino-2-phenylindole; ANOVA: Analysis of variance; IFN: Interferon; NMDS: Nonmetric multidimensional scaling; LDA: Linear discriminant analysis; LEfSe: LDA effect size; MFI: Mean fluorescence intensity.

1. Introduction

Spinal cord injury (SCI) is the main reason for the disability and death of traumatic diseases in the central nervous system [1]. Recently, survival rates in SCI patients have been promoted, yet these patients still suffer from extremely low quality of life and are more likely to die. Consequently, it burdens society with high socioeconomic costs due to labor loss [2-4]. In the pathophysiologic process, the pro-inflammatory response induced by microglia is mainly associated with the prognosis of SCI [5-7]. It was found that suppressing the microglial activation after SCI results in a better prognosis in mice [8, 9]. Besides, many previous studies on SCI have focused on inhibiting the microglial activation by gene regulation [10], pharmacological intervention [6], and cell therapies [3, 4, 11]. In recent years, it was surprisingly found that intestinal microbiota can affect the microglial activation [12]. Much attention thus has been paid to regulating intestinal microbiota to mediate the microglial activation.

The gastrointestinal tract contains many microorganisms, including beneficial and maleficent bacteria, thus forming an extremely complicated network. This network has a close relationship with the host immune system: intestinal immunity and intrinsic microbiota interact with numerous organs such as the spinal cord, maintaining a dynamic balance [13]. Previously, it was acknowledged that the symbiotic microbiota, closely correlated with the development of the nervous system [14], is involved in neurogenesis [15], myelination [15], microglia maturation [16], the maintenance of the blood-brain barrier (BBB) [15], neuronal self-renewal, and differentiation signals [15]. Recently, it was found that intestinal dysbiosis and gut diseases are pivotal in many neurological diseases, such as stroke [17], autism [18], multiple sclerosis [19], and amyotrophic lateral sclerosis [20]. Some studies also showed that intestinal flora imbalance is involved in the pathophysiological process of SCI [21, 22]. Metabolites of intestinal microbiota, including lipopolysaccharide and short-chain fatty acids, are believed to be related to SCI prognosis [12, 22, 23]. These components are supposed to mediate the immunoinflammatory response after SCI. Therefore, the application of regulating intestinal microbiota in the study of acute inflammation after SCI has become a research hotspot in recent years [24, 25].

Resveratrol, a natural polyphenol, is particularly high in red wine and grape [26]. Resveratrol has the properties of antioxidation [27], anti-inflammation [28], anti-bacteria [29], and anti-neurodegeneration [30]. More importantly, innate and adaptive immunity *in vivo* are regulated and modified by resveratrol [31]. Its anti-inflammatory effects are based on the capacity to clean reactive oxygen species [32], inhibit cyclooxygenase [33], and activate many anti-inflammation pathways [34]. It was interestingly found that resveratrol has protective effects on the ischemia-reperfusion injury and has an antioxidant effect on the spinal cord [35]. Previous studies reported that resveratrol significantly suppressed the microglial activation

[36, 37]. Furthermore, many researchers found that intestinal microbiota might possibly be involved in the prognosis of various diseases in resveratrol treatment [38, 39]. In this involvement of intestinal microbiota, SCFAs might play an important part to mediate the resveratrol-induced protective effects [40]. Since the effect of resveratrol on intestinal microbiota is completely different in various pathological background, its effect on intestinal microbiota composition and corresponding metabolites may be quite diverse.

Until now, no evidence has shown that resveratrol can ameliorate the prognosis of SCI through gut microbiota and their metabolites. Furthermore, whether resveratrol-treated microbiota and corresponding metabolites suppress the microglial activation after SCI is unknown. Therefore, we have explored these questions in this study. We hypothesize that resveratrol inhibits the inflammatory response by suppressing the microglial activation in the model of SCI. We assumed that the anti-inflammatory effects of resveratrol might be mediated by intestinal microbiota and corresponding metabolites. For this exploration, 16S rRNA sequencing and SCFAs detection were performed in SCI mice with and without resveratrol treatment. Lastly, we performed fecal microbiota transplantation and fecal supernatant transferring to examine the effects of microbiota-mediated anti-inflammation after resveratrol treatment in SCI mice.

2. Methods

2.1. SCI modeling and drug administration

All experiments referring to animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee and approved by the Ethics Committee of Zhejiang University (Permit No. ZJU20210183). Female C57BL/6 mice (20~25g, 8~12 weeks, specific pathogen-free) were purchased from the Laboratory Animal Center at

Zhejiang University. The Sterile Experimental Animal Feed (Slacom, Cat#P1101F) was used and ad libitum in our pre-experiments and formal animal experiments, comprising water ≤ 10%, crude protein ≥ 20.5%, crude fat ≥ 4%, crude fiber ≤ 5%, crude ash ≤ 8%, calcium 1~8%, phosphorus 0.6~1.2%, lysine ≤ 1.32%, methionine+cystine 0.78%, and sodium chloride 0.4%. All experimental mice were divided into Sham, SCI+Veh, and SCI+Res groups. Mice were housed in a 12h/12h light-dark cycle, around 24~27˚C and 55~60% humidity. The SCI model was performed in accordance with a previous method [41]. Mice were anesthetized with 2% sevoflurane and 40% oxygen (RWD, Cat#R640). A 5g weight was dropped from 25 mm height, resulting in tail reflex and local congestion of the spinal cord. Mice in the Sham group were treated merely with laminectomy. Bladders were carefully voided twice per day for 14 days. Resveratrol (Sigma, Cat#V900386) was dissolved in PBS to prepare a 20 mg/ml suspension. Referencing previous reports and our preliminary experiments, we modified the concentration and the treatment duration for resveratrol in our study [42, 43]. Resveratrol was administered intragastrically at 200 mg/kg/day for 14 days for the SCI+Res group. PBS was administered as the control vehicle with the same volume for the SCI+Veh group. Bladders were carefully voided twice per day until the autonomic rhythm of the neurogenic bladder resumed.

2.2. Basso mouse scale

The standard of Basso mouse scale (BMS) scores followed our previous study [44]. The motor ability of the rear limb was evaluated by BMS from 0 (paralysis) to 9 (health) at 1, 3, 7, 14, 21, 28, and 35 dpi. Sub-BMS from 0 (paralysis) to 11 (health) were also used to access mice simultaneously. Mice were placed on a flat floor and adapted to the environment. Mice were free to walk, and their movement was recorded. BMS and Sub-BMS scores were evaluated by 2 trained observers blinded to experimental conditions.

2.3. Footprint analysis

The method of footprint analysis was performed after SCI at 35 dpi as in a previous study [45]. Footprint analysis as another motor ability index was used as well to assess the motor coordination precisely. Fore and rear limbs were dyed with blue and red inks, respectively. Then mice were placed on the channel floored with white paper and allowed to pass through. The stride length of ipsilateral limbs in the middle parts was counted in. The shorter interval of ipsilateral limbs indicated a better recovery of motor coordination.

2.4. Pressure pain threshold

The standard of pressure pain threshold was conducted after SCI at 35 dpi following our previous study [44]. Pressure pain threshold was used, with Von Frey Hairs (RWD, CAT#Aesthesio), to evaluate paw withdrawal mechanical thresholds (PWMT) that indicate the pain sensitivity of the sensory function. Mice were placed on a metal net for 10 min. Von Frey Hairs with various forces were used to stimulate their rear paw, starting from 0.4 g. The paw withdrawal response caused by Von Frey Hairs with specific bending strength was observed and recorded. At least 5 times were required in each force until no reactions appeared.

2.5. Heat pain threshold

The standard of heat pain threshold was conducted after SCI at 35 dpi following our previous study [44]. Heat pain threshold as another index for sensory functions was employed to appraise the tail flick latency of mice. Mice were placed in the cylinder box with holes through which their tails could pass. The mice were calmed for 3~5 min, then 1/3 tail was put into 49 °C water. The time mice started to flick their tail was recorded.

2.6. 16S rRNA sequencing of intestinal microbiota

Fecal pellets in Sham, SCI+Veh, and SCI+Res groups were collected at 35 dpi and stored in -80˚C. All fecal samples were transported to Wuhan Benagen Tech Solutions Company Limited. After extracting the total DNA of samples, specific primers with Barcodes were synthesized according to the full-length primer sequence. The polymerase chain reaction (PCR) amplification was then performed. The products were purified, quantified, and homogenized to form a sequencing library. Qualified libraries were sequenced with PacBio Sequel. The Usearch software was used to cluster Reads at a 97% similarity level to obtain operational taxonomic units (OTUs). The complexity of Order and Genus and difference analysis were analyzed based on OTUs and species annotations.

2.7. Determination of the contents of SCFAs and BCFAs

Fecal pellets in Sham, SCI+Veh, and SCI+Res groups were collected at 7 dpi and stored in -80˚C. The contents of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) in feces were determined by ultra-performance liquid chromatography (UPLC) I-Class, Waters XEVO TQS-Micro, with calculating curves of standards as in a previous study [12]. Chromatographic conditions for fecal samples were as follows: chromatographic column -- Waters UPLC BEH C8 (2.1 mm × 100 mm, particle size 1.7 μm); mobile phase -- A phase (water and 0.01% formic acid), B phase (methanol: isopropyl alcohol = 8:2); flow rate -- 0.3 mL/min; injection volume -- 5 μl; column temperature -- 45 ˚C. Mass spectrometry conditions for fecal samples were as follows: ion source voltage -- 3 kV; temperature -- 150 ˚C; solvation temperature -- 350 ˚C; solvated gas flow rate -- 1000 L/h; cone-hole gas flow rate -- 10 L/h.

The contents of SCFAs in blood were determined by gas chromatography (GC) Trace 1310, Thermo ISQ LT, with calculating curves of standards as a previous study [46]. Chromatographic conditions for blood samples were as follows: chromatographic column -- Agilent HP-InnoWAX capillary column (30 m × 0.25 mm × 0.25 μm); split injection, shunt injection volume -- 1 μl and shunt ratio -- 10:1; injection port temperature -- 250˚C; ion source temperature -- 300˚C; transmission line temperature -- 250˚C; initial temperature -- 90˚C, then heated up to 120˚C at 10˚C/min, continually heated up to 150˚C at 5˚C/min, finally heated up to 250˚C at 25˚C/min and lasting for 2 min; carrier gas - helium; carrier gas flow rate -- 1.0 mL/min.

2.8. Fecal microbiota collection and transplantation

Fecal microbiota transplantation (FMT) was performed in accordance with a previous method [47]. Mice were divided into 4 groups, SCI+Veh (donor), SCI+Res (donor), SCI+FV (recipient), and SCI+FR (recipient) groups. Before SCI modeling, recipient groups, including SCI+FV (receiving FMT from the donor SCI+Veh group) and SCI+FR (receiving FMT from the donor SCI+Res group) groups, received 200 μl antibiotic cocktails (ampicillin, neomycin, and metronidazole, 1 mg/ml; and vancomycin, 0.5 mg/ml. Sangon, Cat#A610028, A610366, A600633, and A600983, respectively) via oral gavage per day for 3 weeks to remove their intrinsic gut microbiota. Fecal pellets from SCI-Veh and SCI-Res groups were collected and stored in -80˚C. 100 mg fecal pellets were mixed and suspended in 600 μl sterile PBS. The suspensions of fecal pellets were then smashed, vortexed for 30 s, and filtered through 5-layer sterile gauzes. Each recipient mouse in SCI-FV and SCI-FR groups was intragastrically administered with 200 μl fecal filtrate containing gut microbiota and corresponding

metabolites once a day. FMT treatment was prepared and performed within 2 h after fecal pellets were collected. FMT treatment continued for 7 days until behavioral tests.

