Electroacupuncture Treatment Ameliorates Post-Stroke Intestinal Inflammation Injury via Regulating the Balance of Treg / γδ T Cells

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Research article

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Abstract

Much evidence suggests that gut immunity homeostasis plays a vital role in regulating neuroinflammatory response under ischemic stroke. Acupuncture is an effective treatment for post-stroke rehabilitation. However, little is known about the effect of acupuncture on intestinal injury after stroke. Herein, we observed the intestinal inflammatory response and barrier's impairment of the post-stroke rats to explore the mechanism of electroacupuncture (EA) against cerebral ischemia injury from the brain-gut axis. Rats were randomized to the sham operation group (SHAM), the middle cerebral artery occlusion group (MCAO), and the MCAO plus EA treatment group (MEA). Triphenyl tetrazolium chloride (TTC) staining and Longa neurologic score were performed to evaluate the outcomes after ischemic stroke. Inflammatory factors expression levels in the serum, ischemic hemisphere brain, and small intestine were detected. Additionally, the morphology change of small intestine was evaluated via analyzing villus height and smooth muscle thickness. Meanwhile, the expression of tight-junction proteins, including Zonula Occludens-1(ZO-1), Occludin and Claudin-1, was detected to evaluate the impact of EA on mucosal permeability in the small intestine. The percentage of Treg cells ($CD45^+CD4^+Foxp3^+$) and γδ T cells ($CD45^+CD4^-γδ\ T^+$) were measured to assess the effect of EA on T cells. Our data showed that EA could significantly decrease the brain infarction volume and intestine barrier injury of the ischemic stroke rats. At the same time, it effectively suppressed the post-stroke inflammation response of the ischemic brain, small intestine, and peripheral serum. More importantly, EA treatment increased the percentage of Treg cells in the small intestine while reducing the rate of γδ T cells, and ultimately increased the ratio of Treg/ γδ T cells. These results suggested that EA could ameliorate intestinal inflammation damage by regulating the Treg/ γδ T cell polarity shift and improving the intestine barrier integrity post-stroke. It may be one of the mechanisms underlying the anti-ischemic injury effects of acupuncture on stroke.

Introduction

A stroke occurs due to blood supply disorder, ischemia and hypoxia in the brain region, leading to the death of cerebral tissues and neurological dysfunction (Khoshnam et al. 2017). It is a growing global health problem with a high risk of morbidity, disability and mortality. The immune response is involved in ischemic events, from early injury resulting from arterial occlusion to the late stage of tissue restoration, which plays a critical role in ischemic stroke pathology.

Accumulating evidence supported that the intestine immune state correlated with the pathological process of stroke (Liu et al. 2017; Benakis et al. 2016; Dou et al. 2019). Under physiologic conditions, intestinal microorganisms coordinate with the immune system to promote a tolerant, anti-inflammatory response to maintain immune balance. Yet, ischemia cascade can lead to flora disturbance and gut inflammation, resulting in the breakdown of the dynamic equilibrium in the intestine (Singh et al. 2016; Brown, Kenny, and Xavier 2019). Xu reported that ischemic stroke caused gut microbiota dysbiosis, represented by the overgrowth of Enterobacteriaceae, which exacerbated systemic inflammation and cerebral infarction (Xu et al. 2021). In addition, ischemic brain damage could develop intestinal inflammation characterized by lymphocyte changes (Zhang et al. 2020). For instance, it caused the

preferential polarization of intestinal T cells toward T helper cell 17 (Th17), interleukin-17-producing gamma delta T cells (IL-17^{+}γδ T), and other pro-inflammatory T cells. Moreover, ischemia can interfere with the function of regulatory T cells (Treg), which contribute to promoting immune response balance by secreting moderate cytokines, such as IL-10 (Feng et al. 2017; Benakis et al. 2016; Singh et al. 2016; Cramer, Benakis, and Liesz 2019). Similarly, Dou's team reported that acute cerebral ischemia up-regulated inflammation of the small intestine by inhibiting the Th2 and Treg cells while promoting the Th1 and Th17 (Dou et al. 2019; Singh et al. 2016). And the disordered intestinal immune system and excessive intestinal bacteria could increase intestinal permeability resulting in the invasion of intestinal toxins, such as antigens or microorganisms, through the mesenteric lymph nodes or blood circulation. These endogenous danger signals could cause severe infiltration of pro-inflammatory factors and glial cells in the brain and aggravated inflammatory injury (Dou et al. 2019; Singh et al. 2016; Brown, Kenny, and Xavier 2019). Growing evidence demonstrated that modulating intestine inflammation injury could alleviate the inflammatory response of the Central Nervous System, thus relieving the cerebral ischemia damage for the interactions within the brain-gut axis. Benakis and his colleagues have found that the alteration of intestinal microbiota to restore intestinal immune homeostasis could protect mice from MCAO-induced brain injury. The mechanism was related to the inhibition of intestinal IL-17^{+}γδ T differentiation and migration of IL-17^{+}γδ T from the gut to the brain (Benakis et al. 2016). Recently, Dou's team has proved that resveratrol treatment could balance T cells-related intestinal inflammation in regulating the polarity shift of Th17 / Treg cells and Th1 / Th2 cells, which was contributed to inhibiting brain ischemia damage (Dou et al. 2019). These pieces of evidence strongly suggested the amelioration of intestinal inflammation injury might be an effective strategy for relieving pathological brain damage after stroke.

As one of the most famous Traditional Chinese Medicine treatment methods, acupuncture has attracted more and more attention for its effectiveness and safety in treating ischemia stroke. It exerted significantly neuroprotective effects on acute stroke by inhibiting post-ischemic inflammation, oxidative and nitrative stress, excitotoxicity (Sun et al. 2020; Chen and Hsieh 2020) and maintaining the integrity of the blood-brain barrier (Feng et al. 2017; Lu et al. 2016). Meanwhile, acupuncture has also been reported to reduce pathological intestine damage during ischemia stroke and mitigate secondary cerebral injury. It was reported that acupuncture could inhibit the expression of pro-inflammatory factors IL-23, IL-17, IL-6 and TNF-α in the gut and brain injury (Wu et al. 2019). Meanwhile, it could also improve the disturbance of gut flora caused by ischemic stroke (Feng et al. 2020). These indicated that ameliorating intestinal inflammation might help the prognosis of cerebral ischemia. Nevertheless, few studies explored the therapeutic effect of acupuncture on post-stroke intestinal inflammation injury caused by Treg/γδ T cells. Herein, we originally hypothesized that because EA could restore the T cell ratio in the small intestine, EA would be effective against intestinal injury and inhibit its secondary cerebral injury in MCAO rats. Our data revealed that acupuncture played a protective role in post-stroke intestinal damage by promoting Treg / γδ T cells polarization towards Treg cells in the small intestine and improving intestinal barrier integrity.

Materials And Methods

Animals and Grouping

Male Sprague-Dawley rats (eight/nine-week-old) were purchased from the Experimental Animal Center of Nanjing University of Chinese Medicine. All rats were housed under a 12-hour light cycle in specific pathogen-free conditions and had free access to food and water. After acclimatizing for one week, rats were randomly divided into three experimental groups: the SHAM group, the MCAO group, and the MEA group. All animal experiments were performed following the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

MCAO surgery

Cerebral ischemia-reperfusion (I/R) injury was induced by MCAO surgery described previously (Fu et al. 2014). In short, the rats were anesthetized with isoflurane, and the rectal temperature was maintained at 37 ± 0.5^{o}C during surgery. A 3/0 monofilament nylon suture was inserted into the left external carotid artery, entered the anterior cerebral artery through the internal carotid artery, and finally blocked the middle cerebral artery to induce ischemic injury. After 2 hours of middle cerebral artery occlusion, the nylon suture was removed to induce reperfusion. Rats in the sham operation group received the same surgical procedures but without suture insertion.

