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Original Article

Resolvin D1 protects against sepsis-associated encephalopathy in mice by inhibiting neuro-inflammation induced by microglia

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Abstract: Objectives: Neuro-inflammation induced by microglia is crucial in the pathogenesis of sepsis-associated encephalopathy (SAE). The endogenous lipid mediator, Resolvin D1 (RvD1), which is synthesized from docosahexaenoic acid, has been extensively reported to attenuate inflammation in various diseases by its anti-inflammation and pro-resolving functions. However, the effect of RvD1 on SAE remains unclear. In this study, we aimed to ex the function and mechanism of RvD1 on SAE mice. Methods: In our study, the SAE mice model was established by the method of cecal ligation and perforation (CLP). C57BL/6J mice were randomly divided into three groups: the Sham group, the CLP group and the CLP+RvD1 group. Cognitive impairment of the mice was assessed by Morris water maze. Iba1 immunohistochemistry was conducted to observe the activation of microglia in hippocampus of the mice from different groups. The production of cytokines, including TNF-α, IL-6 and IL-1β, and their mRNA levels were evaluated by ELISA and Q-PCR. The expression of the molecules from inflammatory signaling pathways was assessed by Western blot. Results: xaRvD1 treatment significantly improved the learning and cognitive ability of SAE mice. The activation of microglia and the production of inflammatory cytokines in hippocampal tissues were inhibited in CLP+RvD1 group. We also found that the inflammation of microglia was attenuated by RvD1 treatment both *in vivo* and *in vitro*. Moreover, the activation of NF-κB, MAPK and STAT signaling pathways were inhibited by RvD1 treatment, which partly explained the anti-inflammation function of RvD1 on SAE mice. Conclusions: RvD1 could improve the learning and cognitive ability of SAE mice by inhibiting the systemic and local inflammation. It could attenuate the inflammation in microglia by inhibiting the activation of inflammatory signaling pathways and then decreasing the production of cytokines. These findings are helpful to better understand the pathophysiology of SAE, which also provide a novel therapeutic method in clinic.

Keywords: Resolvin D1, sepsis-associated encephalopathy, inflammation, microglia

Introduction

Sepsis is one of the central public health problems that need to be solved urgently. Sepsis can be defined as the life-threatening organ dysfunctions induced by dysregulated host responses to infection [1]. It is also one of the main causes of mortality in patients with critical illnesses [2], which always leads to severe organ injuries, including lung, liver, encephalon, kidney and so on [3]. The brain plays an important role in sepsis, which is involved not only as a mediator in innate immune responses, but also as a target organ affected by the process of sepsis. The diffuse dysfunction of brain induced by sepsis is called sepsis-associated encephalopathy (SAE) [4], the clinical manifestations of which are mainly neurological symptoms such as amentia, delirium or even long-term impairment of cognition [5, 6]. Moreover, obvious infections or structural abnormalities in central nervous system do not usually present in patients with SAE [7, 8]. The clinical process of SAE has not been specified yet. Whether it is a result from the cascade reaction of other organ dysfunctions or it is originally caused by the acute inflammation induced by sepsis at the beginning course of disease is still uncer-

tain. The morbidity of SAE in septic patients is reported from 9% to 70% due to its unclear definition [6] and it usually leads to the deaths of patients from intensive care unit (ICU). The pathophysiology of SAE is complicated and may be related to several mechanisms, including the activation of endothelial cells, the injury of blood brain barrier, neuro-inflammation, the changes of neurotransmission and so on [9]. Among these factors, the inflammation of brain mediated by microglia has been reported to be highly involved in the pathogenesis of SAE [10].

Microglia, derived from myeloid cells, are the first and the most important immune defense in central nervous system [11]. Generally, microglia appear as ramified morphology in a resting state. When stimulated by inflammation, toxin or injuries, microglia are activated and transform into amoeboid morphology, to protect neurons from impairment [12]. However, microglia may be over-activated when neurons are damaged severely, and then they would promote the production of inflammatory cytokines, which may conversely lead to the deaths of neurons and the lesion in tissues [13]. It has been found that microglia play a vital role in the pathological processes of various diseases in nervous system, including ischemic injury of the brain and some other cerebral diseases such as Alzheimer's disease, Parkinson's disease and so on [14-18]. Studies have also revealed that repressing the activation of microglia could reduce the neuro-inflammation and the oxidative injuries of the brain [19-21], and improve the long-term cognitive function of mice with sepsis, indicating that microglia take an indispensable part in SAE.