2.9. Fecal supernatants collection and transferring

Fecal supernatants were collected following a previous study [48]. 100 mg fecal pellets from SCI+Veh (donor) and SCI+Res (donor) groups after SCI at 7 dpi were mixed, suspended in 600 μl sterile PBS, smashed, and vortexed for 30 s. And then 0.22 μm filter was used to remove all bacteria in suspensions so that fecal supernatants contain only gut microbiota metabolites for the *in vitro* BV2 microglia experiment. BV2 microglia were divided into 4 groups, Ctrl+SV, Ctrl+SR, LPS+SV, and LPS+SR groups. The fecal supernatant from the donor SCI+Veh mice was transferred to Ctrl+SV and LPS+SV groups, respectively; While the fecal supernatant from the donor SCI+Res mice was transferred to Ctrl+SR and LPS+SR groups, respectively.

2.10. Cell culture

Mouse BV2 microglia were purchased from the National Collection of Authenticated Cell Cultures. BV2 microglia were cultured in DMEM-HG medium supplemented with 10% fetal bovine serum (FBS) (Sigma, Cat#F8318) and 1% penicillin/streptomycin (Gibco, Cat#10378016). When their confluency reached 70~80%, fecal supernatant (3 v/v‰) or butyrate (200 μM) (Sigma, Cat#V900464) was added to the medium for 24 h followed by lipopolysaccharide (LPS, 100 ng/ml) (Sigma, Cat#L5293) stimulation for another 24 h. These treated cells were collected for qPCR and immunofluorescence.

Mouse MODE-K epithelial cells were also purchased from the National Collection of Authenticated Cell Cultures. MODE-K epithelial cells were cultured in RPMI-1640 medium

supplemented with 10% FBS and 1% penicillin/streptomycin. When their confluency reached 70~80%, resveratrol (50 μM) (Sigma, Cat#R5010) was added to the medium for 24 h followed by LPS (100 ng/ml) stimulation for another 24 h. These treated cells were collected for qPCR.

2.11. Real-time quantitative polymerase chain reaction

Quantitative real-time PCR (qPCR) was performed using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Cat#RR047A) and TB Green Premix Ex Taq II (Takara, Cat#RR820A), respectively. In reverse transcription PCR, the total RNA from BV2 microglia, MODE-K epithelial cells, and small intestine tissues was extracted with RNAiso Plus (Takara, Cat#9109) and regulated to 1 μg as the template. cDNA was then synthesized and regulated to 100 ng for qPCR. The standard procedure for two-step PCR amplification was the same as the instruction of TB Green Premix Ex Taq II. The process was performed using Roche LightCycler 480II System. The quantity of the target gene was normalized to *Actin* or *Gapdh* and calculated by the ΔΔCT method. Target primers are shown in Table 1.

Table 1. Primers for qPCR in cells and small intestine tissues.

Primers	Forward (5'- 3')	Reverse (5'- 3')
Tnf	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT
Il6	CACTTCACAAGTCGGAGGCT	CTGCAAGTGCATCATCGTTGT
Inos	GGAGTGACGGCAAACATGACT	TAGCCAGCGTACCGGATGA
Mct1	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT
Smct1	AAGACAATCAGGCCATCAGCA	TGGACCTGTGGGTTGTTGAC
Gpr41	CACTTCACAAGTCGGAGGCT	CTGCAAGTGCATCATCGTTGT
Gpr43	ACCTGGTAGAAGTGATGCCC	CCACTGCCTTGCTCTTATTTT
Gpr109a	CAGGTGTCCCAAAGAAGCTGTA	CATTTGGTTCCGATCCAGG
Actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA

Gapdh	TGGCCTTCCGTGTTCCTAC	GAGTTGCTGTTGAAGTCGCA

2.12. Enzyme-linked immunosorbent assay

Endotoxin metabolite level of LPS were detected by mouse lipopolysaccharide-binding protein (LBP) enzyme-linked immunosorbent assay kit (ELISA) (Nanjing Jiancheng Bioengineering Institute, Cat#H253) with instructions attached. Blood samples were collected in sterile tubes from orbital veins of Sham, SCI+Veh, and SCI+Res groups. All blood samples stood for 2~4 h at room temperature (RT) for coagulation and were centrifugated at 4000 g for 10 min. Serum supernatants were carefully collected and stored at -80 ˚C. Colon samples were cut into small pieces and put in tubes with WB&IP lysis buffer (Beyotime, Cat#P0013) on ice for 30 min, followed by centrifuging at 13000 g for 5 min. Colon supernatants were collected and stored at -80˚C. As for ELISA, the procedure was in accordance with the instruction. Bio-antigen was added to 50 μl serum or colon supernatants in the antibody-precoated well, incubating for 30 min at 37˚C. HRP-conjugated avidin was added, incubating for 30 min at 37˚C. Lastly, all samples were reacted with the chromogenic solution and stop solution, followed by the detection of 450 nm OD values within 10 min. Experimental results were analyzed using CurveExpert v1.4.

2.13. H&E and Nissl staining

Hematoxylin-eosin (H&E) and Nissl staining were performed in accordance with our previous method [44]. All spinal cord samples were fixed in formaldehyde for 24 h at RT and dehydrated by 70%, 80%, 90%, and 95% ethanol in sequence, followed by an embedment in paraffin. Paraffin-embedded spinal cords were sectioned into 4 μm thick slices. Hematoxylin/Eosin solutions and cresyl violet solution were used for staining. Olympus VS200 Slide Scanner and Olympus OlyVIA v3.2.1 were used for photographing. The lesion

area for H&E staining and the positive area for Nissl staining were measured and analyzed by ImageJ v2.1.0.

2.14. Immunofluorescence

Immunofluorescence was performed in accordance with a previous method [41]. Samples of spinal cords from Sham, SCI+Veh, SCI+Res, SCI+FV, and SCI+FR groups at 7 dpi were extracted, then fixed in 4% formaldehyde for 24 h at RT and dehydrated by 15% and 30% sucrose in sequence, followed by an embedment in Sakura O.C.T. Compound. Spinal cord tissues were sectioned into 8 μm thick slices. After serum blocking, spinal cord slices were incubated with GFAP (Boster, Cat#M00213-8) and IBA-1 (Abcam, Cat#ab178846), then incubated with corresponding secondary antibodies and 4', 6-diamidino-2-phenylindole (DAPI).

As for BV2 microglia, samples were fixed in 4% formaldehyde for 15 min at RT and then blocked with serum for 1 h at RT. BV2 microglia were incubated with IBA-1 (Abcam, Cat#ab178846) and iNOS (Abcam, Cat#ab49999), then incubated with corresponding secondary antibodies and DAPI.

Olympus VS200 Slide Scanner and Olympus OlyVIA v3.2.1 were used for photographing and processing. Samples were analyzed by ImageJ v2.1.0.

2.15. Determination of the contents of inflammatory cytokines

Cytometric bead array (CBA) Mouse Inflammation Kit (BD, Cat#552364) was used as described in a previous study [49] to detect the concentrations of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 in spinal cord tissues. All the related reagents, the flow cytometry

protocol, and the result analysis were implemented according to kit instructions. The standards of inflammatory cytokines were prepared for the manufacturing standard curve. The capture beads of above 6 inflammatory cytokines were mixed rigorously to prepare the mixture of capture beads before 50 μl of the mixture were added to each sample and standard, closely followed by adding 50 μl of PE detection reagent. All the array tubes were incubated for 2 h at RT protected from light. All the array tubes were rinsed once with wash buffer, centrifuged, and resuspended before flow cytometry analysis. BD LSR Fortessa Multicolor Flow Cytometer was used for examination. Experimental results were analyzed using FCAP Array v3.1, FlowJo v10.4, and CurveExpert v1.4.

2.16. Statistical analyses

All data in the study are presented as mean ± SEM. Student's *t*-test, one-way ANOVA, and two-way ANOVA were used to compare multiple groups. 16S rRNA sequencing analysis of relative abundance in gut microbiota was performed using Tukey's HSD test via the R package. Pearson correlation coefficient was used to analyze the correlation between differential bacteria and behavioral tests. Clustering correlation heatmap with signs was performed using online R package https://www.omicstudio.cn. we also used online R package http://www.bioinformatics.com.cn/en to analyze the correlation between IBA-1 positive area/positive number and butyrate/isobutyrate. Statistical significance was considered as $p<0.05$.

3. Results

3.1. Resveratrol improves the neurological functions after SCI

To evaluate the action of resveratrol on the recovery of neurological functions after SCI, we divided the experimental mice into 3 different groups (Sham, SCI+Veh, and SCI+Res). BMS,

Sub-BMS, footprint analysis, pressure pain threshold, and heat pain threshold were evaluated as shown in Fig. 1A. The SCI+Res group showed both higher BMS and Sub-BMS scores than the SCI+Veh group from 7 dpi (BMS 1.86 ±0.127 *vs* 1.27±0.136; Sub-BMS 1.00±0.14 *vs* 0.59±0.12) to 35 dpi (BMS 4.58±0.49 *vs* 3.50±0.48; Sub-BMS 4.14±0.70 *vs* 2.67±0.56) (Fig. 1B). This comparison suggested that resveratrol could enhance the recovery of motor function. Furthermore, footprint analysis, a test that evaluates the coordination of motor ability, showed that the SCI+Res group had better coordination with the lower stride length compared to the SCI+Veh group (1.11±0.05 *vs* 1.31±0.02) (Fig. 1C). For the recovery of sensory functions, as shown in Fig. 1D and 1E, the SCI+Res group showed higher PWMT and higher tail flick latency than the SCI+Veh group, which indicated that resveratrol could improve the pain sensitivity to a normal level. As shown in Fig. 1F and 1G, the lesion area in the H&E staining was significantly reduced in the SCI+Res group after SCI at 35 dpi. Furthermore, Nissl staining showed that SCI+Res mice had a higher neuronal survival. Collectively, the behavioral results, lesion area, and neuronal survival indicate that resveratrol significantly promoted the recovery of neurological functions after SCI at 35 dpi.