EA Treatment

Rats in the MEA group were treated with EA stimulation in the "Baihui" acupoint (GV20). when the cerebral blood flow was restored, They received the first EA intervention. The second EA treatment was applied two hours before being sacrificed. GV20 is located at the line midpoint joining the tips of left and right ears. The needles were connected to Han's electroacupuncture instrument (Beijing Huawei Technologies Co. Ltd, LH402A). The stimulation current was 1 mA, and the frequency was 2/15 Hz, 20 min for each time. The SHAM and the MCAO groups were only fixed in the same way for 20 min, without EA stimulation (Fig.1).

Behavioral Test

Longa Neurologic Score was applied to assess the neurologic function: a score of 0 indicated no neurologic deficit; a score of 1 (failure to extend right forepaw fully) indicated a mild focal neurologic deficit; a score of 2 (circling to the right) indicated a moderate focal neurologic deficit; a score of 3 (falling to the right) indicated a severe focal deficiency; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness; a score of 5 suggested rats has died(Longa et al. 1989). The rats with a score from 2 to 3 were included in the experiments for follow-up research.The rats scoring outside of this range was sacrificed.

TTC staining

TTC staining was applied to evaluate the cerebral infarction area.Rats were sacrificed by intraperitoneal injection with excess pentobarbital. After cardiac perfusion with 0.85% saline, brains were isolated and frozen at -20^{o}C for 20 min. Then they were cut into five sections and incubated in 2% TTC (2,3,5-triphenyl tetrazolium chloride, Sigma Chemical Co., 298-96-4) for 20 min at 37^{o}C. The normal hemisphere exhibited red while the infarcted tissues exhibited pale. After that, the slices were immersed in 4% paraformaldehyde overnight. The sections were photographed, and the infarcted area was evaluated by Image-Pro Plus 6.0.

Hematoxylin and eosin staining (H&E staining) microscope

The ileum was fixed in 4% paraformaldehyde. The tissue was embedded in paraffin and cut into 5μm slices followed by Hematoxylin and eosin staining. The morphology damage of the small intestine was observed under a microscope (Nikon TE2000, Japan). The villous height and smooth muscle thickness in the ileum were determined using Image J software. Ten intestinal villi and the thickness were detected in each rat by pathologists blinded to the animal grouping.

Immunohistochemistry (IHC)staining

The ileum tissue wax blocks were sectioned at 5μm thickness, followed by the routine immunochemistry procedure. They were incubated with flowing antibodies, ZO-1 (1:250, Invitrogen,617300), Occludin (1:250, Invitrogen, #71-150) ,Claudin-1 (1:300, Invitrogen,51-9000) at 4oC overnight. Anti-IL-1β (1:300,Abcam,ab9722),Anti-TNF-α (1:300,Abcam,ab66579).The expression of the related proteins was observed from three matched sections at least containing the small intestine from each group (n=four/group) under the microscope, and the average optical density value (integrated optical density value/area) was calculated with Image-Pro Plus software.

Immunofluorescence analysis

The ileum sample was embedded in 4% paraformaldehyde for 48 h, at 4°C. 2mm-long tissues were then dehydrated with 30% sucrose and frozen in OCT for 2 hours at -20^{o}C. Then, they were cut into 10μm slices and treated with PBS containing 0.05%Triton (Sigma, T8787) and blocking solution (Thermo Fisher Scientific,37527). Then, slices were incubated with CD4 (1:200, BD,554843) and TCR-γ/δ (1:200,Biolegend,202605) at 4^{o}C overnight. Then the slices were incubated with secondary antibodies at 37^{o}C for 1 h, followed by DAPI (Absin, abs47047616) staining at room temperature (RT) for 10min. Finally, four small intestine slices were accessed per mouse and the microscope captured 3 fields for further analysis by pathologists blinded to the animal grouping.

Western Blot Analysis

After 24 h of ischemia-reperfusion, the small intestine of each group was harvested. The total protein of the tissues was extracted with RIPA buffer. Equal amounts of denatured protein samples were subjected to SDS-PAGE analysis, transferred onto a PVDF membrane and blocked with the blocking solution

(5%BSA) for 1.5 h. The membrane was then incubated with primary antibodies ZO-1(1:1000, Invitrogen,617300), Occludin (1:1000, Invitrogen,#71-150) and Claudin-1 (1:1000, Invitrogen,51-9000), and β-actin (1:1000, Abcam,ab8826) under 4^{o}C overnight and followed by a reaction with the corresponding secondary antibodies at RT. Bands were captured by Chemiluminescence imaging instrument and quantified usingImage J software.

Real-time (RT) -qPCR

Total RNA was extracted from the ischemic brain or the small intestinal using Trizol. The cDNA was produced by Revert Aid First Strand cDNA Synthesis Kit (Thermo, k1622). The two-step PCR cycling protocol was as follows: 40 cycles of 5s at 95^{o}C and 5 s 60 ^{o}C using QuantiNova SYBR Green PCR Kit (QIAGEN,2008054). Data were analyzed by the $2^{-\Delta\Delta CT}$ method. The primers are shown in Table 1,and GAPDH was used as the housekeeping gene.

Table 1 **Sequences of the primers for real-time PCR.**

	Primer sequence (5′- 3′)	
Gene names	Forward	Reverse
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
TNF-α	GCTACGGGCTTGTCACTC	CCACGCTCTTCTGTCTACTG
IL-1β	AGGTCGTCATCATCCCAC	TTCAAATCTCACAGCAGCAT
CXCL1	GGGACACCCTTTAGCATCTT	ACCCAAACCGAAGTCATAGC
CXCL2	GTCACCGTCAAGCTCTGG	ACCCAAACCGAAGTCATAGC

ELISA

50μl standard or sample was added to 96 well polystyrene microplates and incubated for 30 min at 37^{o}C. Then 50μl HRP-Conjugate reagent was added and incubated at 37^{o}C for 30 min. Next, 50μl chromogen solution and Tetramethylbenzidine were added in turn. The above reagents come from Nanjing Jinyibai Biotechnology Institute. Finally, the absorbance of every well was read at 450 nm.The serum sample was added to detect the IL-10,DAO and D-LAC expression. The whole small intestinal was used for evaluating IL-10 level.

Isolation of intestinal lamina propria lymphocytes Cell

Rats were sacrificed by pentobarbital overdose, a 15 cm-long piece of the terminal small intestine was excised and washed in PBS. After removal of Peyer' 's patches, mesenteric fat, and intestinal contents, the intestine was opened longitudinally, cut into 1 cm pieces, placed into 20 ml calcium- and magnesium-free

DMEM with 10 mM EDTA, and then put in a shaking incubator set at 80 r.p.m and 37^{o}C for 20 min. After incubation, tissue pieces were washed by vortexing four times with calcium-free PBS until the supernatant was clear. Next, the intestinal pieces were cut into 1mm fragments and digested in a separation solution composed of 5 mL DMEM containing Liberase TL (1 Wünsch unit/mL, Roche,05401020001) and DNase I (1 U/mL) at 37^{o}C for 20 min with constant agitation (80 r.p.m.). After 20 min, we collected supernatants and added an equal volume of DMEM containing 10% bovine serum. The remaining tissue pieces were continued to be digested by adding 5mL new separation solution. We repeated the above steps three times for a total of 60 min. Subsequently, we separated the intestinal fragments mechanically on a 100 μm nylon net with the soft plug head, washed all the above cell suspensions twice with DMEM, and then filtered the cell suspensions with 40μm nylon cell strainer. Finally, the cells were resuspended in DPBS. Cells were stained for flow cytometry analysis.

Flow cytometry analysis

Cell suspensions were incubated for 10min with Fixable Viability Stain 780 (1:1000, BD. Horizon,565388) to clarify the dead and living cells. Then, cell suspensions were stained with CD45 (1.0 μg /100 μl, Biolegend, 202220) ,CD4 (0.25 μg /100 μl,BD,554843) and TCR-γ/δ (0.25 μg/100 μl,Biolegend ,202605) for extracellular staining. After 40 min, we used Fixation and Permeabilization buffers (Invitrogen,00-5523-00) to fix and permeabilize at 4℃ for 40min. For intracellular staining, the Foxp3 (1.0 μg /100 μl, eBioscience,17-5773-82) was used. Cells were washed and resuspended in 200 μl of PBS buffer and analyzed with a cytometer. Analysis was performed with CytExpert software.

