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ORIGINAL RESEARCH

Neuroprotection of Resveratrol Against Focal Cerebral Ischemia/Reperfusion Injury in Mice Through a Mechanism Targeting Gut-Brain Axis

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Abstract

Increasing evidences have shown that resveratrol could protect the brain from ischemic injury; the mechanisms underlying its neuroprotective effects are multifactorial and not fully understood. It remains unclear whether resveratrol could exert neuroprotection through modulating gut-brain axis, which plays important roles in stroke pathology. In this study, C57BL/6 mice underwent middle cerebral artery occlusion (60 min) followed by reperfusion for 3 days. Resveratrol, when applied immediately after MCAO onset for 3 days, promoted Th1/Th2 balance towards Th2 polarization and skewed Treg/Th17 balance towards Treg in the small intestinal lamina propria (SI-LP), and decreased small intestinal pro-inflammatory cytokines expression through modulating intestinal flora at 3 days post-ischemia (dpi). Resveratrol attenuated cerebral ischemia-induced increase in the epithelial and vascular permeability of small intestine as evidenced by reduced evans blue extravasation and decreased protein leakage by feces/plasma albumin ratio at 3 dpi. The blood levels of pro-inflammatory cytokines at 3 dpi were also attenuated by resveratrol due to inhibiting intestinal pro-inflammatory immunity and decreasing epithelial and vascular permeability. Resveratrol robustly protected against post-stroke inflammation-induced blood–brain barrier disruption not only in the cortex but also in the striatum at 3 dpi. Furthermore, resveratrol mediated smaller cerebral infarcts and less neurological deficits via decreasing the levels of pro-inflammatory cytokines in the peri-infarct area at 3 dpi. Our results for the first time demonstrated that resveratrol may inhibit systemic post-stroke inflammation and neuroinflammation via modulating intestinal flora-mediated Th17/Tregs and Th1/Th2 polarity shift in SI-LP, which may be one of the mechanisms underlying the neuroprotective effects of resveratrol.

Keywords Gut-brain axis · Inflammation · Intestinal immunity · Resveratrol · Stroke

Abbreviations

dpi	Days post-ischemia
BBB	Blood–brain barrier
Treg	Regulatory T
γδ T	Gamma delta T
Th17	T helper type 17
SI-LP	Small intestinal lamina propria
IFN-γ	Interferon-γ
IL-17A	Interleukin-17A
MCAO	Middle cerebral artery occlusion
TTC	2,3,5-Triphenyltetrazolium chloride
LPL	Lamina propria lymphocytes
VAN	Vancomycin
AC	Amoxicillin and clavulanic acid
ZO-1	Zonula occludens 1

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Introduction

Immune system response and inflammation play key roles in the pathophysiology of acute stroke (Iadecola and Anrather 2011). The peripheral immune system (including innate and adaptive immune cells) plays essential roles in the brain injury during and after ischemic stroke by releasing pro-inflammatory cytokines (Iadecola and Anrather 2011).

In the human body, the intestine is the largest peripheral lymphoid organ. Recent researches have provided evidences that microbiota-gut-brain axis exerts important roles in the pathogenesis of ischemic stroke injury (Benakis et al. 2016; Spychala et al. 2018). Altered composition of intestinal flora, or intestinal dysbiosis is responsible for age-related systemic upregulation of pro-inflammatory responses and exerts detrimental effects on acute ischemic stroke (Spychala et al. 2018).

Gut commensal bacteria could regulate lymphocyte development and function in the small intestine, including regulatory T (Treg), gamma delta (γδ) T, T helper type 17 (Th17), T helper type 1 (Th1), and T helper type 2 (Th2) (Bauche and Marie 2017; Belkaid and Harrison 2017; Denes et al. 2010; Honda and Littman 2016; Iadecola and Anrather 2011; Littman 2018; Luo et al. 2017; Ruiz et al. 2017). Changes of the intestinal flora causes small intestinal immune dysfunction, leading to upregulation of Treg and downregulation of interleukin (IL)-17-positive γδ T or Th17 cells in the small intestinal lamina propria (SI-LP), which may suppress the trafficking of γδ T and/or Th17 cells from the small intestine to the peripheral blood and subsequently to the ischemic brain parenchyma, thus reduces systemic inflammation and exerts neuroprotection after acute ischemic stroke (Benakis et al. 2016; Honda and Littman 2016). Small intestinal Treg could inhibit γδ T cells and thus confer neuroprotective effects by secreting the anti-inflammatory cytokine IL-10 after stroke (Benakis et al. 2016). Therefore, intestinal immune cells-mediated small intestinal and systemic inflammatory response may play important roles in the modulation of neuroinflammation and acute stroke injury. However, whether the immunophenotypic shift of Th cells from interferon-γ (IFN-γ)-expressing Th1 to IL-4-expressing Th2 response and Treg-mediated suppression of IL-17A producing Th17 immune response in the small intestine could exert neuroprotection after acute ischemic stroke remain unclear.

Resveratrol, a natural polyphenol found in grapes, berries, peanuts, and other plants, has received widespread attention for its potential use as a preventive and therapeutic agent in many diseases. Resveratrol can modulate the composition of intestinal flora, be biotransformed to active metabolites by the intestinal microbiota, and affect intestinal barrier function (Bird et al. 2017; Zhao et al. 2017). Resveratrol could ameliorate experimental acute ileitis by inhibiting Th1-type cellular immune responses and preventing intestinal bacterial translocation by maintaining intestinal barrier function (Bereswill et al. 2010). Resveratrol could also regulate the balance of Th17/Treg through reducing the number of Th17 cells, increasing the number of Treg in the intestine, and regulating the level of plasma and intestinal mucosal cytokines including IL-10 and IL-17A (Yao et al. 2015). Previous studies have shown that resveratrol plays important roles in the protective effects against acute stroke injury via inhibition of neuroinflammation (Jeong et al. 2016; Lin et al. 2013a, b). However, whether resveratrol could protect against acute stroke injury via the suppression of intestinal immune cells-mediated intestinal and systemic inflammation remains unknown.

This study aimed to explore the hypothesis that the modulation of gut-brain axis is involved in the protective effects of resveratrol on acute ischemic stroke. Our results revealed that resveratrol could improve cerebral ischemia through modulating the immune balance of Th17/Tregs and Th1/Th2 as well as the expression of their related cytokines (IL-17A, IL-23, IL-10, IFN-γ, and IL-4) in the small intestine, and subsequently decreasing the ischemia-induced transfer of cytokines from the small intestine to the blood via attenuating the increase in the small intestinal epithelial and vascular permeability. The resulting decrease in neuroinflammatory response due to attenuating post-ischemic inflammation-mediated blood–brain barrier (BBB) injury was eventually involved in the protective effects of resveratrol on acute stroke injury.

Methods

Experimental Animals

Adult male C57BL/6 mice (8–10 weeks) weighing 23–25 g were purchased from Beijing Vital River Laboratory Animal Technology Company. Mice were housed in the specific pathogen-free conditions and were given ad libitum access to food and water. All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and approved by the Animal Care and Use Committee of The First Affiliated Hospital of Zhengzhou University.

Middle Cerebral Artery Occlusion (MCAO) Procedure

The model of transient focal cerebral ischemia was induced by occlusion of middle cerebral artery for 60 min, followed by reperfusion as described previously (Zhang et al. 2014). Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (ketamine: 100 mg/kg; xylazine: 8 mg/kg). The rectal temperature of the mice was maintained at 37 ± 0.5 °C. A silicone-coated nylon-6 monofilament with a rounded tip was introduced into the internal carotid artery (ICA) through a small incision in external carotid artery (ECA) and advanced until mild resistance was felt under an operating microscope right middle cerebral artery. The regional cerebral blood flow (rCBF) during MCAO was monitored by a laser-Doppler probe (Periflux 5000, Perimed

AB, Sweden) positioned at 5 mm lateral and 2 mm posterior to the bregma. Mice with a drop in cCBF < 75% after occlusion and < 80% cCBF increase upon reperfusion were excluded. Sham-operatedmice received same surgical procedures but without ischemia.

Experimental Groups and Drug Administration

The mice were randomly divided into nine groups (Fig. 1): (1) Sham-operation group (sham); (2) Ischemia/reperfusion group (MCAO); (3 and 4) Resveratrol (RES) or 1% DMSO treatment group (MCAO + RES, MCAO + DMSO): RES (200 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) or 1% DMSO (Sigma-Aldrich, St. Louis, MO, USA) was intraperitonealy administered to mice every 24 h for 3 days starting immediately after MCAO onset; (5 and 6) Antibiotics vancomycin (VAN) or amoxicillin and clavulanic acid (AC) pre-treatment group (MCAO + VAN, MCAO + AC): VAN (0.5 g/L; Gold Biotechnology, St. Louis, MO) or AC (1 g/L; West-Ward Pharmaceutical Corp., Eatontown, NJ) was given to mice via drinking water 2 weeks prior to MCAO. The antibiotic solution was changed every 3 days. Administration of vancomycin or AC was discontinued 3 days before MCAO; (7) Fecal microbiota transplantation group (FMT) (MCAO + VAN + FMT): Stroke mice following 3 days of reperfusion were used as donor mice. Cecal contents were harvested from two donor mice, suspended in 2.5 mL PBS/cecum, and then filtered through a 40 μm cell strainer. FMT was performed by gastric gavage with 200 μL of cecal extract every 24 h for 3 days starting immediately after MCAO onset; (8) FMT plus RES treatment (MCAO + VAN + FMT + RES): RES was intraperitonealy administered to mice 30 min after FMT; (9) RES plus recombinant IL-17A (rIL-17A) and rIFN-γ treatment group (MCAO + RES + rIL-17A + rIFN-γ): Mice were injected intravenously with 2 μg mouse rIL-17A (R&D System, Minneapolis, MN) plus 0.5 μg mouse rIFN-γ (eBioscience, San Diego, CA, USA) 30 min after RES treatment.