3.2. Resveratrol reduces the inflammatory response and suppresses the microglial activation after SCI

The occurrence of SCI is accompanied by a severe inflammatory response in the early phase. To examine the effects of resveratrol on the inflammatory response and the microglial activation after SCI in acute stage, we mainly chose 7 dpi as the time point of interest (Fig. 2A). Firstly, we tested the inflammation-related cytokine levels by CBA as shown in Fig. 2B. We found that IL-6, MCP-1, and TNF concentrations were strikingly reduced in the SCI+Res group compared to the SCI+Veh group at 3 and 7 dpi after SCI (Fig. 2C and 2D). In addition, resveratrol treatment also decreased the concentration of IFN-γ and IL-12p70 at 3 dpi after

SCI despite no significant differences (Fig. 2C). However, IL-10, a protective and anti-inflammatory cytokine in neuroinflammatory response, showed an opposite trend. IL-10 level was lower at 3 dpi but higher at 7 dpi in the SCI+Res group than the SCI+Veh group (Fig. 2C and 2D). To further see if these elevated inflammation-related cytokines in the spinal cord were related to the microglial activation after SCI, we examined the expression of IBA-1 by immunofluorescence in the spinal cord as shown in Fig. 2E. It was found that the SCI+Res group had fewer IBA-1 positive areas and positive cell numbers compared with the SCI+Veh group (Fig. 2F), which indicated that resveratrol strikingly inhibited the microglial activation after SCI at 7 dpi. As expected, these results indicated that resveratrol inhibited the microglia-induced inflammatory response in the acute stage after SCI.

3.3. Resveratrol alters the intestinal microbiota composition after SCI

Apart from the direct pharmacological actions of resveratrol, the possibility of alleviating inflammation in the spinal cord by changing intestinal microbiota has caught our attention because resveratrol and its corresponding metabolites were reported to hardly pass through BBB. To identify how the intestinal microbiota composition after SCI responds to resveratrol treatment after SCI, 16S rRNA sequencing was performed on intestinal microbiota in feces. Based on a 97% similarity threshold, the effective reads were clustered into 375 OTUs that included 130 species, 100 genus, 39 families, 20 orders, 14 classes, and 10 phyla. The species complexity and difference of bacteria between groups used species annotations and the above OTUs.

The Shannon index illustrating the α diversity showed no statistical difference between the SCI+Veh and the SCI+Res groups, although both groups have a higher α diversity than the Sham group (Fig. 3A). Consistent with the above result, the Venn diagram demonstrated that

the SCI+Veh and the SCI+Res groups acquired more differential OTUs than the Sham group (Fig. 3B), confirming that SCI elevated the diversity and richness of intestinal microbiome regardless of resveratrol treatment. To examine the extent of similarity of microbial communities, the nonmetric multidimensional scaling (NMDS) and sample heat map with Bray-Curtis analysis were then performed, both illustrating the β diversity. These data unveiled that the gut microbiota pattern was changed in the SCI+Veh group, and resveratrol could alter the microbiota composition, as shown in Fig. 3C and 3D. Further species distributions in order and genus levels were analyzed to compare the relative abundance of the dominant species. Compared to the Sham group, the SCI+Veh group showed distinct microbiota in the order level, in which there was an increase in the abundance of *Clostridiales* and a decrease in that of *Erysipelotrichales* after SCI. And these changes were reversed by resveratrol treatment (Fig. 3E and 3F). As for genus level, it was demonstrated that the SCI+Veh group increased the relative abundance of *uncultured_bacterium_f_Lachnospiraceae* and *Lachnospiraceae_NK4A136_group* and decreased that of *Dubosiella* and *Parasutterella*, and these changes were also restored in the SCI+Res group (Fig. 3E and 3F). Interestingly, the SCI+Res group increased the relative abundance of *Lactobacillales* and *Lactobacillus* (Fig. 3E and 3F), the recognized probiotic. These results showed that SCI could change the intestinal microbiota composition after SCI, while resveratrol could reverse this microbiota composition.

3.4. Altered microbiota composition is associated with the recovery of neurological functions after SCI

Based on the above apparent alteration in intestinal microbiota composition, we assumed that the differential species might have an association with behavioral tests after SCI. Therefore, we performed the ANOVA and the Pearson correlation analysis to resolve the relationship

between the bacterial species with significant differences and the neurological recoveries after SCI in mice. It was found that *Clostridiales*, *uncultured_bacterium_f_Lachnospiraceae*, and *Lachnospiraceae _NK4A136_group* were significantly negatively associated with the recovery of neurological functions after SCI. Meanwhile, *Erysipelotrichales*, *Dubosiella*, and *Parasutterella* showed a positive association with motor and sensory functions, as shown in whole Fig. 4. As a result, ANOVA and correlation analysis demonstrated that the altered intestinal microbiota composition by resveratrol treatment has positive correlations with the recovery of neuronal functions after SCI.

3.5. Altered microbiota composition reduces the infiltration of endotoxin metabolite after SCI

To see whether the altered microbiota composition can reduce LPS production by minimizing the proportion of LPS-producing gram-negative bacteria, we performed linear discriminant analysis (LDA) effect size (LEfSe, based on LDA score > 4 [significant effect]) and Cladogram (based on maximum relative abundance difference in each level) to find LPS-related microbiota biomarkers with a statistical difference (Fig. 5A and 5B). *Bacteroidetes*, *Bacteroidia*, *Bacteroidales*, *Desulfovibrionales*, *Desulfovibrionaceae*, *g_uncultured_bacterium_f_Desulfovibrionaceae*, and *s_uncultured_bacterium_f_Desulfovibrionaceae* were the LPS-producing bacteria with high impact (LDA score > 4) in the SCI+Veh group, while fewer high impact LPS-producing bacteria (*Enterobacterals* and *Enterobacteriaceae*) were in the SCI+Res group. LEfSe analysis confirmed the differences of the LPS-producing bacterial species between the SCI+Veh and the SCI+Res groups (Fig. 5A). In addition, Cladogram analysis further indicated that the abundance of *Bacteroidales*, *Desulfovibrionaceae*, *g_uncultured_bacterium_f_Desulfovibrionaceae*, and

s_uncultured_bacterium_f_Desulfovibrionaceae in the SCI+Veh group was significantly predominating (Fig. 5B).

To verify the effect of resveratrol on microbiota composition alteration, and subsequently on LPS production, we collect the colon tissue and blood samples to detect LBP (in line with the concentration of LPS) by ELISA. As shown in 5C and 5D, LPB levels showed no significant differences in colon and serum at 3 dpi albeit a noticeable trend in the colon. But in 7 dpi, the concentrations of LPB both in the colon and serum showed striking differences. Resveratrol significantly reduced LBP levels in the colon and serum after SCI. These results demonstrated that resveratrol could minimize the effect of LPS-producing bacteria and then reduce the infiltration of LPS into blood in the acute stage after SCI.

3.6. Altered microbiota composition elevates the content of butyrate after SCI

The profile of intestinal microbiota-derived metabolites is supposed to be altered to some extent when intestinal microbiota composition changes after SCI. Of all intestinal microbiota-derived metabolites, apart from LPS, SCFAs are the most important anti-inflammatory molecules [46]. To verify how the SCFA's profile responds to the altered microbiota composition by resveratrol treatment after SCI, we used UPLC to assess the concentrations of SCFAs in feces after SCI at 7 dpi. In the concentration of total SCFAs, no significant difference was found between the Sham, the SCI+Veh, and the SCI+Res groups (Fig. 6A). However, it was interesting to see that the SCI+Veh group (19.47±0.79 ng/mg) had lower butyrate concentration when compared to the Sham group (25.21±2.36 ng/mg), which was significantly restored by resveratrol treatment (24.63±1.53 ng/mg) at 7 dpi. Moreover, isobutyrate, one member of BCFAs, showed a contrary tendency. Resveratrol treatment lowered the content of isobutyrate (SCI+Veh *vs* SCI+Res: 2.41±0.17 ng/mg *vs* 1.77±0.14

ng/mg) (Fig. 6B). The other SCFAs, such as acetate, propionate, valerate, and caproate, demonstrated no significant differences in feces between the SCI+Veh and the SCI+Res groups (Fig. 6A).

Given that butyrate needs to be absorbed into the blood by intestinal epithelial cells by SCFA-related transporters, small intestine tissues *in vivo* at 7 dpi and MODE-K epithelial cells *in vitro* were prepared to detect the expressions of SCFA-related transporters and receptors by qPCR. The results showed that resveratrol treatment markedly enhanced the gene expressions of SCFA-related transports and receptors both *in vivo* (Fig. 6C) and *in vitro* (Fig. 6D).

After the high expressions of SCFA-related transporters with resveratrol treatment were confirmed, we then used GS and performed a target analysis for butyrate and isobutyrate concentrations in serum after SCI at 7 dpi. Similarly, it was found that in comparison with the SCI+Veh group (0.046±0.004 ng/μl), the content of butyrate was strikingly higher in the SCI+Res group (0.074±0.007 ng/μl), indicating that resveratrol treatment could significantly raise the concentration of butyrate in the blood after SCI (Fig. 6E). Yet the concentration of isobutyrate in both groups was undetected (data not shown). In general, these findings indicated that the altered intestinal microbiota composition by resveratrol after SCI further changed the SCFA's profile. Moreover, the resveratrol treatment increased the content of butyrate but did not significantly affect other subtypes of SCFAs in feces after SCI.

3.7. Altered microbiota-derived metabolites suppress the microglial activation

To explore the actions of post-SCI microbiota-derived metabolites on LPS-stimulated microglia, fecal supernatant (only metabolites retained) transferring was performed as shown in Fig. 7A. We transferred the fecal supernatant from the donor SCI+Veh mice to Ctrl+SV

and LPS+SV groups. We transferred the fecal supernatant from donor SCI+Res mice to Ctrl+SR and LPS+SR groups. And then, we examined the expression of pro-inflammatory genes in BV2 microglia by qPCR. As expected, the mRNA expressions of pro-inflammatory *Il6*, *Tnf*, and *Inos* were significantly downregulated in the LPS+SR group compared to the LPS+SV group. No significant difference was observed in the expressions of these pro-inflammatory genes between the Ctrl+SV and the Ctrl+SR groups, which were also treated with fecal supernatants from the SCI+Veh and the SCI+Res groups, respectively (Fig. 7B). Moreover, IBA-1 and iNOS immunofluorescence were also used to verify the microglial activation *in vitro*. It was found that the mean fluorescence intensity (MFI) of IBA-1 and iNOS were strikingly lower in the LPS+FR group than that in the LPS+FV group (Fig. 7C and 7D). No significant difference was found in the MFI of IBA-1 and iNOS between the Ctrl+SV and the Ctrl+SR groups.

Apart from fecal supernatant, single metabolite butyrate was used to treat BV2 microglia to see whether it has a similar inhibitory effect as fecal supernatant has on the microglial activation. As shown in Fig. 7E, qPCR results *in vitro* further confirmed that pro-inflammatory genes *Il6*, *Tnf*, and *Inos* were downregulated in the LPS+But group as compared to the LPS+Veh group. Yet no significant difference was observed in *Inos*. These data indicated that altered microbiota-derived metabolites by resveratrol inhibited the microglial activation *in vitro*. Furthermore, among all mixed metabolites in fecal supernatants, butyrate might play a major role in suppressing the microglial activation.

3.8. Altered microbiota promotes the recovery of neurological functions after SCI

To confirm the relationship of intestinal microbiota and resveratrol-mediated anti-inflammatory actions, we performed the FMT experiment as shown in Fig. 8A. As expected,

the SCI+FR group, receiving intestinal microbiota from the SCI+Res group, showed the favorable prognosis in behavioral tests compared with the SCI+FV group accepting counterparts from the SCI+Veh group. The BMS score and tail flick latency test displayed statistical significances in donor and recipient groups at 7 dpi (Fig. 8B), demonstrating that intestinal microbiota was involved in the resveratrol-mediated neuroprotective effect.