Statistical Analysis

The data were analyzed by one-way ANOVA and were expressed as mean ± SD, performing by Prism 8.0.2, Groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett t3 post hoc test. $P < 0.05$ was considered statistically significant.

Results

EA treatment alleviated cerebral ischemic-reperfusion injury

Male SD rats were subjected to the MCAO and reperfusion for 24 h. The rats of the MEA group were treated with EA at GV20 for two consecutive days. Next, we measured the cerebral infarct size and neurological function score to clarify the EA effect on improving ischemic injury. We observed the percentage of cerebral infarction area, which was pale under ischemia injury because of decreased dehydrogenase activity with TTC staining (Fig. 2A). Our results showed that the infarct area of the MCAO group was increased obviously. Contrarily, EA treatment markedly decreased infarct volumes of the MCAO rats (from 33.80 ± 3.49% to 19.40 ± 2.70%) (Fig. 2B). Then, the neurological function was assessed according to Longa's criteria. The neurological function score of the MEA group was significantly reduced compared with the MCAO group (Fig. 2C). These results suggested that EA has a

neuroprotective effect in MCAO model rats.Traditionally, the inflammatory response has been considered as an essential factor in mediating ischemia injury prognosis (Jayaraj et al. 2019). This study detected the expression of pro-inflammatory factors TNF-α and IL-1β in the ischemic hemisphere and serum by RT-qPCR and ELISA, respectively. The results showed that ischemia-reperfusion injury led to a dramatic increase of TNF-α and IL-1β in the brain and serum. At the same time, EA treatment significantly decreased the expression of IL-1β and TNF-α (Fig. 2D-G). For the anti-inflammatory factor IL-10, its expression was dramatically decreased in the MCAO group, whereas EA intervention increased IL-10 expression in the serum(Fig. 2H). These data demonstrated that EA treatment could ameliorate the ischemic stroke rats' focal and peripheral inflammation response.

EA treatment reduced the inflammation response of the small intestine in ischemic stroke rats

To test I/R injury-induced morphological abnormalities in the small intestine, H&E-stained

sections of ileum were measured. The thickness of smooth muscle (the blue line)and the villous height (the black line) were determined as specific indicators for evaluating intestinal structure. As shown in Fig. 3A-C, compared to the sham group, the villous height of the MCAO group was significantly decreased, and smooth muscle height was increased. Instead, with EA treatment, the villous height of the MEA group was increased, and the smooth muscles' thickness was decreased compared with the MCAO group.For evaluating the expression of inflammatory cell cytokines, we detected the protein expressions of IL-1β and TNF-α with immunochemistry. In the MCAO group, the optical density of IL-1β and TNF-α were significantly increased compared with the SHAM group. After EA treatment, the optical density of IL-1β and TNF-α were obviously decreased(Fig. 3D-G). These results indicated that the EA treatment could suppress intestinal inflammatory cell cytokines and maintain the structure of epithelium and mucosa. In support of this, the mRNA level of inflammatory cytokines IL-1β, TNF-α, CXCL1and CXCL2 in the small intestine were significantly up-regulated after ischemic stroke. Notably, the results showed that EA treatment could substantially inhibit their expression (Fig. 3H-K). At the same time, EA could significantly increase the expression of the anti-inflammation factor IL-10 in the intestine (Fig. 3L). Given the interaction of brain and gut (Malone et al. 2019; Bonsack, Jiang, and Borlongan 2020), we further evaluated the relationship of inflammatory cytokine' s levels in the brain and the small intestine with Pearson Correlation Analysis. In this paper, the correlation coefficient between IL-1β in the brain and the small intestine is 0.59 or 0.81(Fig. 3M and O). Relevance analysis of Btrain' s mRNA and intestinal optical density showed a positive correlation between IL-1β expression in the brain and the small intestine. Simultaneously, TNF-α expression in the brain and the small intestine also showed a positive correlation confirmed by coefficient 0.85 or 0.72(Fig. 3N and P). These results indicated that ischemic stroke could induce intestinal injury, and intestinal inflammation is closely related to the pathological injury of the brain under the ischemic stroke situation. And, EA could reduce the post-stroke intestinal inflammatory response, which may contribute to neuroprotective effect in MCAO rats.

EA enhanced the intestinal barrier integrity of ischemic stroke rats

The integrity of the intestinal barrier is the basis for the transportation of essential molecules and the restriction of harmful substances. The imbalance of inflammatory factors secretion can damage the barrier integrity. And the compromised barrier integrity is also considered to contribute to the activation of the intestinal inflammatory response (Chelakkot, Ghim, and Ryu 2018). Given the data mentioned above, we evaluated mechanical barrier function to elucidate the protective effect of EA on relieving I/R-induced intestinal damage. The expression level of diamine oxidase (DAO) and D-lactate (D-LAC) in the serum is an essential indicator of intestinal barrier dysfunction(Chen et al. 2020; Zhou et al. 2020; Guo et al. 2019). The result showed that I/R injury resulted in an apparent increase of DAO and D-LAC, while EA treatment could significantly decrease their expression in the serum (Fig. 4A and B). Figure 4C and D presented that the correlation coefficient between DAO, D-LAC and infarction volume is 0.50 and 0.81, respectively. Consistent with the correlation analysis of inflammatory factors in the intestine and the brain, the D-LAC level in the serum showed a significant positive correlation with the brain's infarction volume, which indicated that intestinal barrier dysfunction was associated with brain damage in rats with ischemic stroke. To address the possible protective mechanism of EA on defending intestine barrier injury, we detected the protein expressions of ZO-1, Occludin and Claudin-1 with immunochemistry and Western blot analysis. These proteins are responsible for the tight junction structure of the intestinal epithelium barrier (Capaldo, Powell, and Kalman 2017; Suzuki 2020). Our results showed that I/R injury decreased the optical density of ZO-1, Occludin and Claudin-1, whereas EA treatment could markedly enhance them (Fig. 5E-G,5H-J). Consistent with the immunochemistry result, EA effectively increased the protein expressions of ZO-1, Occludin and Claudin-1 in the small intestine of the ischemic stroke rats (Fig. 5K,5L-N). These results indicated that EA could improve intestinal barrier integrity by increasing the expression of tight junction proteins.

EA maintained the immune balance of Treg/ γδ T cells in the small intestine of ischemic stroke rats

Reports were indicated that T cells could shape the intestinal immune system by cooperating with microbes (Furusawa et al. 2013; Geuking et al. 2011). In this study, we also explored whether T cells were involved in the protective effect of EA on improving intestinal inflammation damage in rats with ischemic stroke. From recent reports, CD4 is an antigen coreceptor on the T cell surface (including most Th cells, Treg cells, etc.), participating in T cell activation via its association with the T cell receptor complex. In addition to CD4 positive cells ($CD4^+$ T cells), another rare type of T cells, called γδ T^+ cell, characterized by $CD4^-CD8^-$ on the cell surface, has been proved to play a significant role in immune regulation as well (Mousset et al. 2019; Sebestyen et al. 2020). In this study, $CD4^+$ and $TCR\gamma\delta^+$ T cell rates in the small intestine were first detected by immunofluorescence staining to assess the overall changes of T cells resulting from ischemic stroke. As shown in Fig. 5A-D, more $CD4^+$ cells and $TCR\gamma\delta^+$ cells were detected after I/R injury, and EA decreased the percentage of the $CD4^+$ cells(from 0.80%-0.55%) and $TCR\gamma\delta^+$ cell (from 2.55–1.51%). It was indicated that EA treatment might regulate the differentiation of T cells subsets in the small intestine of ischemic stroke rats. To assess the differentiation of T cells more accurately, we detected the numbers of Treg cells ($CD45^+CD4^+Foxp3^+$) and γδ T cells ($CD45^+CD4^-TCR\gamma\delta^+$) in the intestinal lamina propria by flow cytometry. As shown in Fig. 5E, 5G,

compared with the SHAM group, the MCAO group had a lower Treg cells percentage down to 6.75%, whereas the MEA group had a higher rate up to 11.23%. In contrast, ischemia-reperfusion increased the γδ T cells percentage to 28.86%, and EA intervention remarkably decreased this percentage to 16.04% (Fig. 5F, H). EA treatment could increase the ratio of Treg to γδ T cells of the MCAO rats significantly from 0.235 to 0.708 (Fig. 5I). These results suggested that EA treatment after ischemic stroke could promote the balance of Treg/γδ T cells towards Treg cells in the small intestine via mediating cell differentiation tendency, which accounts for EA's action on repairing intestinal inflammatory injury.