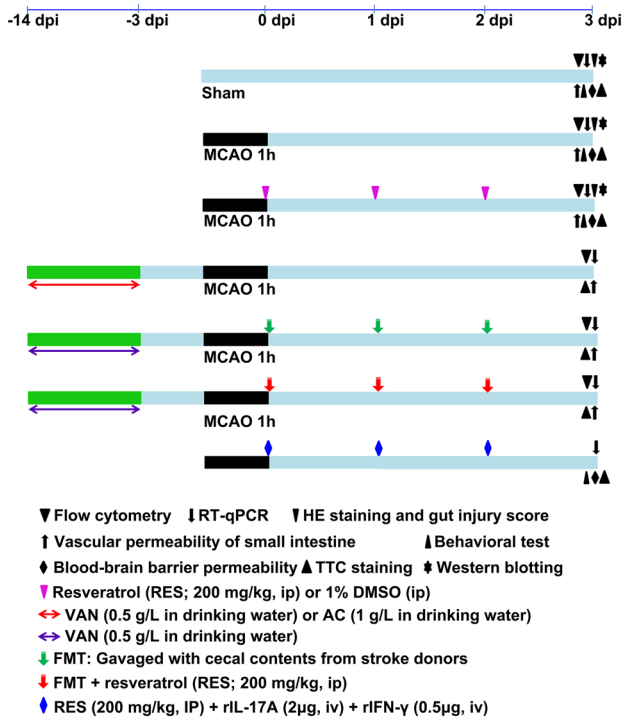


Fig. 1 Experimental procedures and animal groups. Focal cerebral ischemia was induced by transient occlusion of the right middle cerebral artery. $n = 12$/group for behavioral test, and $n = 6$/group for other tests. AC, clavulanic acid; Sham, sham-operation; dpi, days post-ischemia; ip, intraperitoneal injection; iv, intravenous injection; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; VAN, vancomycin; FMT, fecal microbiota transplantation

Behavioral Test

Ischemic stroke-induced functional deficits at 3 dpi were assessed by an 18-point composite neurological score (Lin et al. 2013a, b), which incorporates the observation: (1) spontaneous activity, (2) symmetry in limb movement, (3) forepaw outstretching, (4) climbing, (5) body proprioception, and (6) response to vibrissae touch. Three trials were averaged for each testing, at least 3 min apart. A lower score represents larger neurological deficits.

Mouse Brain Infarction Area Determined with TTC Staining

Mice were euthanized at 3 dpi and their brains were harvested and frozen at − 20 °C for 10 min to keep intact morphology during slicing. Five serial 1-mm coronal sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Amresco, Solon, OH, USA) for 15 min at 37 °C, and then immersed in 4% formalin overnight. The infarct volume was analyzed by Image J software (NIH, Bethesda, MD, USA) outlining infarcted area, the ipsilateral and contralateral hemispheres. The corrected infarct volume (%) = {[Total lesion volume − (ipsilateral hemisphere volume − contralateral hemisphere volume)]/(ipsilateral hemisphere volume + contralateral hemisphere volume)} × 100.

Lamina Propria Lymphocytes Isolation and flow Cytometry

Mice were humanely killed and small intestines were rapidly removed and placed in ice-cold PBS. The small intestine was opened longitudinally after removal of fat and connective tissues. The small intestine was cut into pieces (~ 1.0 cm) after washed in ice-cold PBS and was then incubated twice in 5 mL of 5 mM ethylenediaminetetraacetic

acid (EDTA) in Hank's Buffered Salt Solution (HBSS, Invitrogen, Carlsbad, CA) for 15 min at 37 °C with slow rotation (100 rpm). After the first incubation, the epithelial cell layer was removed by vortexing and filtered through a 100 μm cell strainer. After the second EDTA incubation, the pieces were washed in HBSS and cut into pieces (~ 1 mm^2). The pieces were immersed in 15 mL digestion solution containing 5% FBS (Sigma-Aldrich, St. Louis, MO), collagenase IV (1.75 mg/mL; Roche, Nutley), and DNase I (0.5 mg/mL; Sigma-Aldrich) at 37 °C for 60 min with slow rotation. The digestion procedure was repeated three times. After each digestion, the solution was vortexed and filtered through a 40 μm cell strainer. Supernatants from all three digestions were pooled, washed once with cold flow cytometry (FACS) buffer, and separated by Percoll gradient (Sigma-Aldrich). Lymphocytes in the SI-LP were collected at the interphase of the Percoll gradient, washed once, and resuspended in FACS buffer.

For intracellular cytokine staining, cells obtained from the SI-LP were incubated for 4 h with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience, San Diego, CA) in a tissue culture incubator at 37 °C. The cells were stained for cell-surface expression using optimal concentrations of anti-CD4-FITC, anti-TCR β-PE-cy7, or anti-CD25-PE antibodies followed by intracellular staining of Foxp3-APC, IL-22-PE, or IL-17A-APC using permeabilization buffer (eBioscience, San Diego, CA). All flow cytometry antibodies were purchased from eBioscience (San Diego, CA). Stained cells were measured using FACSverse™ flow cytometer (BD Biosciences) and analyzed using FlowJo version 10 (TreeStar).

Hematoxylin and Eosin Staining

Small intestine was collected at 3 dpi and fixed in 4% formalin for paraffin embedding, sectioning, and hematoxylin and eosin (H&E, Sigma-Aldrich) staining. Villus pictures were obtained using an Olympus light microscope (Olympus BX51, Tokyo, Japan) with an attached digital photograph machine (Olympus E-330, Olympus Optical Co. Ltd., Japan).

Gut Injury Score

The degree of small intestinal injury was quantified using a score scaled at 0 to 2 (Wynn et al. 2016): 0, normal mucosa; 1, development of subepithelial (Gruenhagen's) spaces, vacuolization, or subepithelial lifting limited to the LP or tips of villi; 2, epithelial lifting and vacuolization more than half of the villi, villi distortion, or mucosal ulceration and disintegration of the LP.

ELISA for Albumin Quantification

Mice were humanely killed 3 days after sham or MCAO and feces and plasma samples were collected. Sampled feces (50 mg/mL) and plasma (1:50,000) were diluted in assay buffer containing Tris (50 mM), NaCl (0.1 M) and 0.05% Tween 20. ELISA was performed with corresponding albumin standard dilutions using the mouse albumin ELISA kit (Bethyl Labratories, Montgomery, TX) according to the manufacturer's instructions. Feces/plasma albumin ratio was presented.

Vascular Permeability Assay

2% Evans blue (4 mL/kg; Sigma-Aldrich) in normal saline was injected into the tail vein 3 days after sham or MCAO. 3 h after the Evans blue injection, mice were transcardially perfused with normal saline. For quantitative measurements, each small intestinal segment were weighed and homogenized in 1 mL of *N*,*N*-dimethylformamide (Sigma-Aldrich). After incubation overnight at 55 °C and centrifugation, the supernatants were collected. To evaluate BBB disruption following ischemic stroke, cortex and striatum were harvested and homogenized with 50% trichloracetic acid. The supernatants were also collected after centrifugation. The supernatants were analyzed at 620 nm by spectrophotometry. Each data point represents Evans blue quantification for each mouse.

Western Blotting

Mice were humanely killed and peri-infarct tissue was quickly removed from the ischemic penumbra at 3 dpi. After grinding tissue in RIPA lysis buffer containing protease and phosphatase inhibitors (KeyGen Biotech, Nanjing, China), an equal amount of protein extracts was loaded into each lane of a polyacrylamide–SDS gel, subjected to electrophoresis and resolved proteins transferred to a PVDF membrane (Millipore Co., USA). After blocking with 5% skim milk/TBST, the membranes were incubated with the appropriate primary antibodies overnight at 4 °C. Primary antibodies used were as follows: IL-17A, TNF-α, IL-10, IL-4, IFN-γ, ZO-1, occludin, claudin-1, or β-actin. All primary antibodies were purchased from Abclonal (1:500; Shanghai, China). After washing, the membranes were incubated with the secondary horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse antibodies (1:3000; Proteintech Group, Inc., Wuhan, China) for 2 h. ECL Western Blotting Detection Reagents (Millipore, Billerica, MA, USA) plus BioWest enhanced chemiluminescence (UVP, Upland, CA) were used to detect chemiluminescence. The band intensities were measured with Image J software (NIH, Bethesda, MD, USA) and normalized to those of β-actin.