We then examine the profile of inflammation-related cytokines in the spinal cord after SCI at 7 dpi in FMT groups. FMT from the SCI+Res group reduced the pro-inflammatory cytokine levels of IL-6, MCP-1, and TNF, and simultaneously increased the level of anti-inflammatory IL-10 cytokine despite no significant difference in the spinal cord of the SCI+FR group. However, FMT had little impact on the expressions of IFN-γ and IL-12p70 (Fig. 8C and 8D). These results demonstrated that resveratrol-mediated alteration of intestinal microbiota exerted an anti-inflammatory action on the spinal cord after SCI.

Besides, immunofluorescence was also performed to observe the expression of IBA-1 after SCI at 7 dpi. It was found that the positive area and positive cell number of activated microglia were strikingly lower in the SCI+FR group than that in the SCI+FV group (Fig. 8E and 8F), indicating that resveratrol-mediated alteration of gut microbiota markedly inhibited the microglial activation after SCI at 7 dpi. Both the SCI+FV and the SCI+FR groups were deprived of their intrinsic intestinal microbiota due to combined antibiotic treatment beforehand and were not involved in direct pharmacological action of resveratrol. This demonstrated that only intestinal microbiota and corresponding metabolites in the SCI+Veh and the SCI+Res groups exerted joint effects on the microglial activation.

It was therefore assumed that the SCFAs profile featured with elevated butyrate and reduced isobutyrate in the SCI+Veh and the SCI+Res groups at 7 dpi might correlate with the immunofluorescence expression of microglial activation marker IBA-1 in the SCI+FV and the SCI+FR groups at 7 dpi. We used Pearson correlation analysis to examine the correlation between the concentration of butyrate or isobutyrate in feces and the positive area and positive cell number of IBA-1 expression in immunofluorescence. It was shown that the concentrations of butyrate in feces were negatively associated with the IBA-1 positive area and positive cell number in the spinal cord. On the contrary, isobutyrate showed a positive correlation with the IBA-1 positive area and positive cell number (Fig. 8G). The Pearson correlation analysis indicated that butyrate and isobutyrate might be the key factors for the resolution of microglia-mediated inflammation. In summary, these results indicated that the resveratrol-derived alteration of intestinal microbiota composition was involved in the resolution of the microglia-mediated inflammatory response after SCI.

4. Discussion

The intestinal dysbiosis after SCI in mice could be restored by resveratrol. Our study found that resveratrol improved the recovery of motor and sensory functions after SCI in mice. Some of the altered bacterial species were correlated with the recovery of neurological functions after SCI. Resveratrol altered intestinal microbiota composition and metabolite profile (featured with lower LPS infiltration and higher butyrate content). The microbiota composition and metabolite profile altered by resveratrol could suppress the inflammatory response and the microglial activation in the acute stage after SCI. The fecal supernatant from resveratrol-treated SCI mice had an inhibitory effect on LPS-induced BV2 microglia *in vitro*. Therefore, we concluded that resveratrol could alleviate the inflammatory response at the acute stage and promote the recovery of neurological functions at the later stage after SCI *in*

vivo by improving the dysbiosis of intestinal microecology, decreasing the level of LBP, increasing the butyrate content, and reducing the microglial activation.

Our study confirmed the role of resveratrol in promoting the recovery of SCI, which is consistent with previous reports [35, 43]. The neuroprotective effect of resveratrol on SCI has been well documented [35, 43]. But the therapeutic application of resveratrol has been hampered because scientists and clinicians are uncertain about whether resveratrol and its metabolites could pass through the BBB and exert its direct neuroprotective effects. Recently, it has been reported that resveratrol could be detected in both blood and cerebrospinal fluid at very low concentration [50], yet only resveratrol metabolites instead of resveratrol itself could be detected. Furthermore, there has been no direct evidence that resveratrol can pass through the BBB and reach the spinal cord. We assumed there must be some mediators between resveratrol and its anti-inflammatory effects in the course of SCI. Some previous studies found significant changes in the intestinal microbiota of SCI rodents and clinical SCI patients [21, 51, 52]. Hence, the neuroprotective effects of resveratrol might be mainly dependent on gut microbiota. We then used 16S rRNA microbiota sequencing to confirm the intestinal dysbiosis caused by SCI. Resveratrol treatment, interestingly, could significantly improve intestinal microbiome imbalance. More importantly, we found a significant correlation between the differential bacterial species based on resveratrol treatment and behavioral tests through Pearson correlation analysis.

As for intestinal microbiota composition, resveratrol increased the relative abundance of *Erysipelotrichales* (order), *Lactobacillales* (order), and *Dubosiella* (genus) and decreased that of *Clostridiales* (order) and *Lachnospiraceae* (family member of *Clostridiales*), consistent with recent reports unveiling the alteration of microbiota composition in rodents with SCI [21,

24, 51]. Besides, ANOVA and LEfSe analysis demonstrated the order level of *Erysipelotrichales* and *Lactobacillales* and the family level of *Lactobacillus* and *Dubosiella*, which had a marked increase after resveratrol treatment in SCI groups. Specifically, *Erysipelotrichaceae* (family) and *Erysipelotrichales* (order), as a butyrate-producing bacterium, could produce butyrate [53]. More importantly, adjuvanticity has been reported as another feature of *Erysipelotrichaceae* while interacting with chemotherapy drugs [54], indicating that *Erysipelotrichaceae* may to some extent contribute to enhancing the neuroprotective effects of resveratrol. *Lactobacillales* (order) and *Lactobacillus* (genus) are the group of lactic acid bacteria, which have been verified to modify immune function significantly in the gastrointestinal tract [55]. Besides, *Lactobacillus* and *Dubosiella* both have been identified to influence the intestinal immunologic functions in mice [56, 57]. At present, there are not enough studies to explore the relationship between *Clostridiales* or *Lachnospiraceae* and SCI. But one study has shown that the high abundance of *Clostridiales* and its family member *Lachnospiraceae* are closely interconnected with depression-like behavior, impaired oligodendrocyte differentiation, and myelin gene transcription [58], indicating that *Clostridiales* and *Lachnospiraceae* are involved in the maintenance of neural homeostasis. There was a specific kind of bacteria *Bacteroidetes* (phylum), including its class member *Bacteroidia* and order member *Bacteroidales*, classified into the most significant differences in the SCI+Veh group by LEfSe analysis. The relative abundance of *Bacteroidetes* is considered the main characteristic of intestinal dysbiosis after SCI. The family member *Bacteroideaceae* are the most numerous endotoxin (*i.e.*, LPS) producers in the intestinal tract [59]. Previous research indicated that LPS might induce the microglial activation to aggravate post-SCI behavioral outcomes [5, 6]. Our results demonstrated that the concentrations of the LBP at 7 dpi were significantly decreased after resveratrol treatment after SCI, indicating LPS may exacerbate the inflammatory response in the SCI+Veh group, which is consistent with

LEfSe analysis. In general, these whole changes of microbiota composition after resveratrol treatment in SCI mice improve the prognosis of SCI. Nevertheless, the detailed mechanisms by which *Erysipelotrichaceae*, *Lactobacillus*, *Dubosiella*, *Clostridiales*, and *Lachnospiraceae* interact with SCI are still unknown and await further investigation.

Current evidence has highlighted the importance of polyphenols and other potential phytochemical candidates in the future therapy for SCI [41, 60, 61]. Resveratrol is a non-flavonoid polyphenol organic compound. At present, about 500~1000 different microbial species, especially the colon possessing the highest contents, live in the human gastrointestinal tract. But only a small number of microorganisms (such as *Escherichia coli*, *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and *Eubacterium*) catalyze the metabolism of phenolic substances [62]. However, this phenomenon does not seem to be common because different individuals show a rather high degree of individual difference. The relationship of resveratrol (polyphenol) and intestinal microorganism, therefore, may need to be further studied in the future if resveratrol wishes to be formally applied in clinical adjuvant therapy as a therapeutic agent.

Since resveratrol could alter the gut microbiota pattern, we assumed that resveratrol also changes the corresponding metabolites. Among metabolites, LPS and butyrate in SCFAs are the most important members exerting pro- and anti-inflammatory effects, as reported in previous studies [23, 63, 64]. Consequently, the microbiota-derived metabolites of LPS and SCFAs may be the potential connector for the microbiota-microglia transmission [65, 66]. We used ELISA and detected the contents of LBP in the colon and serum. The results showed that LBP significantly decreased with resveratrol treatment in both the colon and serum after SCI at 7 dpi. Furthermore, we used UPLC and detected the contents of SCFAs in feces. As

expected, resveratrol significantly increased the content of butyrate, although the total amount of SCFAs was not strikingly changed. SCFAs featured with butyrate can enter the blood circulation through the transporters, which could be elevated with resveratrol as well, on intestinal epithelial cells [66]. We then used GC and measured the concentration of butyrate in the blood. Compared with the SCI+Veh group, the serum content of butyrate in the SCI+Res group strikingly increased. Based on the above results, we confirmed that resveratrol improved intestinal dysbiosis and changed the metabolite profile to one featured with lower LBP content in colon and serum and higher butyrate in feces and serum. Besides, it has been previously shown that SCFAs in blood can pass through the BBB and reach the brain by the transporters on endothelial cells [13, 66], and regulate the development and function of the nervous system [65]. These results support our view that the improvement of neurological functions in resveratrol-treated SCI mice may be involved in the increase in butyrate and the decrease in LPS.

Since butyrate in the intestinal metabolites has anti-inflammatory properties, we paid more attention to its inhibitory effects on the microglial activation that aggravates the pro-inflammatory response after SCI. Firstly, LPS-activated BV2 microglia *in vitro* were treated with fecal supernatant (containing altered metabolites and no bacteria) from resveratrol-treated SCI mice. It was found that resveratrol-treated fecal supernatant significantly inhibited the expression of pro-inflammatory genes. On the other hand, our *in vitro* results further confirmed that butyrate has a similarly inhibitory effect on microglial activation. We then conducted FMT experiments *in vivo* and found that FMT from resveratrol-treated mice significantly reduced the microglial activation and promoted the recovery of motor and sensory functions. Furthermore, the concentration of butyrate increased by resveratrol treatment in fecal was negatively correlated with the microglial activation.