Discussion

Increasing evidence has demonstrated that intestinal dysfunction is a common complaint among patients with brain damage. In the results of Dourado' reports, 72 patients with stroke were identified, 18% of whom suffered from anal incontinence and 29% suffered from intestinal constipation (Dourado et al. 2012). The su' prospective cohort study of 154 stroke patients suggested that the cumulative incidence of new-onset constipation was 55.2% at 4 weeks post-stroke and the poor outcome was associated with constipation (Su et al. 2009). Olsen AB and their team have been reported that traumatic brain injury could decrease intestinal contractility and transit.Then,they suggested that intestinal inflammation resulted from brain injury exerted a vital role in intestinal dysfunction (Olsen et al. 2013). Notably, intestinal pathological changes also aggravate the stroke injury (Singh et al. 2016; Brown, Kenny, and Xavier 2019), which indicated that intestinal inflammation response should be a potential target of stroke treatment. EA is widely applied in stroke treatment due to its effectiveness and few side effects. It has been extensively reported to ameliorate ischemic stroke damage by reducing the inflammatory response oxidative stress and promoting the regeneration of blood vessels and nerves in the ischemic brain (Chavez et al. 2017; Xing et al. 2018). However, few researchers pay attention to the post-stroke intestinal injury, which plays a vital role in secondary injury and prognosis of ischemic stroke. In this study, we focused on the impact of EA on stroke-induced intestinal injury, more specifically, involving inflammation response and barrier function, to explore the potential mechanism of EA protection effect on stroke from the gut-brain axis.

There is growing evidence indicating that immune reaction played a double-edged role in stroke pathophysiology. The migration of immune cells and cytokines to the ischemic brain could contribute to further disruptive or protective effects in the brain. Previous studies have documented that IL-10 is a neuroprotective cytokine generated by different cells (including Treg cells) and promote the regression of inflammation and repairing surviving cells in the ischemic territory by promoting Treg formation and inhibiting pro-inflammatory cytokines expression (Lu et al. 2016; Chen and Yang 2019; Chen and Hsieh 2020). IL-1β and TNF-α are thought to aggravate immune response in ischemia-related injury(Lambertsen et al. 2012; Ibrahim et al. 2018). In addition, chemokines, such as CXCL1, CXCL2, can mediate the infiltration of leukocytes in ischemic regions and promote inflammation response (Chehaibi et al. 2017; Losy, Zaremba, and Skrobanski 2005). In this study, MCAO models exhibited a dramatic increase in infarct size and neurological scores after 24 h of ischemia-reperfusion. At the same time, the expression of pro-inflammatory factors, namely IL-1β and TNF-α, in the ischemic brain region were up-regulated. EA

treatment could alleviate the I/R injury by reducing the infarction volume, improving neurofunction and restraining the I/R-induced inflammation response in the brain. These are consistent with previous studies (Dou et al. 2019; Lambertsen, Biber, and Finsen 2012; Ibrahim et al. 2020). In addition, I/R injury also induced inflammatory response in peripheral blood manifested by enhanced IL-1β, TNF-α expression and attenuated IL-10 expression. And EA treatment could reverse this trend; it 's suggested EA could also restrain ischemia damage signals-activated systemic immune response and consistent with existing research(Lambertsen, Biber, and Finsen 2012; Dong et al. 2020). It's noteworthy that extensive research showed that intestinal dysfunction and inflammation response in I/R pathogenesis was closely associated with stroke prognosis (Feng et al. 2020; Wu et al. 2019). So, we further explored the effect of EA on intestinal inflammation injury. The intestinal morphology change was detected with H&E staining. We observed acute brain injury led to ileum morphological damage. The analysis results indicated that brain I/R injury increased the smooth muscle thickness and decreased villous height in the ileum. EA intervention could partially improve the intestinal morphology in the smooth muscle and villus. The results of intestinal immunohistochemistry further suggested more inflammatory cytokines such as IL-1β and TNF-α were expressed in the epithelial layer and the mucosa layer in the MCAO group, compared with the sham group. With the injury of intestinal structure, the expression of pro-inflammatory factors, namely TNF-α, IL-1β, CXCL1, and CXCL2 in the small intestine was increased, and the expression of anti-inflammatory factors IL-10 was decreased, which is consistent with the previous study (Feng et al. 2017; Lu et al. 2016; Shang et al. 2015). EA exerted an anti-inflammation effect in the small intestine obviously by inhibiting the expression of these pro-inflammatory factors and promoting the expression of IL-10. These results showed that the intestinal injury accompanied the cerebral I/R injury in morphology and inflammation.EA could improve intestinal morphology and inhibit intestinal pro-inflammatory factors expression.

Inflammation factors TNF-α, Interferon-γ (IFN- γ) and IL-1β were reported to contribute to the destruction of intestinal barrier via damaging tight junction structure during the early phase of acute injury(Dou et al. 2019; Rahman et al. 2018). Conversely, the destruction of the intestine barrier could mediate intestinal inflammatory factors penetrating the barrier to the peripheral blood, bringing secondary damage to the brain (Dou et al. 2019; Zhao et al. 2018). Thus, we also detected the destruction of the intestinal barrier in ischemic stroke rats.

DAO is an intracellular enzyme mainly in the intestine with low concentration in blood under physiological conditions, while D-LAC is intestinal bacterial end metabolites.DAO and D-LAC were reported to drain the mucous layer into blood circulation under intestine damage conditions. Thus, their serum concentrations have been widely used as intestine mucosal permeability indexes(Chen et al. 2020; Guo et al. 2019). In this study, the concentration of DAO and D-LAC in the serum were increased in 24 h post-I/R injury, and EA significantly decreased the permeation of DAO and D-LAC from the intestine. It's indicated that EA could improve the integrity of the intestinal barrier. In this paper, the Pearson correlation between IL-1β in the brain and the small intestine demonstrated a positive relationship. A similar correlation was obtained for TNF-α. At the same time, the serum concentrations of DAO, D-LAC and the expression level of brain IL-1β and TNF-αalso showed a positive correlation. These results indicated an interactive relationship

between cerebral injury with post-stroke intestinal inflammation and barrier dysfunction. The above data suggested that maintaining the intestinal barrier integrity and inhibiting the intestinal inflammation response might contribute to EA's anti-ischemic injury effect. However, the detailed mechanism underlying intestinal inflammation associated with EA's anti-ischemic injury effect remains unknown. Previous studies have shown that the tight junction structure consisting of ZO-1, Occludin, and Claudin-1 surrounds and closes to the intercellular space of epithelial cells to form a natural physical barrier with selective permeability extracellular substances. It is an essential mechanical barrier that can inhibit the leakage of toxic substances in the intestinal lumen under acute intestinal stress (Capaldo, Powell, and Kalman 2017; Suzuki 2020). Consistent with the most current reports, our data showed that the expression of tight junction proteins ZO-1, Occludin and Claudin-1 in the intestine decreased significantly under I/R injury(Ye et al. 2021; Chen et al. 2019). Moreover, EA treatment could increase these protein expressions and attenuate the disruption of intestinal tight junction structure. These results indicated that EA could maintain the intestinal barrier integrity by reducing the disruption of the tight junction.