RNA Extraction and (RT-qPCR)

Total RNA from the small intestine, serum, and peri-infarct tissue was isolated using Trizol Regent (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Reverse transcription was then performed using Taqman reverse transcriptase (Applied Biosystems, Foster City, CA) to obtain cDNA. cDNA was amplified using Power SYBR Green (Applied Biosystems, Foster City, CA). Two-step real-time PCR was performed (95 °C for 15 s, 60 °C for 60-s extension and detection, 40 cycles) with specific primers for IL-17A (forward: 5′-TGTGAAGGTCAACCTCAAAGTCT-3′; reverse: 5′-GAGGGATATCTATCAGGGTCTTCAT-3′), TNF-α (forward: 5′-GACAAGGCTGCCCCGACTACG-3′; reverse: 5′-CTTGGGGCAGGGGCTCTTGAC-3′), IL-10 (forward: 5′-CCAAGCCTTATCGGAAATGA-3′; reverse: 5′-TTTTCACAGGGGAGAATCG-3′), IL-4 (forward: 5′-TGGGTCTCAACCCCCAGCTAGT-3′; reverse: 5′-TGCATGGCGTCCCTTCTCCTGT-3′), IFN-γ (forward: 5′-TTTAACTCAAGTGGCATAGATGTGG-3′; reverse: 5′-TGCAGGATTTTCATGTCACCAT-3′), and β-actin (forward: 5′-AAGGCCAACCGTGAAAAGAT-3′; reverse: 5′-GTGGTACGACCAGAGGCATAC-3′). The relative abundance of each mRNA and the relative changes in mRNA expression were calculated using the Delta Delta cycle threshold (Ct) method (ΔΔCT), where ΔCT is equal to the difference between the target Ct and the reference Ct (C_T target gene $- C_T$ β-actin), $\Delta\Delta CT = \Delta C_T$ sample $- \Delta C_T$ control, and relative quantification $= 2^{-\Delta\Delta CT}$ that represents fold changes compared to control.

Statistical Analysis

All experiments were expressed as the mean ± SEM. Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test. Two groups were compared by two-tailed Student's *t* test. $P < 0.05$ was defined as significant. All tests were performed using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, United States).

Results

Regulatory Effects of Resveratrol on Th17/Treg and Th1/Th2 Balance in the Small Intestine

Acute ischemic stroke increased the differentiation of $CD4^+TCR\beta^+IL\text{-}22^+IL\text{-}17A^+$ Th17 cells in the small intestinal lamina propria (SI-LP) at 3 days post-ischemia (dpi) ($P < 0.05$; Fig. 2a). As the primary cytokine secreted from Th17 cells, IL-17A mRNA expression was increased in the small intestine at 3 dpi ($P < 0.01$; Fig. 2b). IL-23, which is a pro-inflammatory cytokine that promotes the maintenance of Th17 response, is also increased in the small intestine at 3 dpi ($P < 0.01$; Fig. 2b). In contrast, the differentiation of $CD4^+CD25^+Foxp3^+$ Treg cells was decreased in SI-LP at 3 dpi ($P < 0.05$; Fig. 2c). IL-10, a novel inhibitory cytokine secreted by Treg cells, was reduced in the small intestine at 3 dpi ($P < 0.05$; Fig. 2d).

Apart from Th17 and Treg response, classical Th1 and Th2 responses may be also changed after acute ischemic stroke. To examine this, we measured mRNA levels of the cytokines IFN-γ and IL-4 in the small intestine, representing the Th1 and Th2 effector phenotypes, respectively. We found preferential elevation of IFN-γ expression and a decline in IL-4 expression at 3 dpi (Fig. 2e). Th1 cytokine TNF-α, besides IFN-γ, was also increased in the small intestine at 3 dpi ($P < 0.01$; Fig. 2f).

Resveratrol treatment attenuated cerebral ischemia-induced increase in Th17 cell differentiation ($P < 0.05$; Fig. 2a) and decrease in Treg cell differentiation ($P < 0.05$; Fig. 2c) in SI-LP at 3 dpi. Th17-related cytokines IL-17A and IL-23 levels were decreased ($P < 0.05$ and $P < 0.05$, respectively; Fig. 2b), whereas Treg cytokine IL-10 was increased ($P < 0.05$; Fig. 2d) in the small intestine at 3 dpi after the treatment with resveratrol. In addition, resveratrol treatment diminished cerebral ischemia-induced increase in IFN-γ ($P < 0.05$; Fig. 2e) and TNF-α ($P < 0.05$; Fig. 2f) expression and decrease in IL-4 expression ($P < 0.05$; Fig. 2e) in the small intestine at 3 dpi. The IL-4 to IFN-γ mRNA ratio at 3 dpi was increased after the treatment with resveratrol ($P < 0.05$; Fig. 2e).

Resveratrol Decreased Small Intestinal Epithelial and Vascular Permeability After Ischemic Stroke

Consistent with previous research (Stanley et al. 2016), acute cerebral ischemia could induce dysfunction of the epithelial barrier accompanied by an increase in vascular permeability to Evans blue dye in the jejunum and ileum ($P < 0.05$ and $P < 0.05$, respectively; Fig. 3a). We measured a significantly increased protein leakage by feces/plasma albumin ratio compared with sham-treated controls at 3 dpi ($P < 0.01$; Fig. 3b), suggesting a functionally impaired intestinal barrier after ischemic stroke. Resveratrol treatment decreased the vascular permeability to Evans blue dye in the jejunum and ileum ($P < 0.05$ and $P < 0.05$, respectively; Fig. 3a) and reduced protein leakage by feces/plasma albumin ratio at 3 dpi ($P < 0.05$; Fig. 3b).

We next examined the histology of the small intestine to determine whether resveratrol could attenuate stroke associated disruption of the intestinal architecture. In contrast to ileum in sham-treated controls, the ileum after stroke showed histologic evidence of progressive separation of the

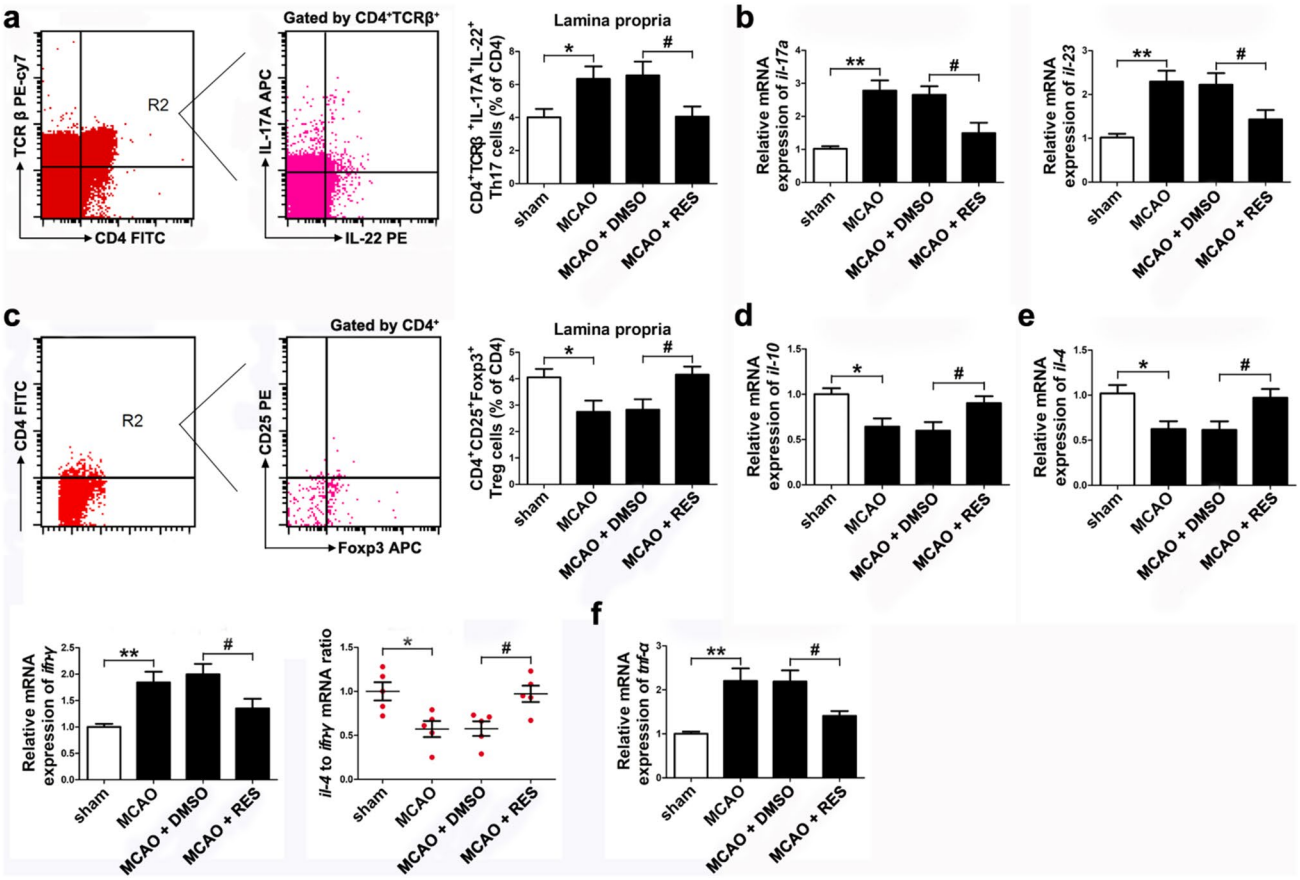


Fig. 2 Resveratrol relieved intestinal pro-inflammatory immune responses and reduced intestinal epithelial and vascular permeability of small intestine after ischemic stroke. **a**, **c** T lymphocyte subsets of small intestinal lamina propria lymphocyte (LPL) in each group as measured by flow cytometry. The mononuclear cells were harvested from LP and strained with various combinations of mAbs (Th17: $TCR\beta^+CD4^+$ $IL\text{-}22^+IL\text{-}17A^+$; Treg: $CD4^+CD25^+Foxp3^+$) at 3 days post-ischemia (dpi). Data represent mean ± SEM, $n = 5$/group; $^*P < 0.05$, $^{\#}P < 0.05$. **b**, **d**–**f** Levels of IL-17A, IL-23, IL-10, IL-4, IFN-γ, and TNF-α in the small intestine were measured by RT-qPCR at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^*P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$

villi from the thickened basement membrane without substantial changes in villus structure (Fig. 3c, d). Resveratrol treatment to stroke mice reduced histopathological damage ($P < 0.05$; Fig. 3c, d).