Our data demonstrated that resveratrol exerted neuroprotective effects against the inflammatory response, in line with previous studies [43, 67, 68]. However, we still have some limitations that need to be improved in future research: 1) Although it has been reported that the concentration of resveratrol and its metabolites in the central nervous system is extremely low, we did not directly detect the contents of butyrate and resveratrol in cerebrospinal fluid and spinal cord tissue to confirm this phenomenon. As a result, we could not completely exclude the direct pathway of resveratrol that reaches the spinal cord to exert anti-inflammatory and neuroprotective effects. This needs to be investigated in future experiments. 2) We focused on butyrate among SCFAs. However, we speculated that it was probable that the whole mixture of SCFAs, not only butyrate itself, played a neuroprotective role. This speculation was supported by a former study reporting that the appropriate content of SCFAs mixture could inhibit the pro-inflammation profile of BV2 microglia [63]. This phenomenon was confirmed in our results, as shown in the results of fecal supernatant (containing altered metabolites). Besides, it was found that isobutyrate concentration was reduced by resveratrol. The possibility that resveratrol may change the metabolite profile of other neuroprotective non-SCFAs *in vivo* cannot be completely excluded; thus, this needs to be further studied. 3) We did not explore how resveratrol regulates intestinal microbiota composition. We hypothesized that resveratrol might change the microbiota composition by regulating the immune cells in the intestinal epithelium after SCI. It has been reported that resveratrol, as an immunomodulatory agent, can directly regulate the immune homeostasis of intestinal epithelium [69, 70], which may indirectly affect the microbiota composition. Consequently, we cannot determine whether resveratrol alters the intestinal microbiota composition directly or indirectly after SCI. In the following experiments, we will use tail vein and intrathecal administration as methods. Tail vein administration makes resveratrol

interact with intestinal immune cells through the blood and avoid direct contact with intestinal microbiota, therefore eliminating the influence of intestinal microecology. Intrathecal administration enables resveratrol to interact directly with neurons and microglia in the spinal cord and avoid direct contact with peripheral immune cells, thereby determining the effect of direct action on the recovery of SCI and eliminating the indirect effect of intestinal microbiota and corresponding metabolites.

The clinical application of resveratrol has been gradually increasing over the past 20 years [71]. Our study revealed that resveratrol could improve the prognosis in mice with SCI by altering the composition of intestinal microbiota, which results in changes in metabolite levels (*e.g.*, increased butyrate content). Furthermore, small molecules, such as butyrate, could pass through the BBB more easily than resveratrol and its metabolites, thus exerting anti-inflammatory effects. As a result, this finding has great significance for the clinical application of resveratrol in the treatment of SCI.

5. Conclusion

To sum up, this study revealed that resveratrol could promote recovery of locomotor and sensory functions after SCI by improving the intestinal dysbiosis, reducing LBP content, elevating the content of intestinal metabolite butyrate, and then inhibiting the microglial activation in the spinal cord in SCI mice. The intestinal microbiota composition and metabolite are associated with the anti-inflammatory property of resveratrol in SCI mice.

Author contribution statement

Dr. Linlin Wang, Dr. Mingzhi Zheng and Dr. Ying-ying Chen mainly participated in the design, interpretation, and proof. Ning He and Gerong Shen not only performed the

experiment but also were involved in data analysis and drafting the manuscript together. Xinghui Song, Shuangshuang Liu and Xiaoqin Jin participated in immunofluorescence experiments together. Heyangzi Li, Jingyu Wang, Chunyan Fu, Lintao Xu, Jun Chen, Xi Cao, Yanwei Li, Jinjie Zhong, and Yueliang Shen took part in interpretation and inductive reasoning together.

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Data availability

16S rRNA sequence data can be accessed from NCBI PRJNA791504. Our analysis use standard, open source software - R packages available on CRAN (cran.r-project.org).

Declaration of competing interest

The authors declare no any competing conflicts of interest.

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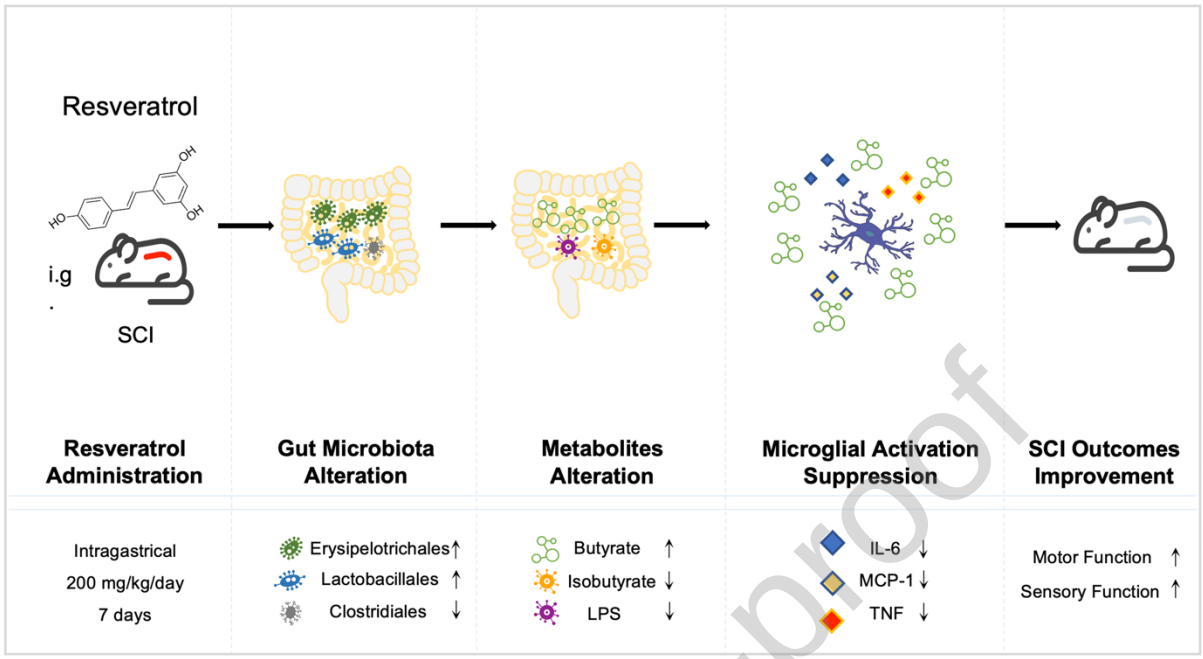
Figures and Legends



Graphic abstract. Schematic diagram of the suppressive mechanism of resveratrol on the microglial activation in SCI mice. Resveratrol promoted the neurological recovery after SCI by altering intestinal microbiota and corresponding metabolites, and reducing the microglia-derived pro-inflammatory cytokines.

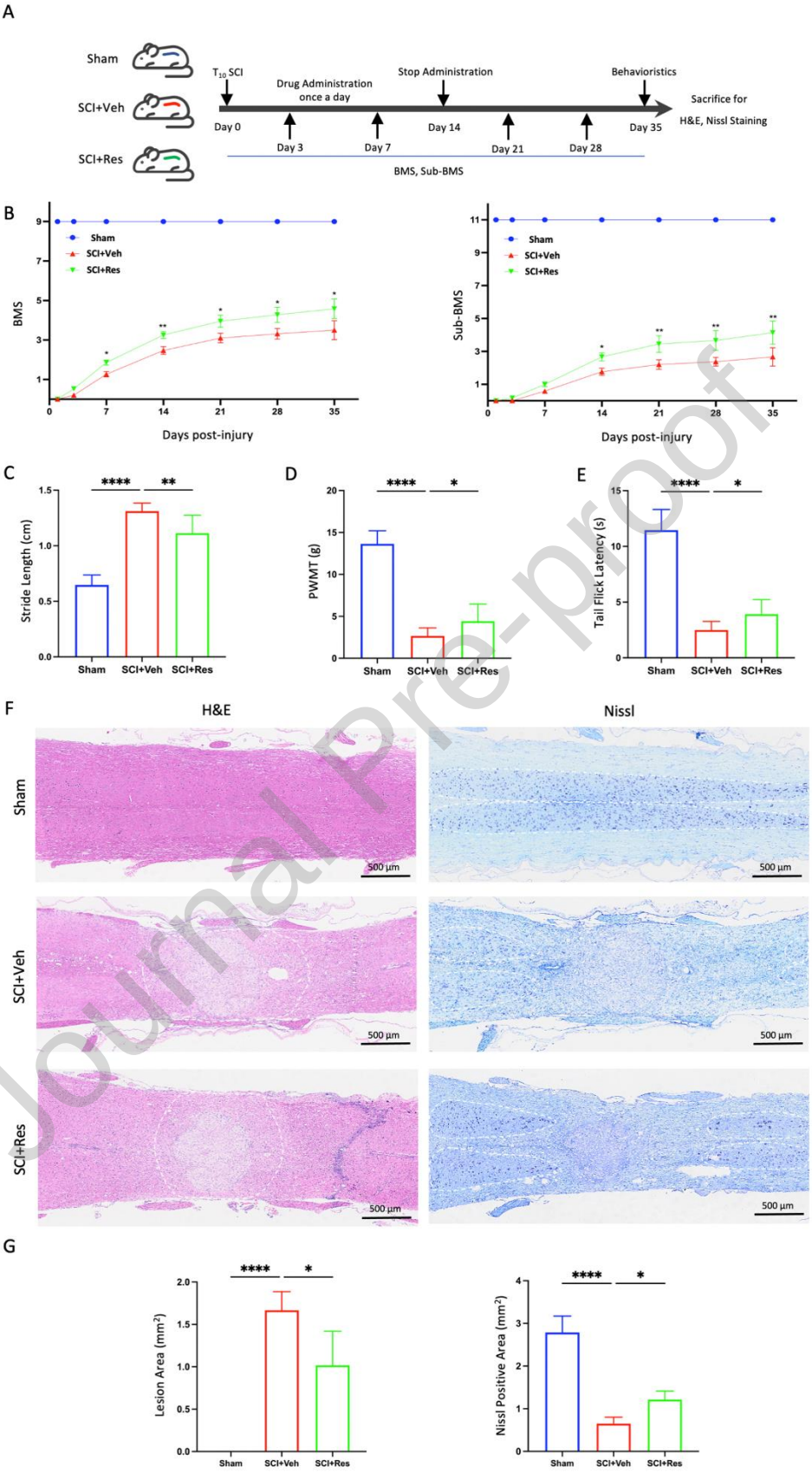


Fig. 1. Resveratrol improved neurological function after SCI in mice. (**A**) Schematic representation of the experiments for behavioral tests and histological detection. (**B**) BMS and Sub-BMS at sequential time points showed that resveratrol treatment promoted the recovery of motor function after SCI from 7 to 35 dpi. n=12/group. (**C**) Footprint analysis showed that resveratrol treatment improved the coordination of motor function after SCI at 35 dpi. n=12/group. (**D** and **E**) Pressure and heat pain threshold tests showed that resveratrol treatment promoted the recovery of PWMT and tail flick latency after SCI at 35 dpi. n=12/group. (**F**) Representative images of H&E and Nissl staining displayed lesion area and survival neurons after SCI at 35 dpi, respectively. Scale bar = 500 μm. (**G**) Statistical analysis for H&E and Nissl staining showed that resveratrol treatment lowered the lesion area and increased the Nissl staining positive area. n=4/group. Data are represented as mean ± SEM. Statistical analysis was performed with two-way and one-way ANOVA. *p<0.05, **p<0.01, and ****p<0.0001.