Following the acute stroke, the differentiation of T cells will shift to a pro-inflammatory phenotype arising from the disturbance of intestinal flora and activated intestinal inflammation excessively, characterized by the increasement of intestinal Th1 and Th17 cells and reduction of intestinal Th2 and Treg immune responses. Accordingly, IL-1 secreted by Th1 cell and IL-17A secreted by Th17 cell will increase while Th2 cell related cytokines IL-4 and Treg cell related cytokines IL-10 will decrease. In addition, γδ T cells were reported to regulate innate and adaptive immunity in the early stage of organismal injury and involved in exacerbating the intestinal inflammation response after stroke (Whibley, Tucci, and Powrie 2019; Gill and Veltkamp 2016; Chien, Meyer, and Bonneville 2014). Lee reported that IL-17$^+$γδ T /Treg-mediated immune response balance shifted towards IL-17$^+$γδ T cells in post-stroke mice. Interestingly, they also showed that intestinal dysfunction of immune and barrier led to IL17$^+$γδ T migrating from lamina propria of intestinal mucosa to ischemic brain and counter the deleterious effects of ischemia cerebral immune response ultimately (Lee et al. 2020). Thus, we next explore the role of T cells in the small intestine protected by EA treatment in post-stroke mice. Our results showed that EA significantly reduced the number of γδ T cells, which were increased in the MCAO group. Olaf reported that there was no significant difference in intestinal CD4$^+$ cells number after experimental stroke,but their box plots showed an upward trend (Schulte-Herbruggen et al. 2009). Conversely, our results suggested ischemia induced an obvious up-regulation of CD4$^+$ cells in MCAO group and EA treatment could down-regulate CD4$^+$ cells.

Since both effector and regulatory T populations express CD4, it is difficult to clarify the change of individual subsets of CD4$^+$ T cells in ischemia stroke. Thus, we further applied flow cytometry to investigate the differentiation status of Treg and γδ T cells. The results showed that ischemic stroke increased the percentage of γδ T cells and decreased the percentage of Treg cells in lamina propria of the intestinal mucosa. Further, the ratio of Treg/γδ T cells was decreased on I/R injury rats, which was consistent with previous studies (Feng et al. 2017; Lu et al. 2016; Xu, Li, and Jiang 2013). EA treatment significantly reversed this trend compared with the MCAO group. These results showed that I/R injury was sufficient to disrupt Treg/γδ T cells mediated- immune balance in the small intestine. EA could

remarkably up-regulate Treg cells polarization and decrease the γδ T cells expression, further increasing the ratio of Treg/γδ T cell to alleviate this imbalanced immune station and ameliorate intestinal inflammatory injury resulting from ischemia stroke. So far, this research has suggested that the anti-ischemic injury effect of EA treatment is related to the regulation of intestinal T cells. However, the mechanism of EA action on Treg and γδ T cells has not been deeply explored. Recent publications showed that the intestinal microbiome has a dual impact on the prognosis of stroke via mediating the polarization of T cells (Feng et al. 2017; Singh et al. 2018; Sadler et al. 2017). EA was also reported to regulate commensal gut microbiota disturbance and thus inhibit the neuroinflammation(Jang et al. 2020). We speculated that regulating intestinal flora's species and abundance might be severed as a key factor in EA's action on restoring the T cell ratio to balance intestinal inflammation in MCAO rats. Our future research will also be based on the specific mechanism of intestinal flora in T cell differentiation to verify the mechanism of EA therapeutic effect on I/R injury.

Conclusion

Collectively, our study documented, for the first time, one of the mechanisms of EA conferred neuroprotection to ischemia stroke could be modulating by intestinal lamina propria Treg/γδ T cells polarization mediated-inflammatory injury in the small intestine (Fig. 6). These findings will help us understand and further elaborate on the molecular biology of EA against ischemic stroke from the brain-gut axis.

Abbreviations

EA
Electro-acupuncture
SHAM
The sham operation group
MCAO
Middle cerebral artery occlusion
TTC
Triphenyl tetrazolium chloride
Th17
T helper cell 17
IL-17 + γδ T
Interleukin-17-producing gamma delta T cells
Treg
Regulatory T cells
Th1
T helper cell 1
Th2

T helper cell 2
DAO
Diamine oxidase
D-LAC
D-lactate
ZO-1
Zonula Occludens 1 Protein.

Declarations

Ethics approval and consent to participate

All animal experiments were in accordance with the guidelines of the Medical Animal Care & Welfare Committee. The animal study was reviewed and approved by the Institutional Animal Care Unit Committee of the Nanjing University of Chinese Medicine.

Consent for publication

Not applicable.

Availability of data and materials

All raw data used in this manuscript are available on reasonable request.

Competing interests

The authors declare they have no conflicts of interest.

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Authors' contributions

SPF and BX initiated and designed the experiments. YLW, LLM, YLC, TZ, XXL, LOY, BFW and RP performed the experiments. SPF and XYJ performed the analysis of H&E and IHC staining data, QL and SFL performed other data analysis. YLW, LLM, SPF and QL wrote and edited the manuscript. All authors approved the final manuscript.

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Figures

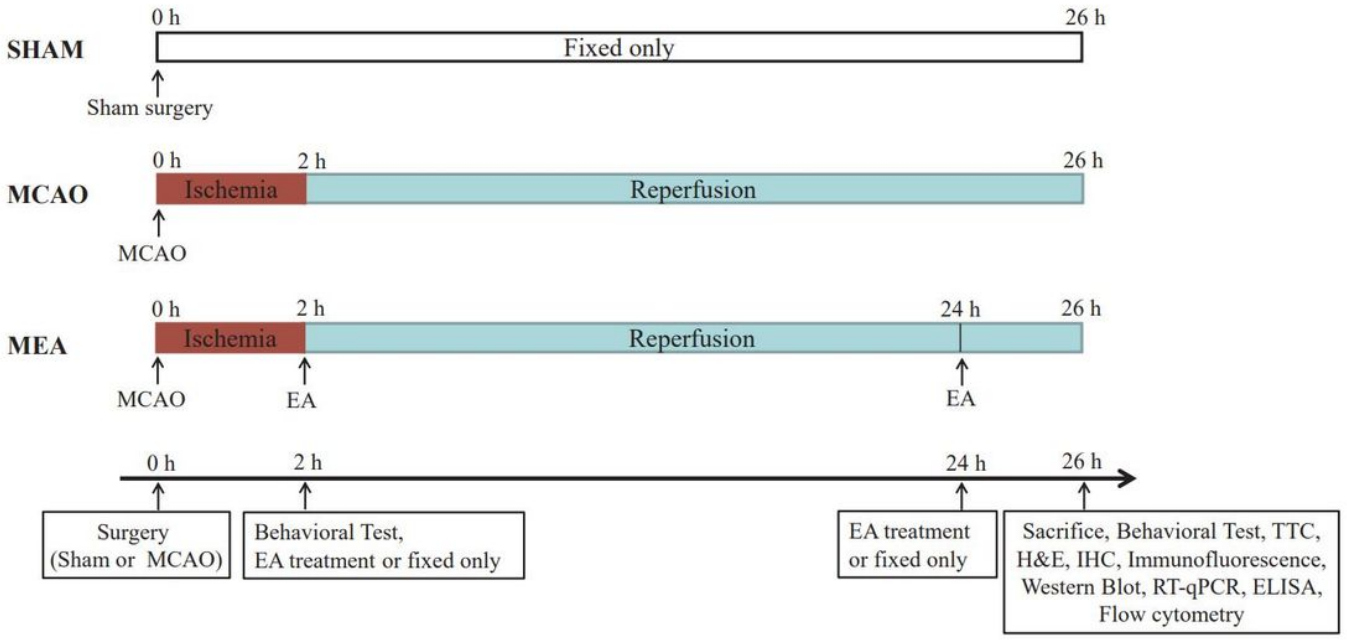

Figure 1

The experimental timeline. In the SHAM group, rats were received sham surgery and only fixed. Other rats were received MCAO surgery to induce ischemia.After 2 hours' ischemia,the nylon suture was removed to induce cerebral ischemia-reperfusion (I/R) injury. Some of them with a Longa's Neurologic Score from 2 to 3 were included and divided into MCAO group and MEA group. The rats in MEA group was performed at "GV20" point and totally experienced two EA treatments. After 24 hours of reperfusion, rats were sacrificed and the inflammation in the ischemic brain and intestine, intestinal barrier and Treg/γδ T cells were evaluated.