We also found that the protein levels of ZO-1, claudin-1, and occludin were significantly decreased in the MCAO group, compared to sham-operated group (all $P < 0.05$; Fig. 3e, f). Resveratrol treatment to stroke mice significantly increased the protein levels of ZO-1, claudin-1, and occludin ($P < 0.05$ or $P < 0.01$; Fig. 3e, f). These data imply that resveratrol could alleviate damaged permeability of the intestinal mucosa.

Resveratrol Modulated Cytokines Expression in Serum After Ischemic Stroke

The mRNA levels of the cytokines IL-17A, IFN-γ and TNF-α were increased, whereas IL-4 and IL-10 mRNA expression levels were decreased in the serum at 3 dpi (Fig. 4a–e). The IL-4 to IFN-γ mRNA ratio was decreased at 3 dpi ($P < 0.05$; Fig. 4f).

Resveratrol treatment decreased the mRNA levels of the cytokines IL-17A, IFN-γ, and TNF-α in the serum of stroke mice at 3 dpi (Fig. 4a–c). The levels of IL-10 ($P < 0.05$; Fig. 4d) and IL-4 mRNA ($P < 0.05$; Fig. e) and the ratio of IL-4/IFN-γ mRNA ($P < 0.05$; Fig. 4f) at 3 dpi were increased after the treatment with resveratrol.

Resveratrol Decreased Serum Pro-inflammatory Cytokines and Infarct Volumes Through Inhibiting Small Intestinal Pro-inflammatory Immune Responses and Decreasing Intestinal Vascular Permeability

To confirm the role of small intestinal immuno-inflammatory response in systemic inflammatory response after acute stroke, mice were pre-treated with the antibiotics vancomycin (VAN) or amoxicillin and clavulanic acid (AC) starting

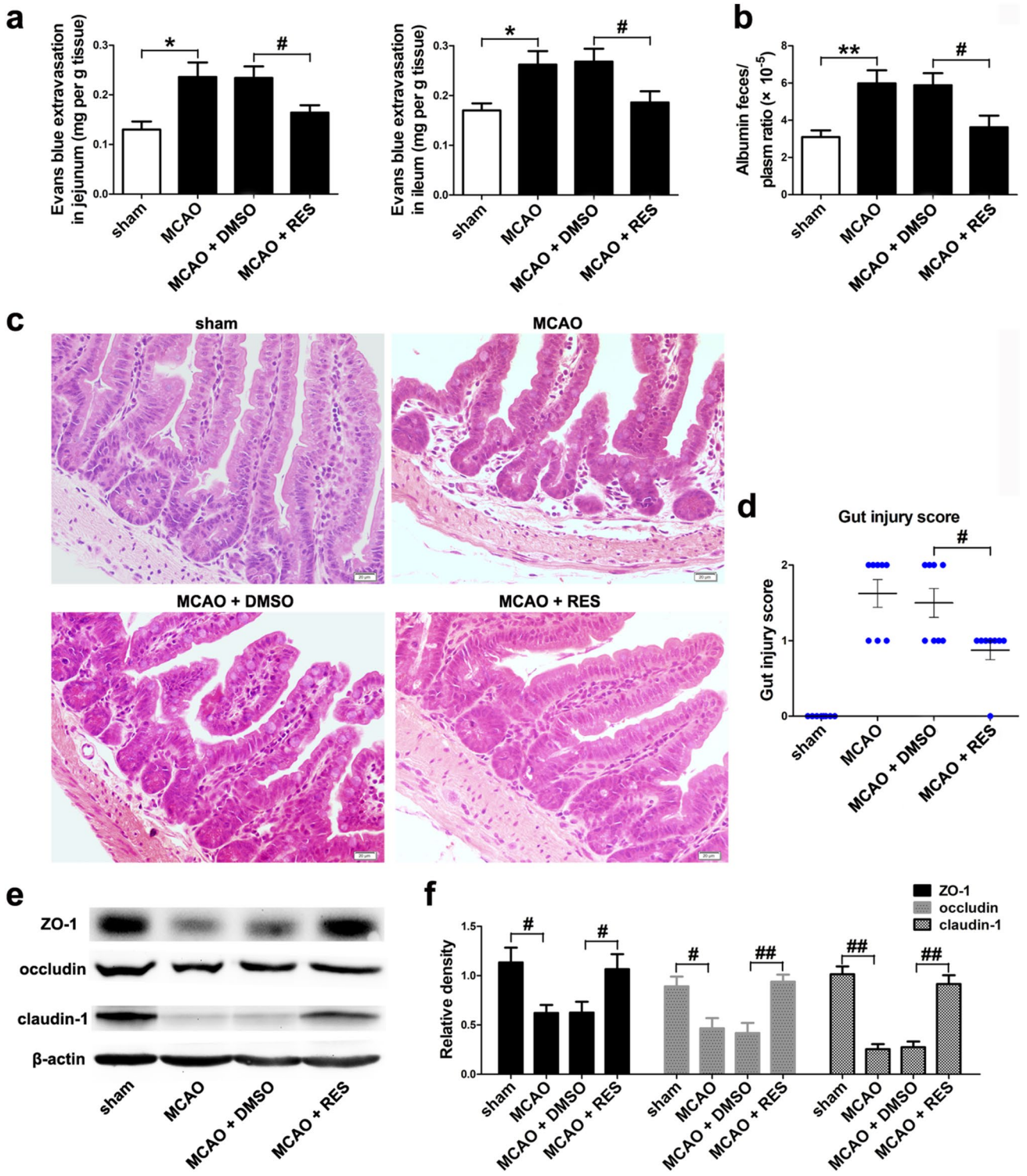


Fig. 3 Resveratrol reduced small intestinal epithelial and vascular permeability and small intestinal histological damage following stroke. **a** Quantification of Evans blue dye extravasation in the jejunum and ileum at 3 days post-ischemia (dpi). Data represent mean ± SEM, $n = 6$/group; $^{*}P < 0.05$, $^{\#}P < 0.05$. **b** Albumin concentrations were determined by ELISA and are represented as the ratio of the concentrations in feces and plasma from each group. Data represent mean ± SEM, $n = 6$/group; $^{**}P < 0.01$, $^{\#}P < 0.05$. **c** Haematoxylin–eosin (H&E) staining (magnification: ×400) of small intestine from each group at 3 dpi. **d** Gut injury score for each respective group at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$. **e**, **f** Western blotting and quantitative data for zonula occludens 1 (ZO-1), occludin, and claudin-1 in the small intestine at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$