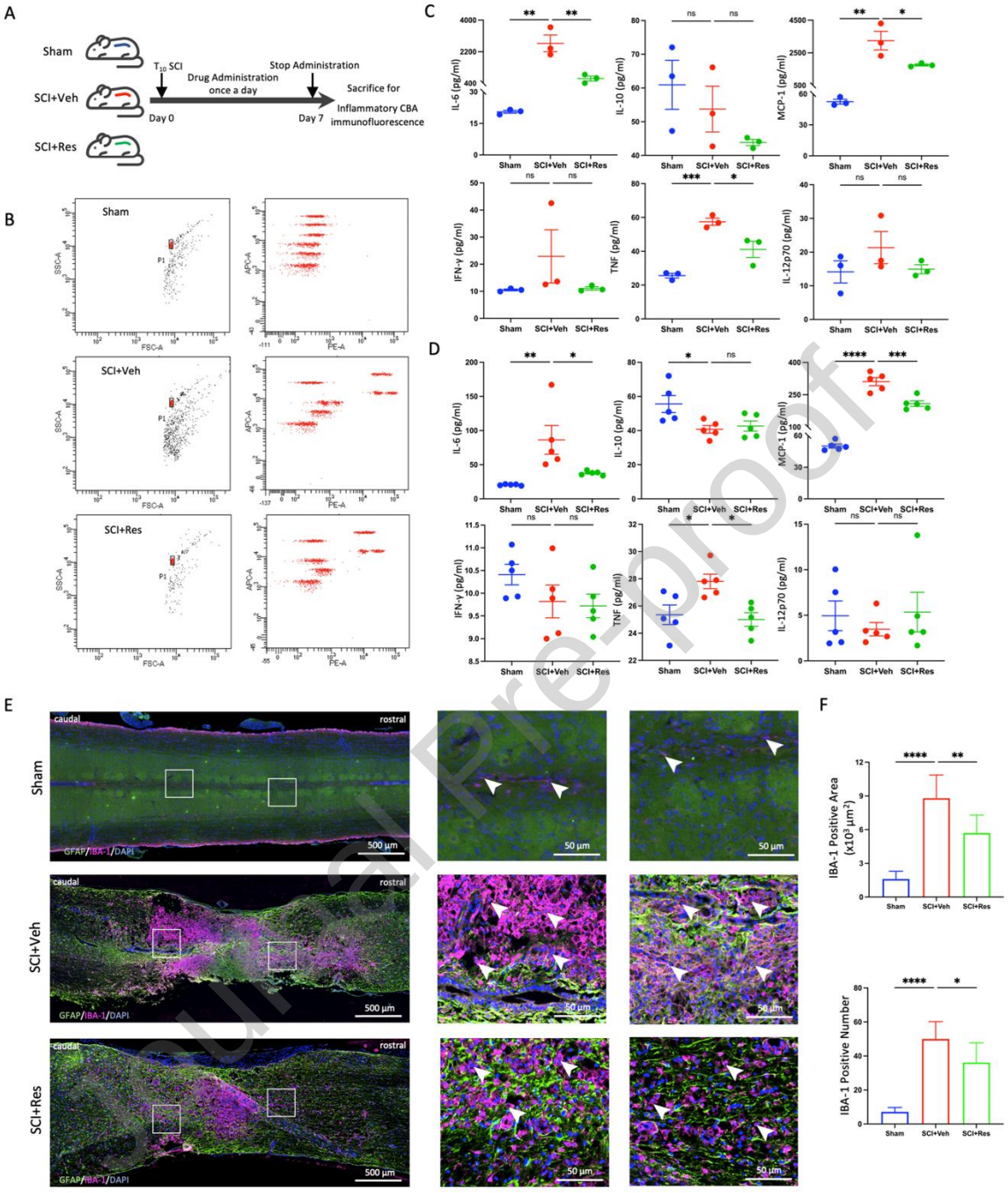


Fig. 2. Resveratrol reduced the inflammatory response and inhibited the activation of microglia after SCI in mice. (**A**) Schematic representation of the experiments for CBA and immunofluorescence. (**B**) Representative images of CBA displayed the fluorescence intensity of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 in the spinal cord. (**C** and **D**) CBA quantitative analysis for IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 showed that resveratrol treatment alleviated the inflammatory response after SCI at 3 and 7 dpi,

respectively. n=3/group at 3 dpi and 5/group at 7 dpi. (**E**) Representative images of immunofluorescence showed microglial activation marker IBA-1 in the spinal cord after SCI at 7 dpi. Scale bar = 500 and 50 μm. (**F**) Statistical analysis for IBA-1 immunofluorescence showed that resveratrol treatment decreased the IBA-1 staining positive area and the number of IBA-1 positive cells after SCI at 7 dpi. n=4/group. All data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

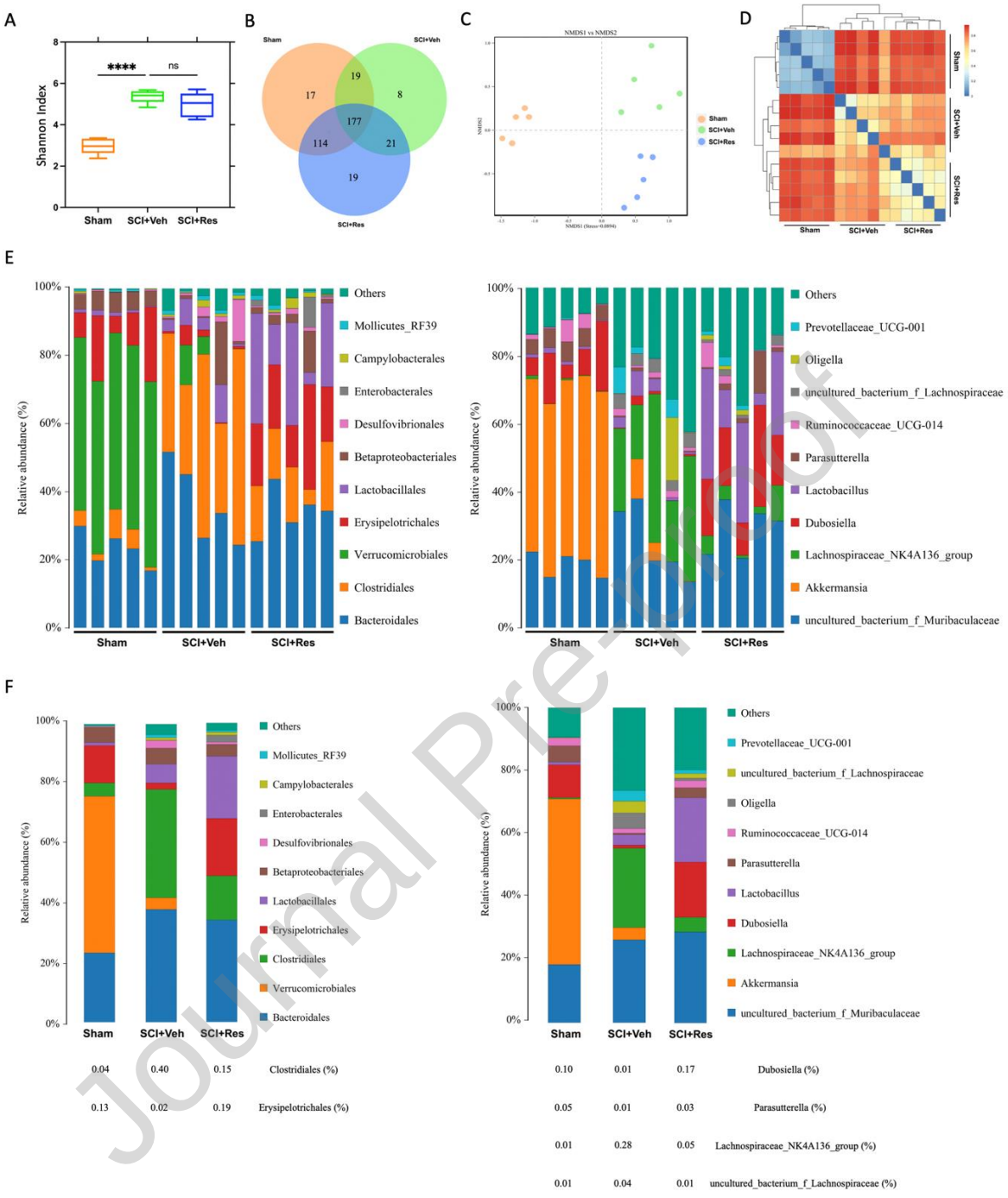


Fig. 3. Resveratrol altered the intestinal microbiota composition after SCI in mice. (**A**) Shannon index showed that both the SCI+Veh and the SCI+Res groups increased alpha diversity, despite no statistical difference between them. (**B**) Venn diagram showed the overlapping OTUs in Sham, SCI+Veh, and SCI+Res groups. (**C**) NMDS represented the beta diversity and the degree of difference within and between Sham, SCI+Veh, and SCI+Res groups. (**D**) Heat map analysis indicated the beta diversity and the degree of different species

between each sample. (**E** and **F**) Histograms of species distribution in order and genus levels, respectively. All data are represented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. ****p<0.0001.