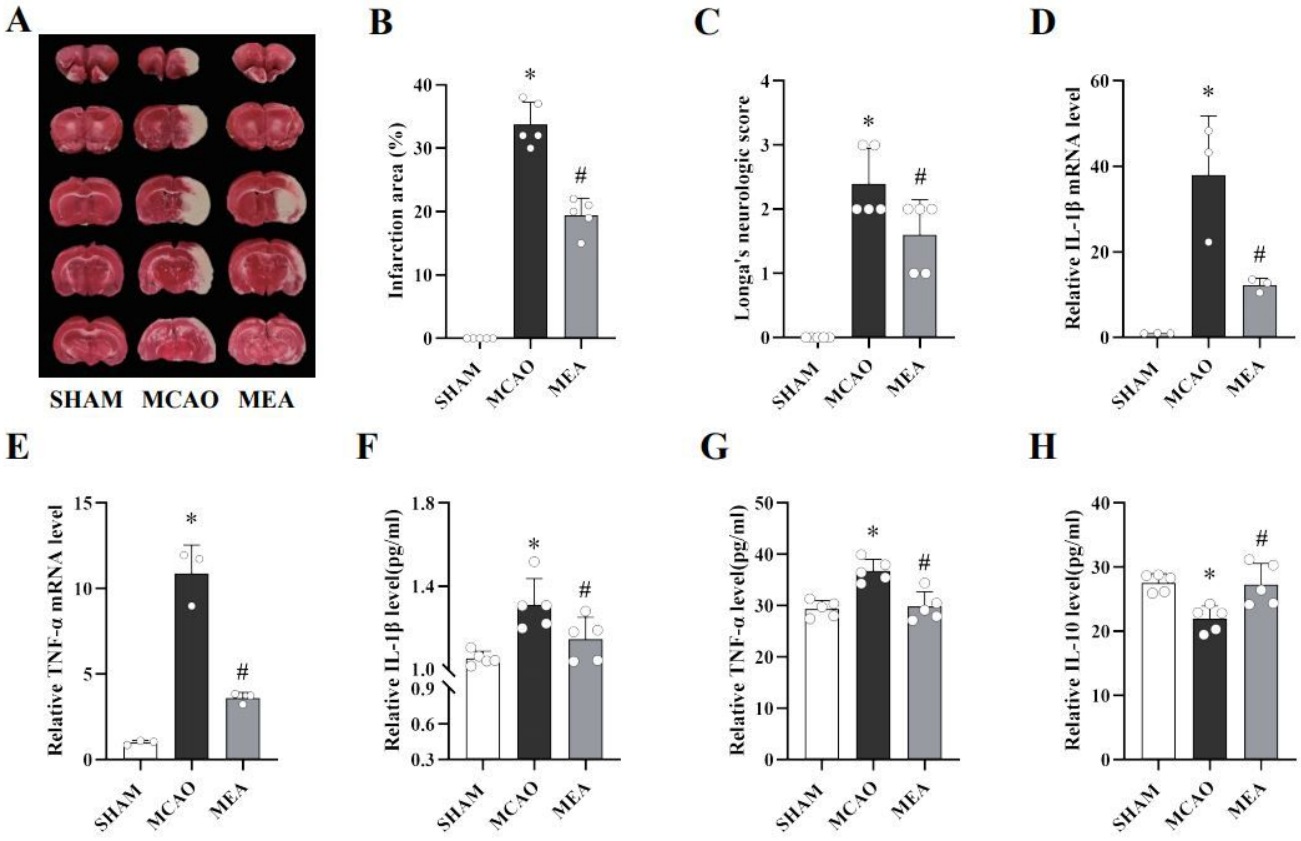

Figure 2

EA treatment alleviated cerebral damage of I/R injury rats. (A-C) EA treatment reduced brain infarction and neurological score induced by ischemia injury. (A) Infarct area was identified by TTC staining. (B) The analysis of infarct area. (C) Neurological score after ischemic. (D-H) EA reduced the expression of relative pro-inflammatory cytokines in Brain and serum. (D) Relative IL-1β mRNA level in Brain. (E) Relative TNF-α mRNA level in Brain. (F) Relative IL-1β protein expression in serum. (G) Relative TNF-α protein expression in serum. (H) Relative IL-10 protein expression in serum. Data were represented as mean ± SD (n=3/5). *P<0.05 vs. the SHAM group, #P<0.05 vs. the MCAO group.

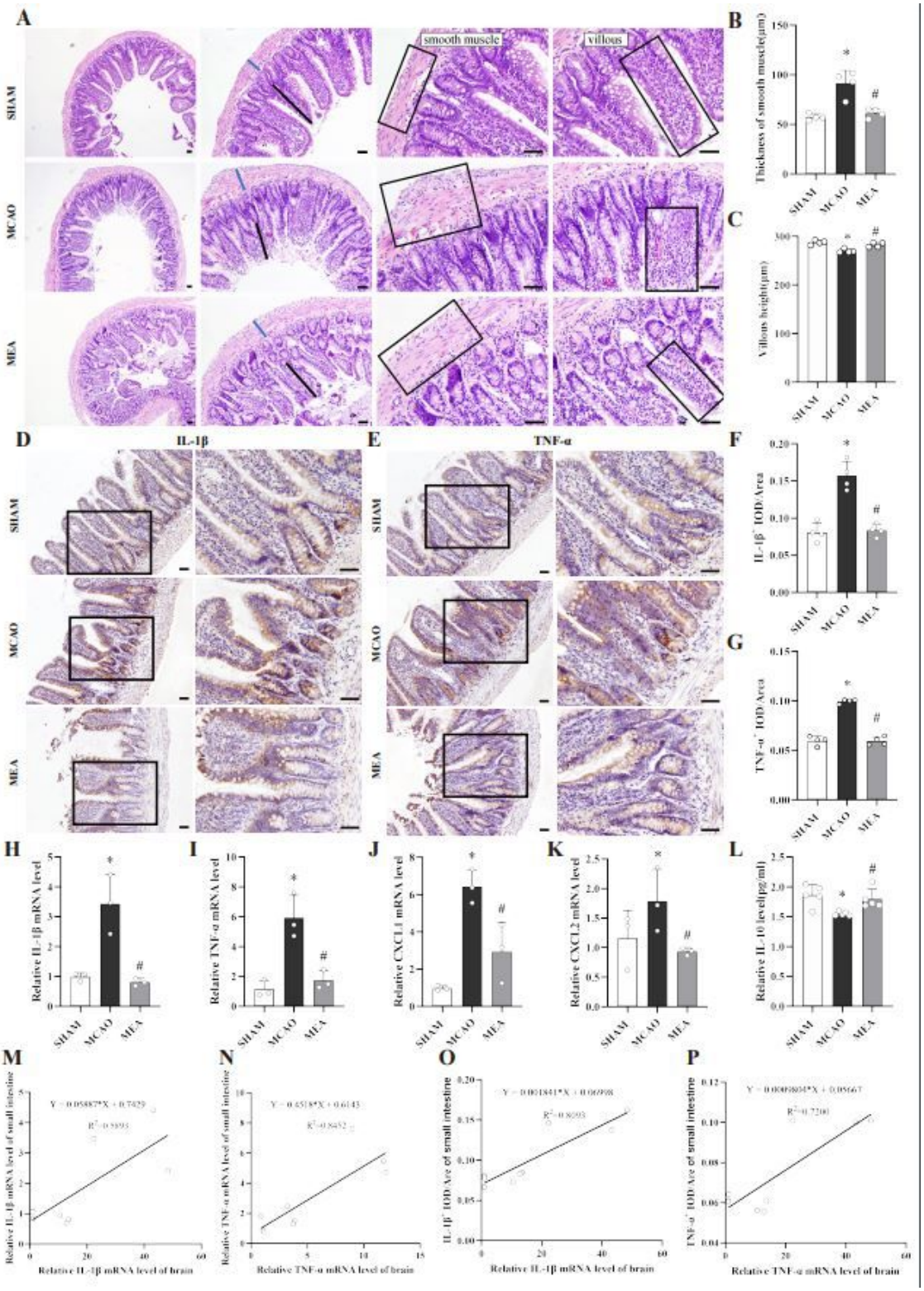

Figure 3

EA inhibitted inflammation in the small intestine. (A-G) EA reduced morphological injury and the expression of inflammatory factor. (A) This figure which consisted of smooth muscle (the blue line) and the villous height (the black line) which was shown by H&E staining. Representative IHC diagrams of IL-1β (D) and TNF-α (E). (F, G) The average optical density value of IL-1β and TNF-α (n=4). (H-L) EA reduced relative pro-inflammatory cytokines mRNA expression and increased IL-10 expression in the small