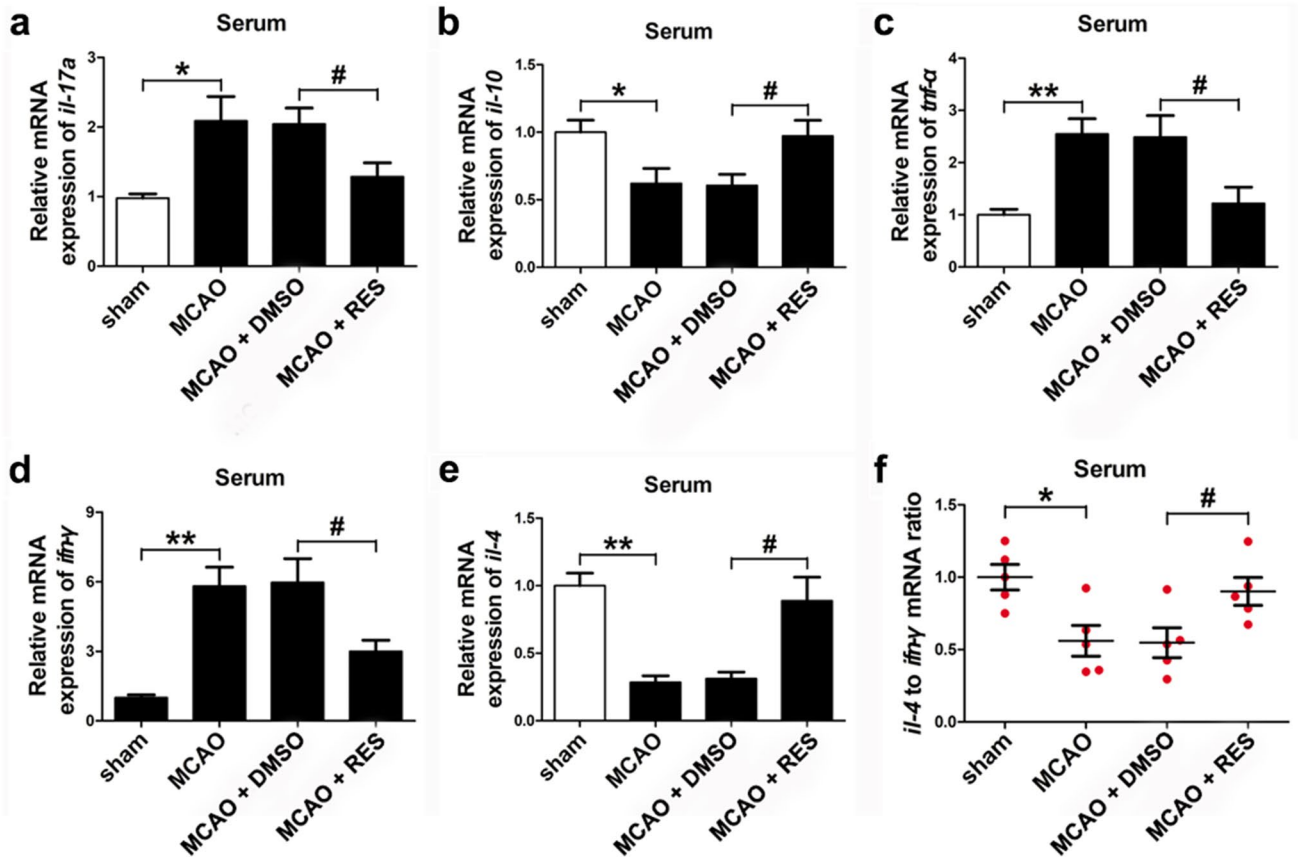


Fig. 4 Resveratrol decreased serum levels of pro-inflammatory cytokines and increased anti-inflammatory cytokines after stroke. **a**–**e** Levels of IL-17A, IFN-γ, TNF-α, IL-10 and IL-4 in serum were measured by RT-qPCR at 3 days post-ischemia (dpi). Data represent mean ± SEM, $n = 6$/group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$. **f** The IL-4 to IFN-γ mRNA ratio in the serum at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{*}P < 0.05$, $^{\#}P < 0.05$

2 weeks prior to MCAO. Consistent with previous results (Benakis et al. 2016), VAN or AC treatment increased the percentage of Treg cells ($P < 0.05$ and $P < 0.05$, respectively; Fig. 5c) and Treg-related cytokine IL-10 ($P < 0.05$ and $P < 0.05$, respectively; Fig. 5f) in the small intestine at 3 dpi, whereas Th17 cells were not affected ($P > 0.05$; Fig. 5a, b). We also found an elevation in IL-4 expression ($P < 0.01$ and $P < 0.05$, respectively) and a decline of IFN-γ expression ($P < 0.05$ and $P < 0.05$, respectively) after the treatment with VAN or AC (Fig. 5g–i). In addition, the pro-inflammatory cytokines IL-17A ($P < 0.05$ and $P < 0.05$, respectively; Fig. 5e) and Th1-related cytokine TNF-α ($P < 0.05$ and $P < 0.05$, respectively; Fig. 5j) were decreased in the small intestine at 3 dpi after VAN or AC treatment.

As expected, the mRNA levels of IL-17A, IFN-γ, and TNF-α were decreased, whereas IL-4 and IL-10 mRNA levels were increased in the serum at 3 dpi (Fig. 6a–e). The IL-4 to IFN-γ mRNA ratio was increased ($P < 0.05$ and $P < 0.05$; Fig. 6f), which may be due to VAN or AC pre-treatment induced decrease in vascular permeability to Evans blue dye in the jejunum ($P < 0.05$ and $P < 0.05$, respectively; Fig. 5g) and ileum ($P < 0.05$ and $P < 0.05$, respectively; Fig. 5l) at 3 dpi. We also found that VAN or AC pre-treatment to stroke mice significantly increased the protein levels of ZO-1, claudin-1, and occludin (all $P < 0.05$; Fig. 6m) and decreased infarct volumes ($P < 0.05$; Fig. 6g, h). Our data further provided evidence that the increase in epithelial and vascular permeability in the jejunum and ileum after ischemic stroke could be directly relevant to the increased pro-inflammatory cytokines in serum and ischemic injury.

To furtherly confirm whether the neuroprotective effects of RES were directly mediated by the intestinal flora, we performed single fecal transplants by transferring the flora from stroke mice to mice that were pre-treated with VAN. RES was administrated 30 min after FMT. We found that FMT significantly decreased the percentage of Tregs ($P < 0.05$; Fig. 5c) in SI-LP at 3 dpi, whereas Th17 cells were not significantly affected in VAN-pre-treated mice ($P > 0.05$; Fig. 5a, b). The mRNA levels of IL-17A, IFN-γ, and TNF-α were increased, whereas IL-4 and IL-10 mRNA expression levels were increased in both small intestine and serum in VAN-pretreated mice that received

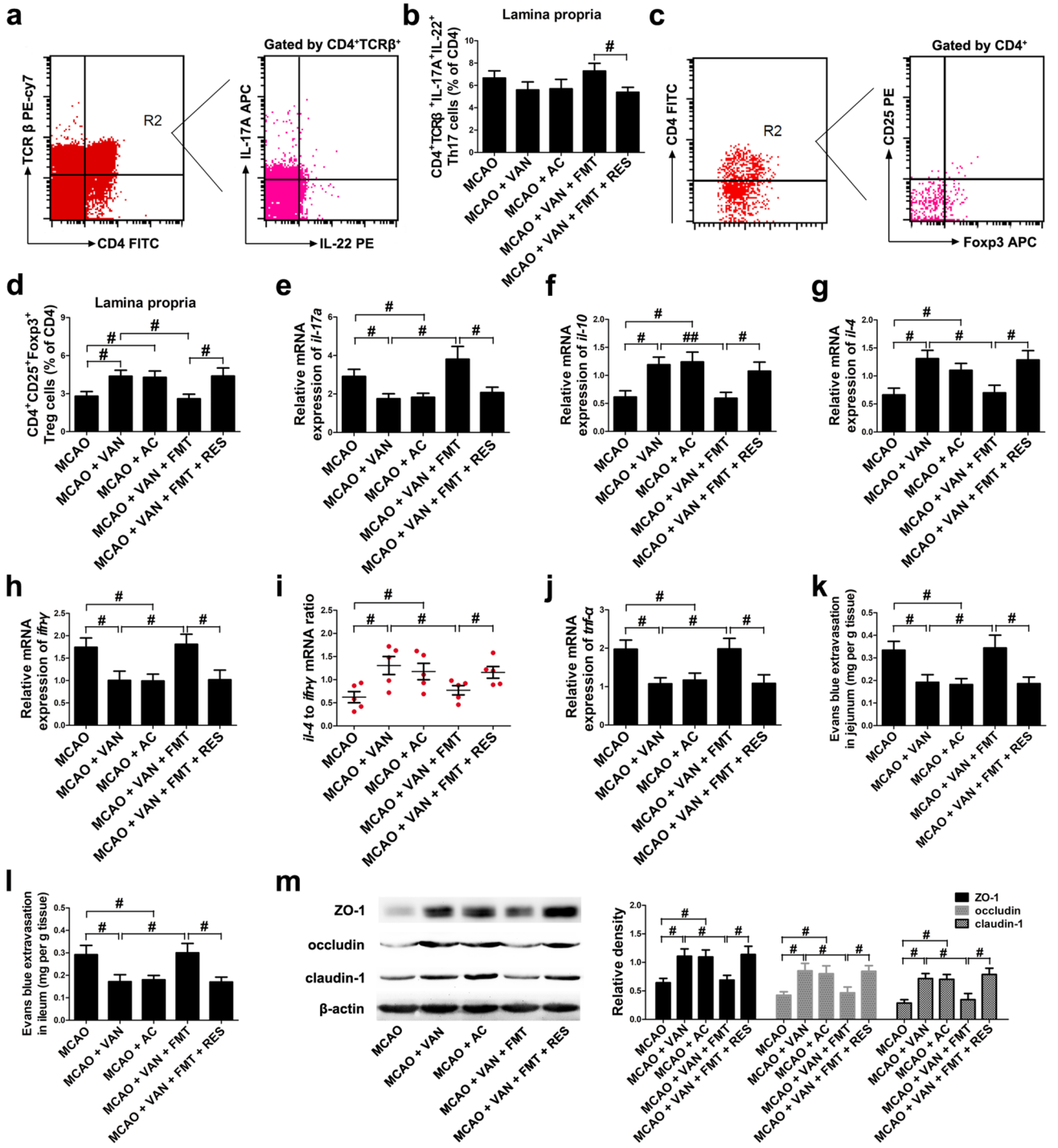
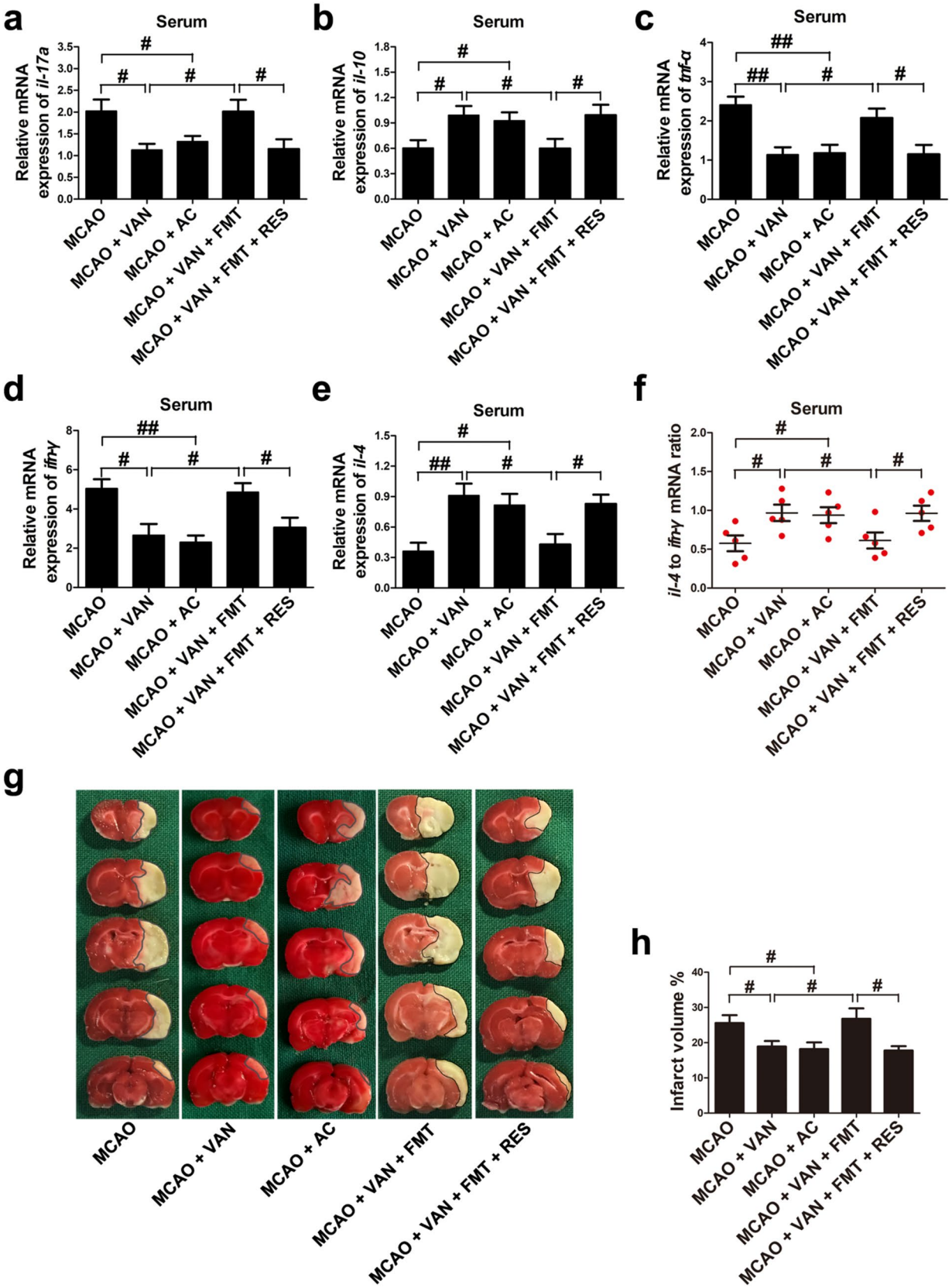