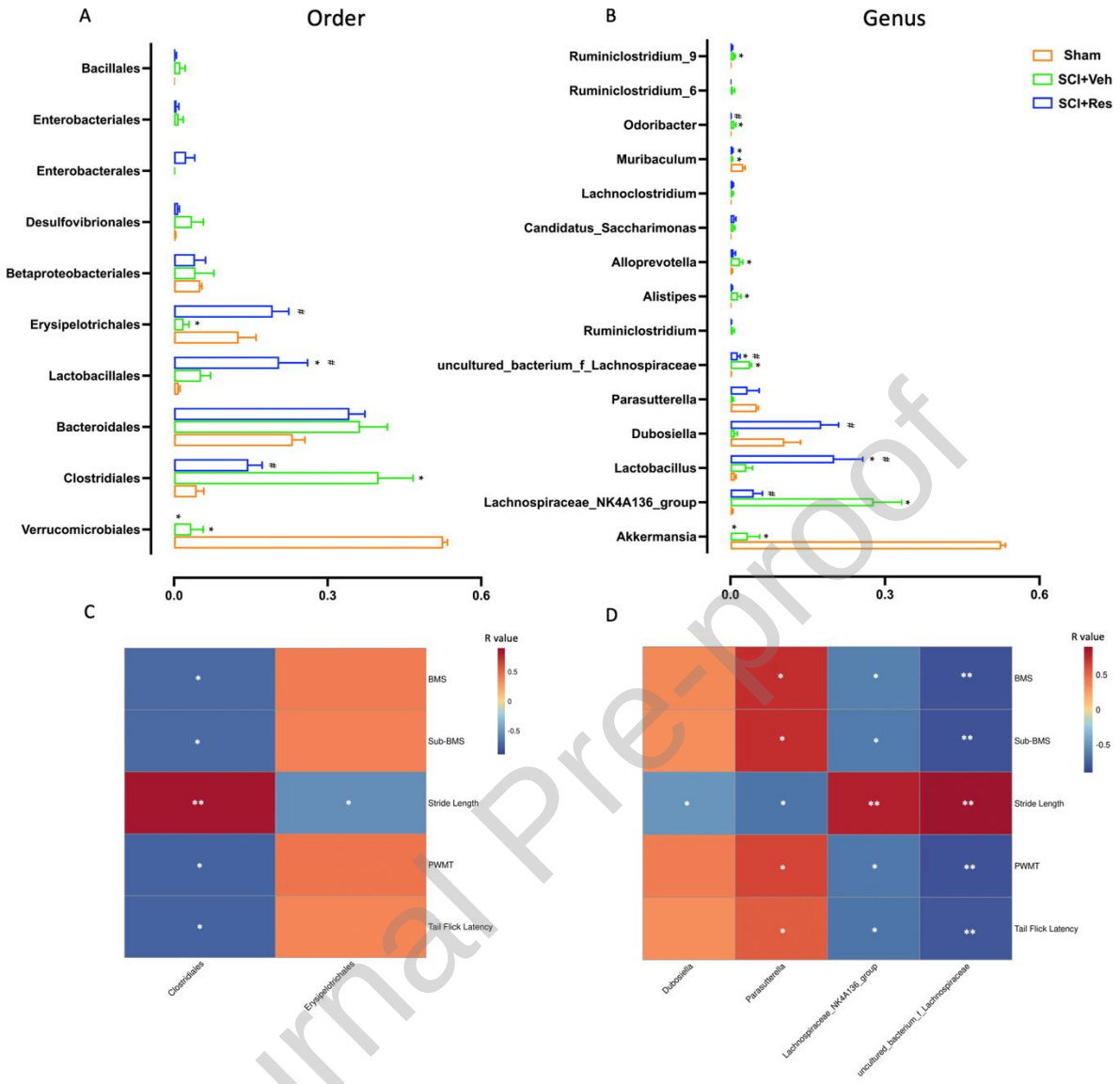


Fig. 4. Resveratrol altered the relative abundance of species in feces after SCI in mice. (**A** and **B**) Differential species in order and genus levels in Sham, SCI+Veh, and SCI+Res groups. n=5/group. **p*<0.05, *vs* the Sham group; $^{\#}p$<0.05, *vs* the SCI+Veh group. (**C** and **D**) Pearson correlation coefficient analysis showed that the relative abundance of 6 different OTUs in order and genus levels had positive or negative associations with the recovery of behavioral tests. n=5/group. All data are represented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. **p*<0.05, and ***p*<0.01.

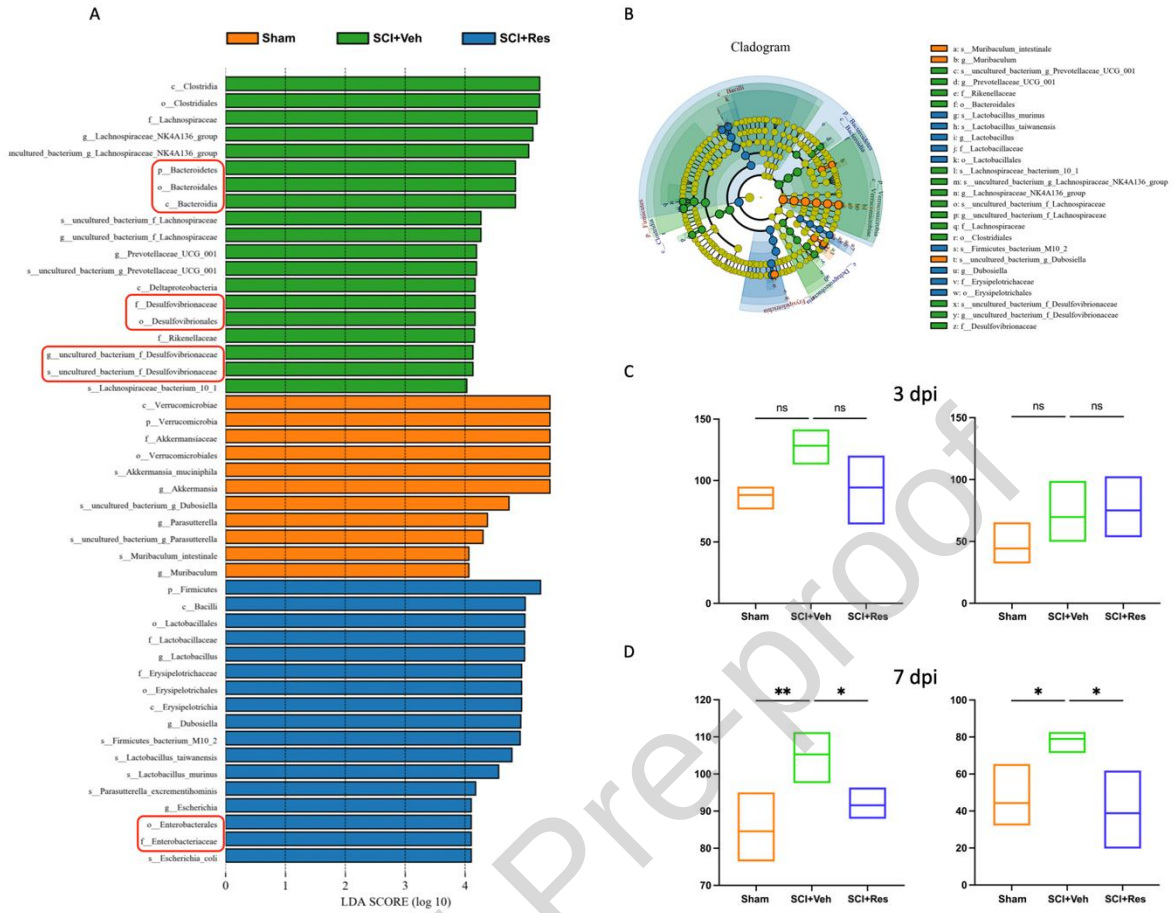


Fig. 5. Resveratrol reduced the LPS-producing bacteria species and reduced LBP concentration after SCI in mice. (**A**) LEfSe illustrated the statistical difference in species in Sham, SCI+Veh, and SCI+Res groups based on LDA Score > 4. The length of the histogram represents the impact of different species (*i.e.*, LDA Score). The species within red frame were endotoxin LPS-producing bacteria. (**B**) Cladogram based on LEfSe analysis represents taxonomic level from phylum to species. The species within red frame were endotoxin LPS-producing bacteria. Species with no significant difference were colored in yellow and other colors stand for different groups. (**C** and **D**) ELISA quantitative analysis for the concentration of LBP in colon and serum in mice after SCI showed that resveratrol treatment reduced the infiltration of endotoxin metabolite LPS at 7 dpi but not at 3 dpi. All data are represented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. *p<0.05, and **p<0.01.

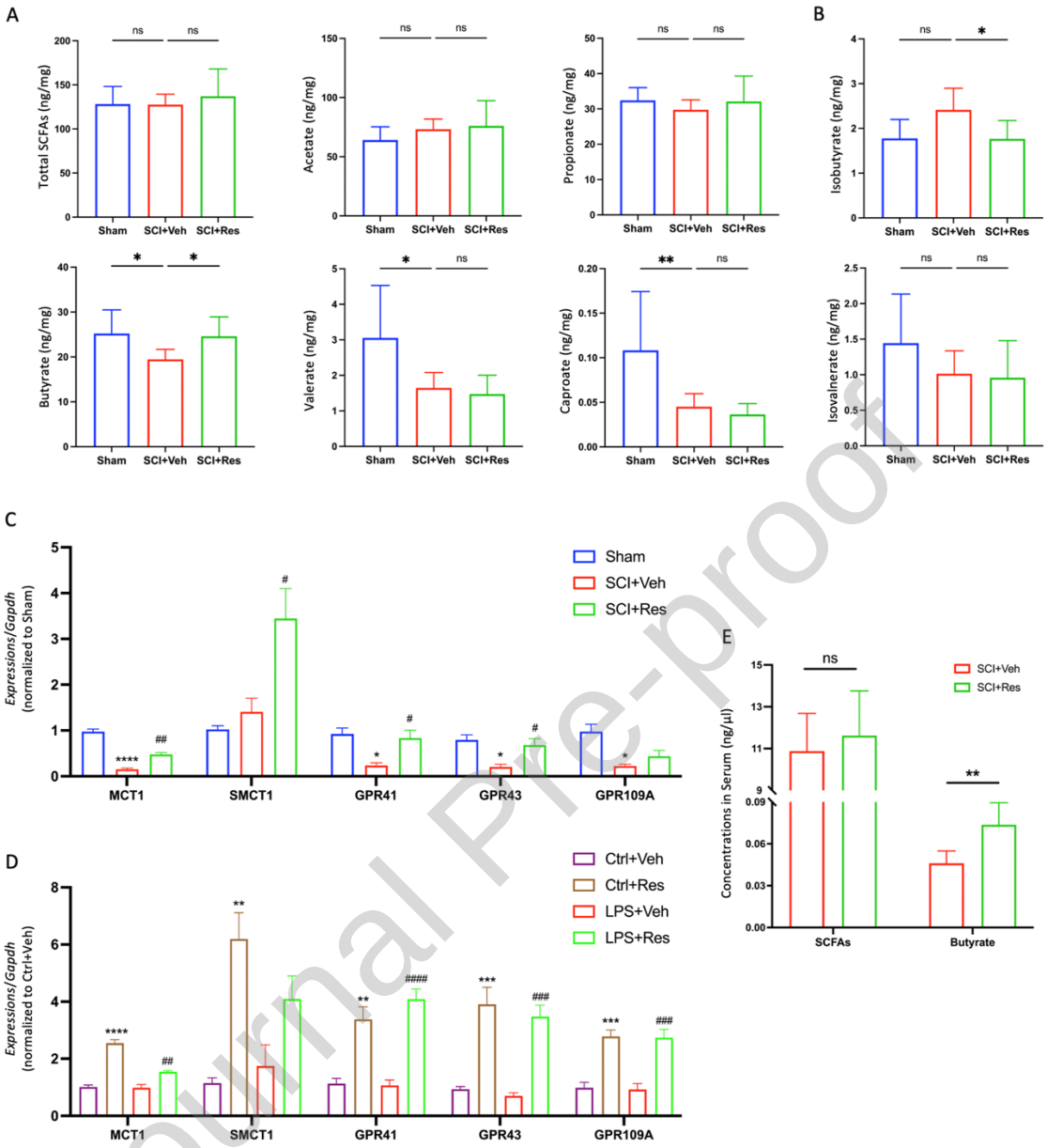


Fig. 6. Resveratrol altered the profile of metabolite SCFAs and BCFAs after SCI in mice. (**A** and **B**) UPLC quantitative analysis showed the subtype concentrations of SCFAs and BCFAs in feces after SCI at 7 dpi. n=5-8/group. (**C**) qPCR analysis showed the expression levels of SCFAs-related transporters and receptors in small intestine in mice. n=3/group. *p<0.05, ****p<0.0001, *vs* the Sham group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, *vs* the SCI+Veh group. (**D**) MODE-K small intestine epithelial cells were pretreated with 50 μM resveratrol for 24 h and then treated with 100 ng/ml LPS for 24h. qPCR analysis showed that resveratrol treatment increased the mRNA expression of SCFA-related transporters and receptors *in vitro*.

n=4/group. **p<0.01, ***p<0.001, ****p<0.0001, *vs* the Ctrl+Veh group; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001, *vs* the LPS+Veh group. (**E**) GC quantitative analysis showed the concentrations of butyrate and total SCFAs in serum after SCI at 7 dpi. n=5/group. Data are presented as mean ± SEM. Statistical analysis was performed with student's *t*-test and one-way ANOVA. *p<0.05, and **p<0.01.