intestine. (H) Relative IL-1β mRNA level. (I) Relative TNF-α mRNA level. (J) Relative CXCL1 mRNA level. (K) Relative CXCL2 mRNA level. (L) Relative IL-10 protein expression (n=3 or 5). (M-P) The relationship of pro-inflammatory cytokines level in the brain and the small intestine (n=9). The X-axis represented the cerebral (M) IL-1β mRNA expression or (N) TNF-α, the Y-axis represented the intestinal (M) IL-1β mRNA expression or (N) TNF-α (n=3/each group). The X-axis represented the cerebral (O) IL-1β mRNA expression or (P) TNF-α (n=3/each group), the Y-axis represented the average optical density of intestinal (O) IL-1β or (P) TNF-α (n=3/each group). Scale bar=50 μm. Data were represented as mean ± SD. * $P<0.05$ vs. the SHAM group, # $P<0.05$ vs. the MCAO group.

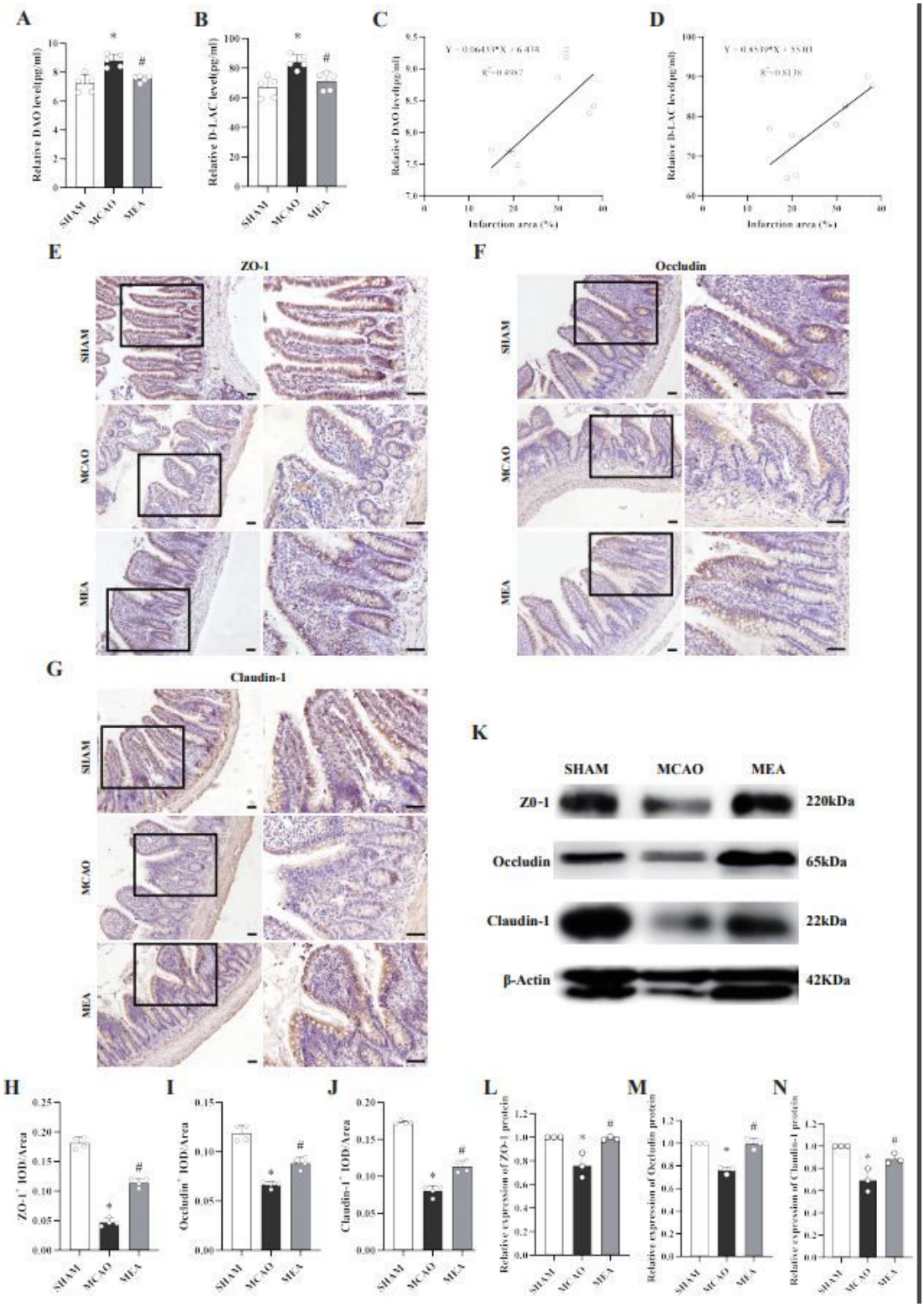

Figure 4

EA treatment protected the intestinal barrier integrity after MCAO. (A-D) EA reduced DAO and D-LAC protein expression in serum. (A) EA decreased relative DAO protein expression. (B) EA decreased relative D-LAC protein expression (n=5). (C) Infarction area and serum DAO level presented a positive correlation. The X-axis represented the infarction area, the Y-axis represented the DAO level (n=5/MCAO, MEA group). (D) Infarction area and serum D-LAC level presented a positive correlation (n=10). The X-axis represented

the infarction area, the Y-axis represented the D-LAC level (n=5/MCAO, MEA group). (E-J) EA increased the expression of ZO-1, Occludin and Claudin-1. Representative IHC diagrams of (E) ZO-1, (F) Occludin and (G) Claudin-1. (H, I and J) The average optical density value of ZO-1, Occludin and Claudin-1 (n=4). (K) Representative western blot image of ZO-1, Occludin and Claudin-1. Relative protein level of (L) ZO-1, (M) Occludin and (N) Claudin-1. Data wererepresented as mean ± SD (n=3). * P<0.05 vs. the SHAM group, # P<0.05 vs. the MCAO group.