Fig. 5 Resveratrol reduced small intestinal pro-inflammatory cytokines and vascular permeability through modulating intestinal flora. **a**–**d** T lymphocyte subsets of small intestinal lamina propria lymphocyte (LPL) in each group as measured by flow cytometry. The mononuclear cells were harvested from LP and strained with various combinations of mAbs (Th17: $TCR\beta^+CD4^+$ $IL\text{-}22^+IL\text{-}17A^+$; Treg: $CD4^+CD25^+Foxp3^+$) at 3 days post-ischemia (dpi). Data represent mean ± SEM, $n = 12$/group; $^{\#}P < 0.05$. **e**–**j** Levels of IL-17A, IL-10, IL-4, IFN-γ, and TNF-α in the small intestine were measured by RT-qPCR at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **k**, **l** Quantification of Evans blue dye extravasation in the jejunum and ileum at 3 days post-ischemia (dpi). Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$. **m** Western blotting and quantitative data for zonula occludens 1 (ZO-1), occludin, and claudin-1 in the small intestine at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$


a
Serum
Relative mRNA expression of il-17a
b
Serum
Relative mRNA expression of il-10
c
Serum
Relative mRNA expression of tnf-α
d
Serum
Relative mRNA expression of ifnγ
e
Serum
Relative mRNA expression of il-4
f
Serum
il-4 to ifnγ mRNA ratio
MCAO
MCAO + VAN
MCAO + AC
MCAO + VAN + FMT
MCAO + VAN + FMT + RES
g
h
Infarct volume %

◂**Fig. 6** Resveratrol reduced serum pro-inflammatory cytokines through modulating intestinal flora. **a**–**f** The mRNA levels of IL-17A, IL-10, TNF-α, IFN-γ, and IL-4 in serum were measured by RT-qPCR at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **g** Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct in the mice brain at 3 days post-ischemia (dpi). **h** Statistical analysis of the percentage of infarct volume was determined for each group. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$

fecal transplants (Figs. 5e–j, 6a–f), which is accompanied by an increase in infarct volumes ($P < 0.05$; Fig. 6g, h). RES treatment significantly increased the percentage of Tregs ($P < 0.05$; Fig. 5c), whereas decreased the percentage of Th17 cells in SI-LP in VAN-pretreated mice that received fecal transplants ($P < 0.05$; Fig. 5a, b). RES treatment also significantly decreased the mRNA levels of IL-17A, IFN-γ, and TNF-α, whereas increased IL-4 and IL-10 mRNA expression levels in both small intestine and serum in VAN-pretreated mice that received fecal transplants (Fig. 5e–j, 6a–f), which may be due to the improvement in FMT-mediated exaggeration of vascular permeability to Evans blue dye in the small intestine (Fig. 5g, l). RES treatment decreased infarct volumes in VAN-pretreated mice that received fecal transplants ($P < 0.05$; Fig. 6g, h).

Resveratrol Protected BBB Integrity and Inhibited Neuroinflammation Following MCAO

Ischemia-induced BBB disruption can be reflected by elevated concentrations of vascular tracer evans blue leaking into the ischemic brain parenchyma. Evans blue extravasation into the ipsilateral cortex ($P < 0.05$) and striatum ($P < 0.01$) was significantly increased in stroke mice than in sham-treated control mice (Fig. 7a). Resveratrol treatment remarkably reduced evans blue leakage into the ischemic cortex ($P < 0.05$) and striatum ($P < 0.01$) at 3 dpi (Fig. 7a).

The mRNA and protein levels of IL-17A, IFN-γ, and TNF-α were increased, whereas the mRNA and protein levels IL-4 and IL-10 were decreased in the peri-infarct region at 3 dpi (Fig. 7b–j). The IL-4 to IFN-γ mRNA ratio was decreased at 3 dpi ($P < 0.01$; Fig. 7k).

Resveratrol treatment decreased the mRNA and protein levels of IL-17A ($P < 0.05$ and $P < 0.05$, respectively; Fig. 7b, c), TNF-α ($P < 0.05$ and $P < 0.05$, respectively; Fig. 7d, e), and IFN-γ ($P < 0.05$ and $P < 0.05$, respectively; Fig. 7g, h) in the peri-infarct region at 3 dpi. The mRNA and protein levels of IL-4 ($P < 0.05$ and $P < 0.01$, respectively; Fig. 7f, h) and IL-10 ($P < 0.05$ and $P < 0.05$, respectively; Fig. 7i, j) and the ratio of IL-4/IFN-γ mRNA ($P < 0.01$; Fig. 7k) in the peri-infarct region were increased after the treatment with resveratrol at 3 dpi.

Resveratrol Reduced Infarct Volumes and Promoted Functional Recovery via Inhibition of Neuroinflammation

Compared to stroke mice, resveratrol treatment significantly decreased infarct volumes at 3 dpi ($P < 0.05$; Fig. 8a, b). Sham-operated rats did not have any deficits. Resveratrol-treated mice had significantly greater scores than stroke mice at 3 dpi ($P < 0.05$; Fig. 8c).

To assess whether these neuroprotective effects were directly mediated by the inhibition of neuroinflammation, rIL-17A and rIFN-γ were administrated intravenously to mice 30 min after RES treatment. We found that exogenous supplementation of IL-17A and IFN-γ abrogated resveratrol-mediated attenuation of BBB disruption in the ipsilateral cortex ($P < 0.05$) and striatum ($P < 0.05$; Fig. 8d), and decrease in the mRNA levels of IL-17A ($P < 0.05$) and IFN-γ ($P < 0.05$) in the peri-infarct region (Fig. 8e). Accordingly, the infarct volumes ($P < 0.05$; Fig. 8a, b) and functional deficits ($P < 0.05$; Fig. 8c) were increased.