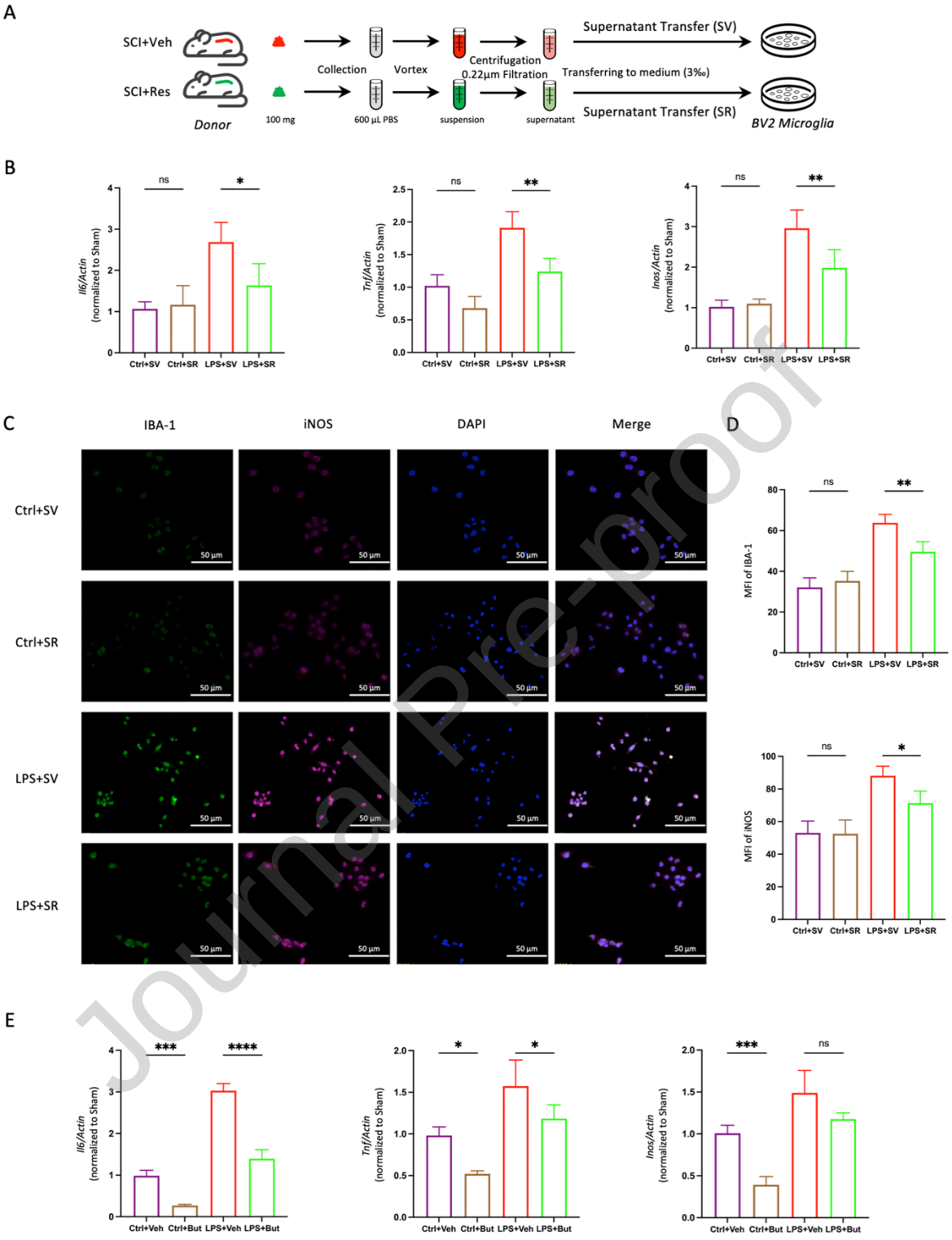


Fig. 7. Resveratrol-treated fecal supernatant suppressed the microglial activation. (**A**) Schematic representation of the experiment for transferring fecal supernatant to BV2 microglia. (**B**) BV2 microglia were pretreated with 3‰ resveratrol-treated fecal supernatant for 24 h and then treated with 100 ng/ml LPS for 24 h. qPCR analysis of BV2 microglia

showed that resveratrol-treated fecal supernatant reduced the mRNA expression of pro-inflammatory genes *in vitro*. n=4/group. (**C**) Representative images of immunofluorescence staining of microglial activation marker IBA-1 and iNOS in BV2 microglia with the treatments of LPS and fecal supernatant. Scale bar = 50 μm. (**D**) Statistical analysis showed that resveratrol-treated fecal supernatant reduced the MFI of IBA-1 and iNOS. n=4/group. (**E**) BV2 microglia were pretreated with 200 μM butyrate for 24 h, then treated with 100 ng/ml LPS for 24 h. qPCR analysis of BV2 microglia showed that butyrate reduced the mRNA expression of pro-inflammatory genes *in vitro*. n=4/group. Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

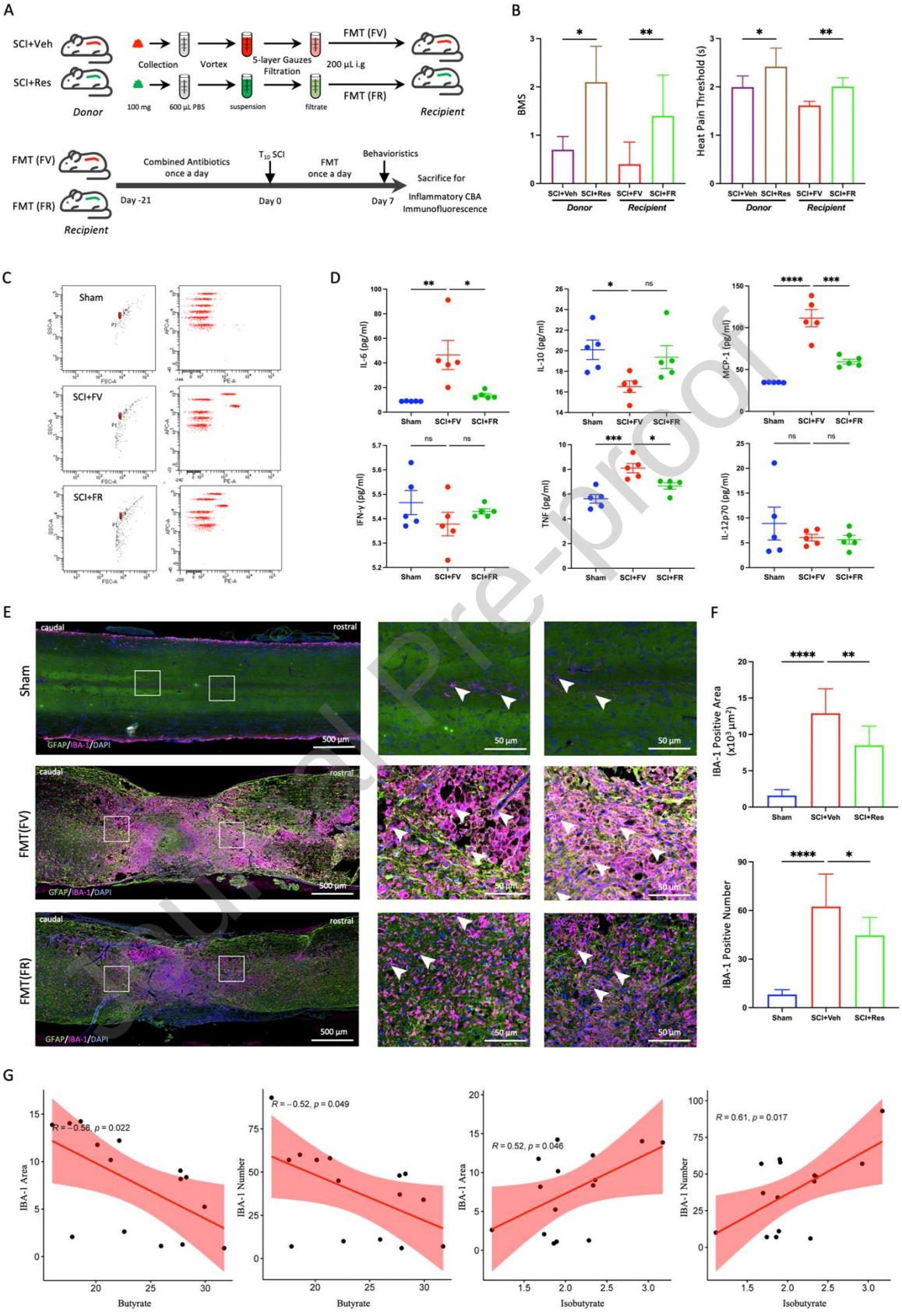


Fig. 8. Intestinal microbiota mediates resveratrol anti-inflammatory effect after SCI in mice. (**A**) Schematic representation of the experiment for FMT to recipient mice. (**B**) Behavioral tests in donor and recipient groups showed that the resveratrol-treated FMT SCI+FR group had better improvement than the vehicle-treated FMT SCI+FV group in neurological functions. n=10/group. (**C**) Representative images of CBA displayed the fluorescence intensity of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 in the spinal cord. (**D**) CBA quantitative analysis showed that the concentrations of pro-inflammatory cytokines of IL-6, MCP-1, and TNF in the spinal cords of the SCI+FR group were decreased after SCI at 7 dpi. n=5/group. (**E**) Representative images of immunofluorescence staining of microglial activation marker IBA-1 in the spinal cords after SCI at 7 dpi. Scale bar = 500 and 50 μm. (**F**) Statistical analysis showed that the area and the number of IBA-1 positive cells were decreased in the SCI+FR group at 7 dpi. n=5/group. (**G**) Pearson correlation coefficient analysis showed the correlation between butyrate or isobutyrate in feces and the IBA-1 staining positive area or number of IBA-1 positive cells in the spinal cord. 2 high-magnification fields were taken from each sample, n=5/group. Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Credit Author Statement

Ning He: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - Original Draft.
Gerong Shen: Investigation, Validation, Formal analysis, Writing - Original Draft.
Xiaoqin Jin: Investigation, Validation.
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Lin-lin Wang: Conceptualization, Supervision, Project administration, Writing - Review & Editing, Funding acquisition.

Declaration of Interest

All authors declare NO conflict of interest in this study.

Highlights

- Resveratrol reduced the inflammatory response and the microglial activation after SCI, and promoted the recovery of neurological functions after SCI
- Resveratrol altered intestinal microbiota composition, decreased the endotoxin infiltration, and increased the concentration of butyrate after SCI
- Both fecal supernatant from resveratrol-treated SCI mice and butyrate suppressed the activation of LPS-stimulated microglia *in vitro*

- Fecal microbiota transplantation from resveratrol-treated SCI mice reduced the microglial activation and the inflammatory response, and promoted the prognosis after SCI