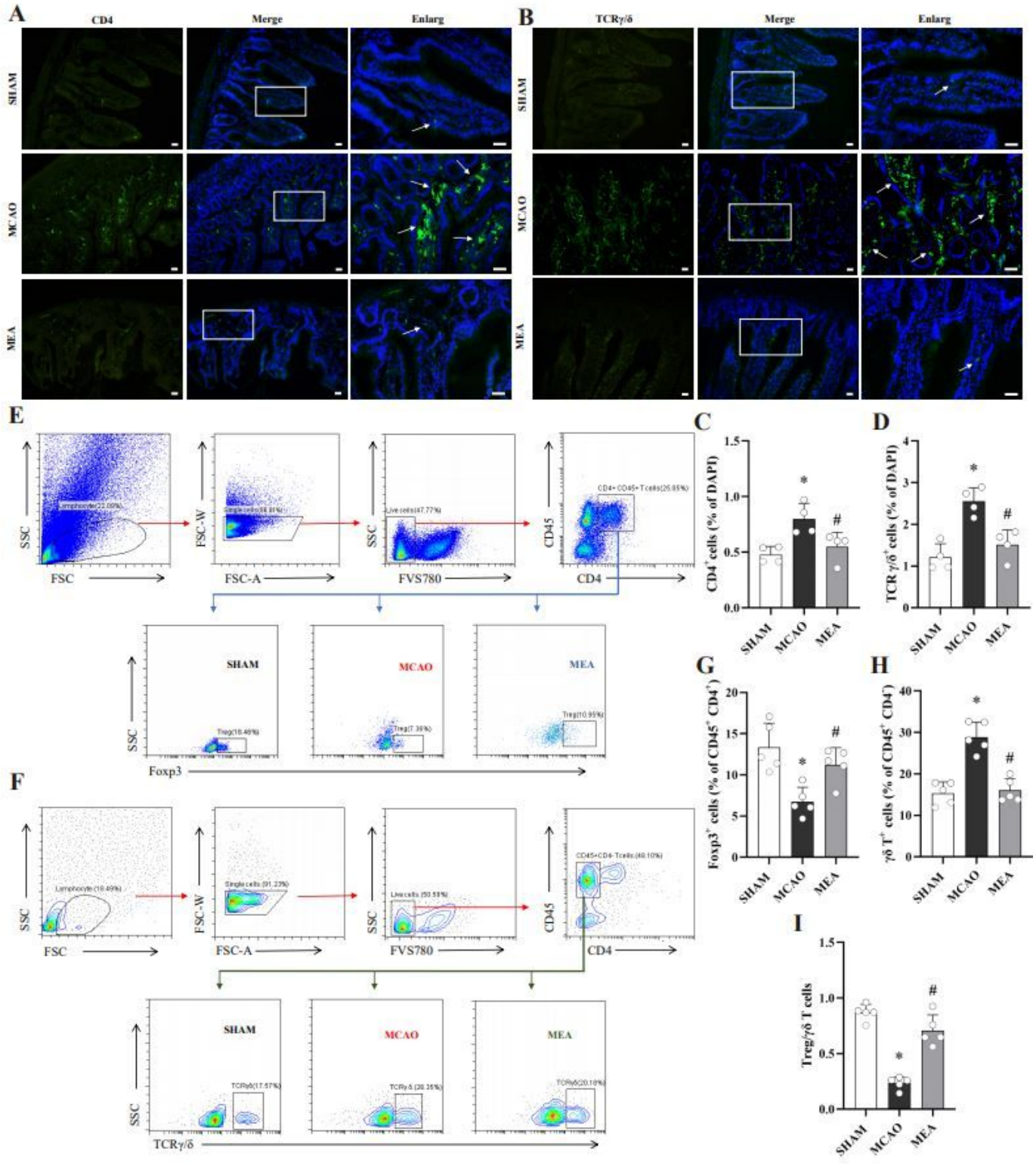

Figure 5

EA maintained the immune balance of Treg/ γδ T cell in the small intestine. (A-D) EA treatment significantly reduced CD4+ cells and TCRγ/δ+ cells in small intestine. (A, B) The nucleus were shown by DAPI expression (blue) and immunostaining was performed for CD4 (green) or TCRγ/δ (green). (C, D) Comparison in the percentage of CD4 or TCR γ/δ cells with DAPI expression was shown in the different groups (n=4). (E-I) EA increased the expression of Treg cells and decreased the expression of γδ T cells. (E, G) Treg cells decreased 24h after ischemic injury in the IPL of small intestine. (E) Flow cytometric gating strategy of Treg cells. Representative flow cytometry pseudo color plots of T cells indicated by forward ascatter (FSC) plots and side scatter (SSC) and FVS780 used to ensure only live cells were gated and CD45+ and CD4+ cells expression from live cells (E,up). Representative flow cytometry pseudo color plots of Treg cells (CD45+CD4+FoxP3+) in SHAM, MCAO and MEA groups (E,down). (G) Flow cytometry analysis of Treg cells reflected by the percentage of FoxP3+ cells of CD45+CD4+ cells using histogram. (F, H) γδT cells in the IPL of small intestinal tissue increased 24h after ischemic injury. (F) Flow cytometric gating strategy of γδT cells. Representative flow cytometry contour plots of CD4+ T cells indicated by FSC plots and SSC and CD45+ and CD4- cells expression from live cells (F,up). Representative flow cytometry contour plots of TCRγδ cells (CD45+CD4-TCRγδ+) in each groups (F,down). (H) Flow cytometry analysis of this cells. (I) The ratio of Treg to γδ T cells. Data were represented as mean ± SD (n=5).

*P<0.05 vs. the SHAM group, #P<0.05 vs. the MCAO group.

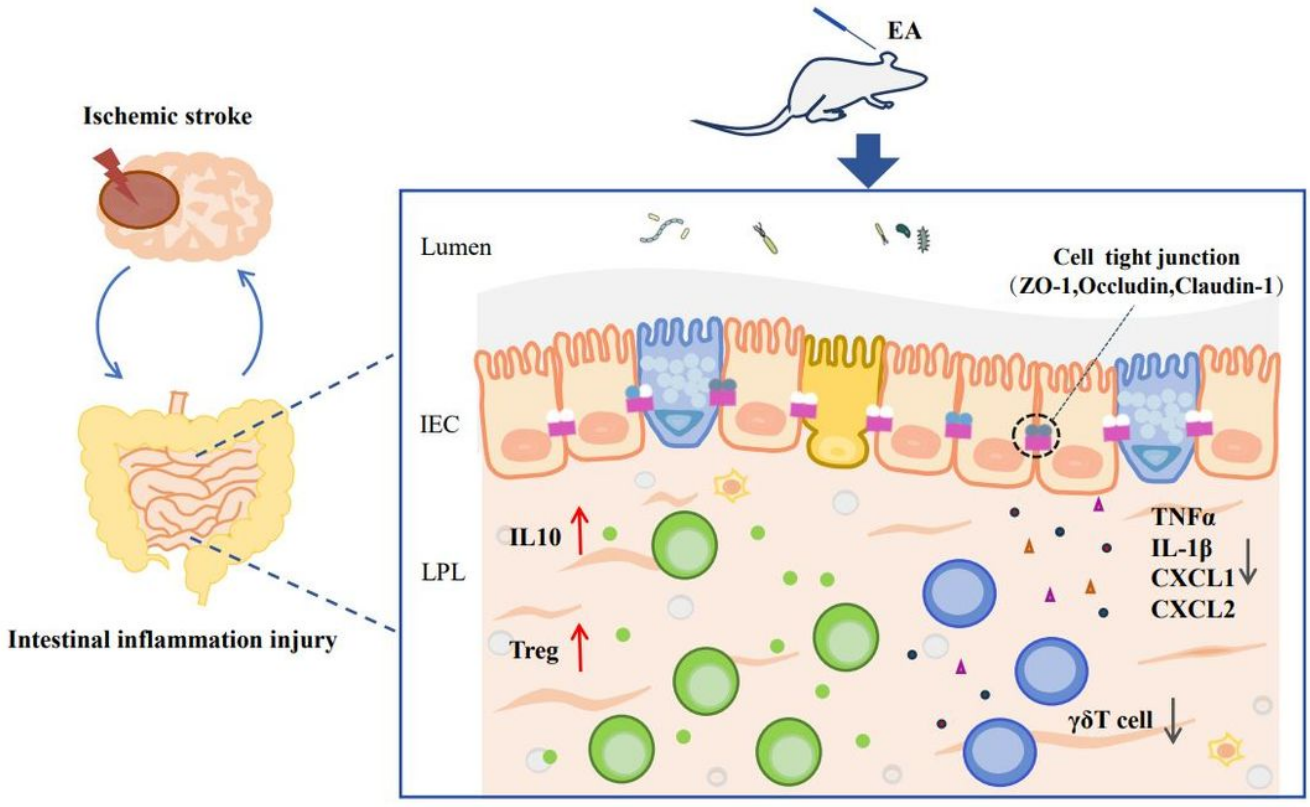

Figure 6

The function of EA treatment on repairing intestinal injury under I/R injury. EA trteatment promotes Treg/γδ T cells polarity shift to Treg cells and improves the intestine barrier integrity to ameliorate intestinal inflammation, subsequently exerting protective role in ischemic stroke. The red rows mean promotion, and the black rows mean inhibition.