Discussion

The major findings in the present study are: (1) Cerebral ischemia-induced increase in the intestinal epithelial and vascular permeability appears to be responsible for the penetration of increased intestinal inflammatory cytokines to the peripheral blood; (2) Resveratrol treatment could attenuate the blood levels of pro-inflammatory cytokines through promoting Th1/Th2 balance towards Th2 polarization and skewing Treg/Th17 balance toward Treg in the SI-LP, as well as decreasing small intestinal pro-inflammatory cytokines and intestinal vascular permeability; (3) Resveratrol attenuates cerebral ischemic injury through attenuation of blood pro-inflammatory cytokines-mediated amelioration of BBB disruption and neuroinflammation.

Resveratrol is associated with anti-inflammatory, antioxidant, and anti-apoptotic properties, which has been used for the treatment of various neuroinflammatory and neurodegenerative diseases such as stroke, epilepsy, spinal cord injury, Huntington's disease, and Alzheimer's disease (Pangeni et al. 2014; Rauf et al. 2017). Previous experimental studies have suggested that administration of resveratrol could reduce infarct volume after acute ischemic stroke (Jeong et al. 2016; Lin et al. 2013a, b; Lopez et al. 2015). Mechanisms underlying resveratrol-induced neuroprotection against cerebral ischemia are multifactorial (Lee et al. 2018). In the present study, the mechanism by which resveratrol confers neuroprotection after stroke could be by modulating intestinal immune cells-mediated inflammatory responses in the small intestine. We found that acute cerebral ischemia inhibited Th2 and Treg immune responses, whereas

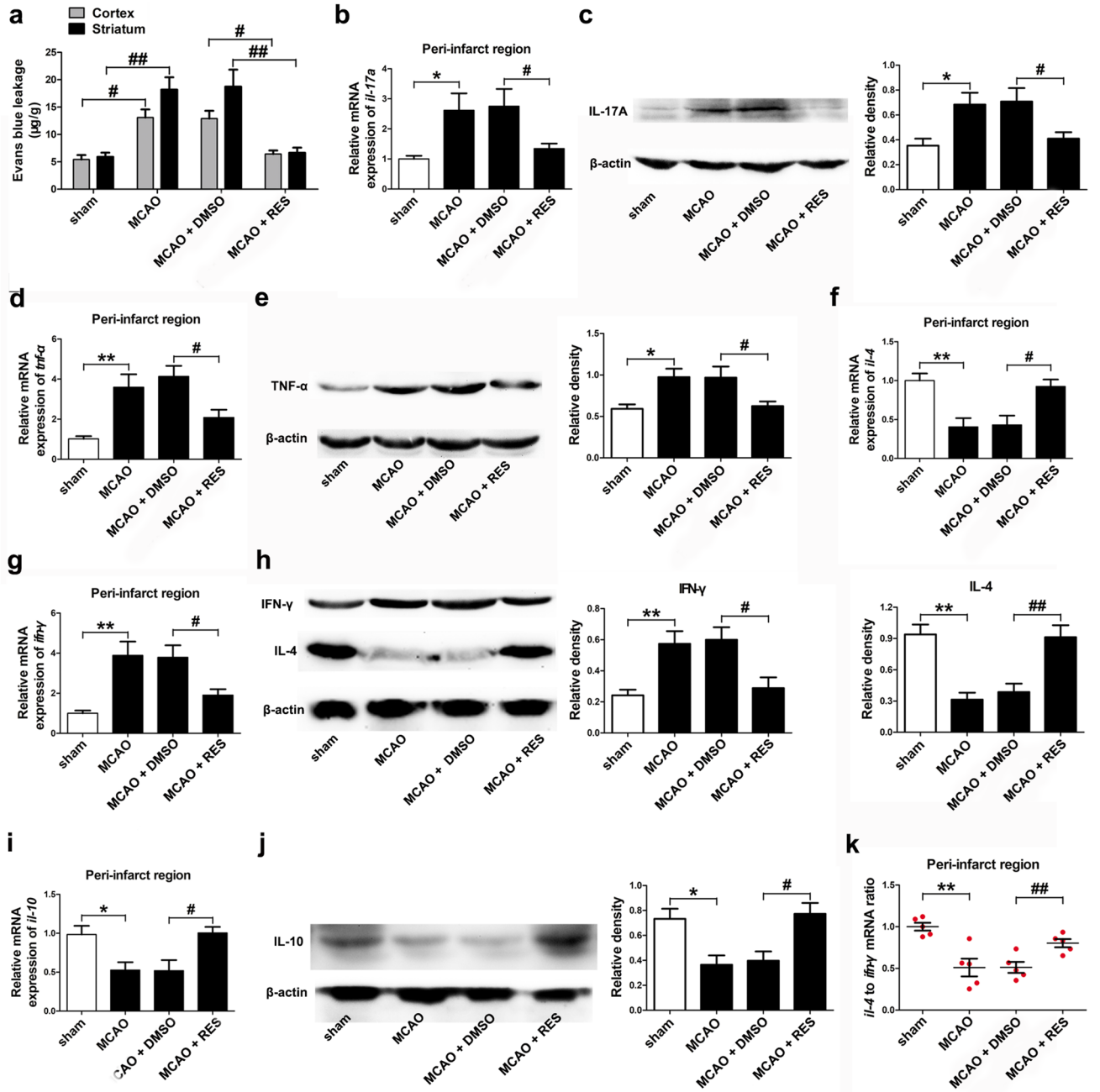


Fig. 7 Resveratrol protected blood–brain barrier (BBB) integrity and inhibited neuroinflammation after ischemic stroke. **a** Tissue concentrations of evans blue leaking into the ischemic (ipsilateral) cortex and striatum at 3 days post-ischemia (dpi). Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **b**, **d**, **f**, **g**, **i** The mRNA Levels of IL-17A, TNF-α, IL-4, IFN-γ, and IL-10 in the peri-infarct region were measured by RT-qPCR at 3 days post-ischemia (dpi). Data represent mean ± SEM, $n = 6$/group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **c, e, k, j** Western blotting and quantitative data for IL-17A, TNF-α, IFN-γ, IL-4, and IL-10 in the peri-infarct region at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **k** The IL-4 to IFN-γ mRNA ratio in the peri-infarct region at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{**}P < 0.01$, $^{\#\#}P < 0.01$

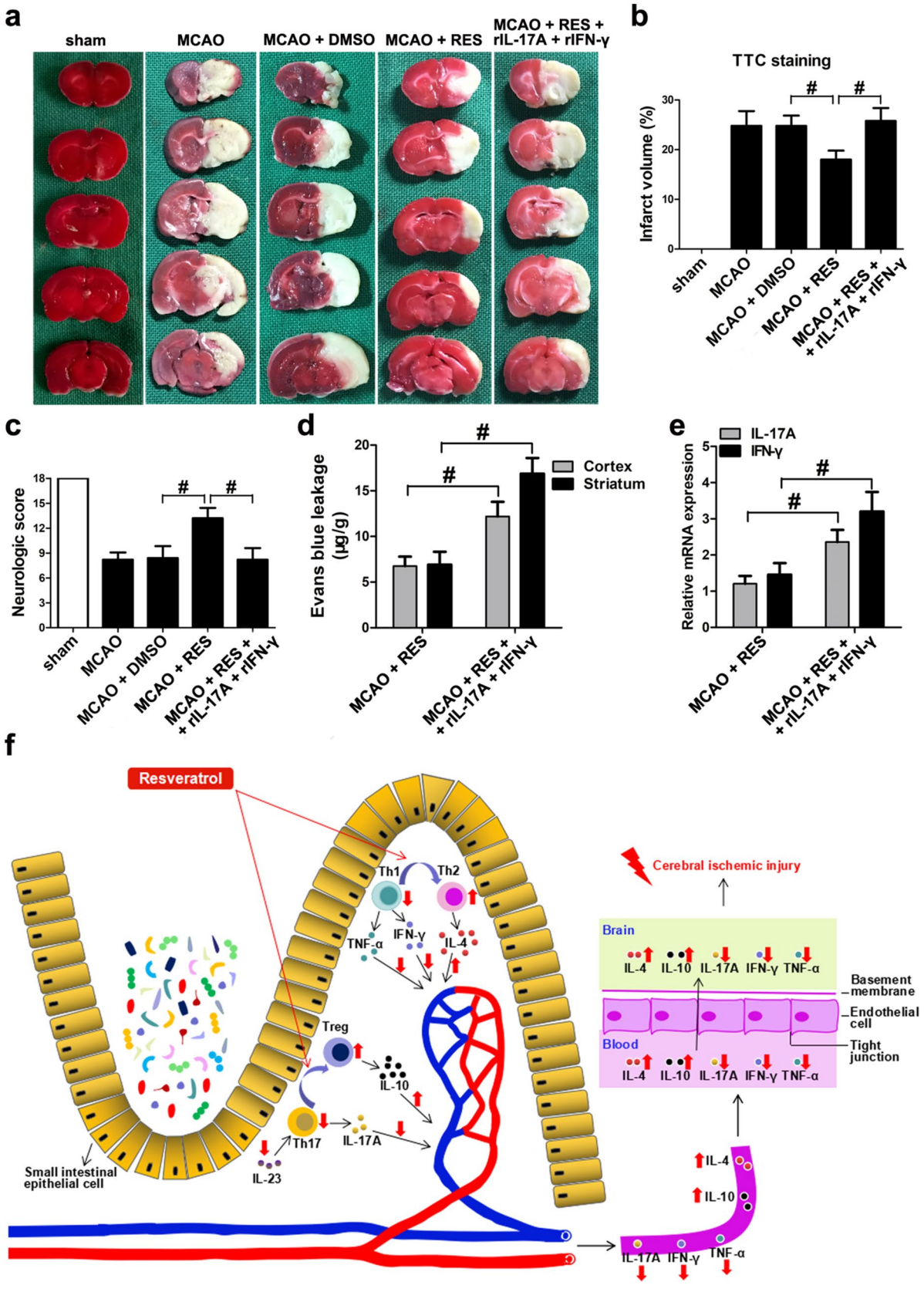
promoted Th1 and Th17 immune responses in the small intestine. Furthermore, we showed that post-stroke treatment with resveratrol promoted Th1/Th2 balance towards Th2 polarization and skewed Treg/Th17 balance towards Treg in the small intestine. Accordingly, Th2-related cytokines IL-4 and Treg-related cytokine IL-10 were increased, whereas Th1-related cytokines (IFN-γ, TNF-α) and Th17-related cytokines (IL-17A, IL-23) were decreased in the small intestine after resveratrol treatment. The neuroprotective effects mediated by small intestinal Treg and Treg-related IL-10 on

ischemic stroke is consistent with a previous report (Benakis et al. 2016). Therefore, our results reveal a previously uncharacterized property of small intestinal Th17/Tregs and Th1/Th2 balance and their related cytokines expression in resveratrol-mediated neuroprotection aganist acute stroke injury.

The change of these cytokines expression in the small intestine after acute ischemic stroke is consistent with that in the peripheral blood. We speculate that the cerebral ischemia-induced increase in the intestinal vascular permeability may be responsible for the penetration of intestinal inflammatory cytokines across the intestine to the peripheral blood. One thing to note is that cytokines could be produced by a variety of activated cell types during cerebral ischemia, including microglia, neurons, platelets, leukocytes, fibroblasts, and endothelial cells (Huang et al. 2006), which are not confined to the intestine. Therefore, we performed further experiments to characterize the roles of ischemia-induced intestinal pro-inflammatory immune response and increased intestinal vascular permeability in the peripheral blood immune response after acute stroke. In our present study, broad-spectrum antibiotics VAN or AC were pre-administrated to mice to alter intestinal flora. Evidence has shown that the neuroprotective effects of VAN or AC were mediated by the altered intestinal flora-induced anti-inflammatory immune response in the small intestine after acute ischemic stroke (Benakis et al. 2016). Our results furtherly indicated that the decrease in intestinal pro-inflammatory cytokines and intestinal vascular permeability mediated by antibiotics pre-treatment was directly relevant to the decreased serum pro-inflammatory cytokines after stroke. VAN-pre-treated mice gavaged with cecal contents from stroke mice have significant increase in small intestinal pro-inflammatory cytokines and small intestinal vascular permeability, which was also accompanied by the increase in the pro-inflammatory cytokines. However, RES treatment abrogated FMT-mediated increase in intestinal pro-inflammatory cytokines, intestinal vascular permeability, serum pro-inflammatory cytokines, and infarct volumes in VAN-pre-treated mice. Therefore, resveratrol treatment may attenuate the blood levels of pro-inflammatory cytokines in part through shifting Th17/Tregs and Th1/Th2 immune responses toward anti-inflammatory responses, and decreasing the pro-inflammatory cytokines expression and intestinal vascular permeability in the acute phase of stroke. However, the mechanisms by which resveratrol modulates small intestinal Th17/Tregs and Th1/Th2 balance by intestinal flora after ischemc stroke are unclear. Resveratrol could mediate changes in small intestinal microbiota and subsequent production of different metabolites produced or synthesized de novo by gut microbes that could exert effects on the immune balance of Th17/Tregs and Th1/Th2 after ischemic stroke (Ladinsky et al. 2019; Postler and Ghosh 2017).

There is ample evidence that the inflammatory response contributes to acute stroke injury (Doyle et al. 2008; Iadecola and Anrather 2011). Pro-inflammatory cytokines (IL-1β, IFN-γ, TNF-α, and IL-6) could aggravate brain infarction both in humans and in experimental stroke models (Doyle et al. 2008; Lambertsen et al. 2012; Wang et al. 2018). Following acute ischemic stroke, one major mechanism underlying BBB disruption is increased systemic inflammatory responses (Pillai et al. 2013). Pro-inflammatory cytokines induced matrix metalloproteinases-9 (MMP-9) can lead to the breakdown of extracellular matrix and direct degrading of tight junction proteins, thus lead to BBB damage and neuroinflammation-mediated acute stroke injury (Asahi et al. 2001; Lakhan et al. 2009; Yang and Rosenberg 2011). Consistently, we observed that acute cerebral ischemia induced robust increase in pro-inflammatory cytokines (IL-17A, IFN-γ, and TNF-α) levels and decrease in anti-inflammatory cytokines (IL-10, IL-4) levels in the peripheral blood with a concomitant increase in BBB disruption in the cortex and striatum. Evidence has shown that resveratrol treatment could prevent the MMP-9 expression and activation after stroke resulting in neuroprotection (Gao et al. 2006). Further, we showed that post-ischemic administration of resveratrol robustly protected against ischemia-induced BBB disruption and attenuated neuroinflammatory response at 3 dpi, as evidenced by reduced evans blue extravasasion and pro-inflammatory cytokines levels in the peri-infarct area. Resveratrol-mediated attenuation of pro-inflammatory cytokines blood levels ameliorated BBB disruption not only in the cortex but also in the striatum. However, resveratrol-mediated attenuation of BBB disruption, decrease in pro-inflammatory cytokines, and improved stroke outcomes were abrogated by exogenous supplementation of IL-17A and IFN-γ. This indicated that improved BBB integrity and decrease inflammation due to reduced systemic inflammatory response could be directly attributed to resveratrol-mediated smaller cerebral infarcts and less neurological deficits.

However, there are some limitations in our present study. We did not measured the microbiota composition using 16S ribosomal RNA gene sequencing analysis. It would be better to perform 16S ribosomal RNA gene sequencing analysis of microbial flora after resveratrol treatment, broad-spectrum antibiotics administration, and (or) fecal microbiota transplantation to further validate the roles of intestinal flora-mediated Th cells differentiation and homeostasis in resveratrol-mediated neuroprotection against acute stroke injury. Further study are needed to determine the changes of intestinal flora following different interventions.


a
sham
MCAO
MCAO + DMSO
MCAO + RES
MCAO + RES + rIL-17A + rIFN-γ
b
TTC staining
Infarct volume (%)
c
Neurologic score
d
Evans blue leakage (μg/g)
Cortex
Striatum
e
Relative mRNA expression
IL-17A
IFN-γ
f
Resveratrol
Th1
Th2
TNF-α
IFN-γ
IL-4
Treg
IL-10
Th17
IL-17A
IL-23
Small intestinal epithelial cell
Cerebral ischemic injury
Brain
IL-4
IL-10
IL-17A
IFN-γ
TNF-α
Basement membrane
Endothelial cell
Tight junction
Blood

◂**Fig. 8** Resveratrol reduced infarct volumes and promoted functional recovery. **a** Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct at 3 days post-ischemia (dpi). **b** Statistical analysis of the percentage of infarct volume was determined for each group. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$. **c** Quantification of neurologic scores at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$. **d** Tissue concentrations of evans blue leaking into the ischemic cortex and striatum at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$. **e** The mRNA levels of IL-17A and IFN-γ in the peri-infarct region were measured by RT-qPCR at 3 dpi. Data represent mean ± SEM, $n = 6$/group; $^{\#}P < 0.05$. **f** Proposed mechanism of protection from ischemic brain injury induced by resveratrol. Resveratrol exerts neuroprotective effects through promoting Th1/Th2 balance towards Th2 polarization and skewing Treg/Th17 balance towards Treg in the SI-LP, and decreasing small intestinal pro-inflammatory cytokines and intestinal epithelial and vascular permeability, which attenuates the blood levels of pro-inflammatory cytokines and alleviate cytokines-mediated BBB disruption and neuroinflammation

Conclusions

The present study demonstrates that post-stroke administration of resveratrol has protective effects against acute stroke injury in mice, and the neuroprotective effects may be attributed to promoting Th1/Th2 balance towards Th2 polarization and skewing Treg/Th17 balance towards Treg in the SI-LP, and decreasing small intestinal pro-inflammatory cytokines and intestinal vascular permeability, which attenuates the blood levels of pro-inflammatory cytokines from small intestine and alleviate cytokines-mediated BBB disruption and neuroinflammation (Fig. 8f). The finding provides further insight into the important role of gut-brain axis in acute ischemic stroke, and therefore represents a novel potential therapeutic target. Our study also provides new mechanism by which resveratrol exerts its neuroprotection and suggest that resveratrol might be of therapeutic value for the treatment of acute ischemic stroke.

Author Contributions Experimental design: YQL; stroke model, behavioral test, flow cytometry, TTC staining, western blotting, permeability assay, ELISA: ZGD, XFR, EXZ; RT-qPCR, hematoxylin and eosin staining, gut injury score: LXZ; imaging tools: ZCD; data analysis: XFR; wrote article: ZCD.

Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and approved by the Animal Care and Use Committee of The First Affiliated Hospital of Zhengzhou University.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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