Resolvins, the endogenous lipid mediators which are generated from omega-3 polyunsaturated fatty acids, have been known to have the function of anti-inflammation and pro-resolution in several diseases in animal models [22]. Among the family of Resolvins, Resolvin D1 (RvD1), which is biosynthesized from docosahexaenoic acid (DHA), could also modify inflammation as previous study reported [23]. It binds to the receptor named G protein-coupled receptor 32 (GPR32) in mankind [24], while it combines with the receptor called Alloxan/formyl peptide receptor 2 (ALX/FPR2) in mice, the latter of which also exist in microglia and astrocytes [15, 25]. RvD1 can inhibit the infiltration of inflammation and reduce the production of cytokines [26, 27]. It can also promote the absorption of inflammation and enhance the elimination of antigen. Numerous researches have revealed the significant protective function of RvD1 in organ injuries of mice, such as acute lung injury (ALI) [28], sepsis induced heart injury [29], acute kidney injury (AKI) [26] and so on. In these studies, RvD1 was found to have strong regression on neutrophils and promote the activities of macrophages [25]. By suppressing the activation of nuclear factor kappa B (NF-κB), RvD1 represses the induction of inflammatory signaling pathways [23, 30], which leads to the reduction of inflammatory cytokines and attenuates inflammation. Furthermore, it was also reported that RvD1 could contribute to the decrease of the mortality of the mice with sepsis and relieve the inflammation of lung injuries induced by sepsis, indicating that RvD1 might play a protective role in sepsis. However, whether RvD1 has functions on SAE has not been illustrated yet.

Based on these studies, we hypothesized that RvD1 might also have positive function in attenuating the inflammation during SAE, and this could be related to its inhibition on the activation of microglia. Thus, we aimed to find out the function of RvD1 on SAE and the potential mechanism of it in this study.

Materials and methods

Animals and cells

This study was carried out under the approval of the Animal Care and Use Committee of Naval Medical University, which was also in line with Chinese legislation on the use and care of laboratory animals. 6-8 weeks male C57BL/6 mice were purchased from the Laboratory Animal Center of the Naval Medical University and raised in the cages free of specific pathogens. The animals were free to get water and food, which were raised in 12/12 day/night cycle. The room temperature was maintained at 22±2°C, and all the mice were kept for at least one week to acclimatize before use.

Mice microglia cell line BV2 was purchased from ATCC and kept in nitrogen canister before resuscitation. BV2 cells were cultured in

Dulbecco's modified Eagle's medium (Gibco Life Technologies, NY, USA) with 10% fetal bovine serum (Gibco).

Regents

Resolvin D1 (RvD1) was purchased from Cayman Chemical Company (CAS Registry No: 528583-91-7, USA). The ELISA kits of TNF-α, IL-1β and IL-6 for mouse were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies for Western blot including β-actin, ERK, p-ERK, JNK, p-JNK, p38, p-p38, p65, p-p65, STAT1, p-STAT1, STAT3, p-STAT3 were purchased from Cell Signaling Technology (CST, USA).

Animal model of SAE

Cecal ligation and puncture (CLP) was performed as reported [31] on mice to establish SAE model. The midline incision (1-1.5 cm) was made after the mice were anesthetized with inhaled sevoflurane to get cecum exposed. Then we ligated the cecum at the junction of colon with 5-0 silk suture and punctured it with a needle (22-gauge). We gently squeezed the end of cecum until a drop of intestinal content was observed. After the abdominal incision was closed, 1 ml sterile normal saline was injected subcutaneously to get the mice fluid-resuscitated.

Grouping and treatment

C57BL/6 mice were randomly divided into different groups (n=5 for each group) as following: the sham-operated group (Sham), the CLP model group (CLP) and the CLP model treated with Resolvin D1 group (CLP+RvD1). In the Sham group, all the surgical procedures of CLP, except the ligation or puncture of the cecum, were conducted to the mice. RvD1 (5 μg/kg) was injected by caudal vein immediately after the operation in the CLP+RvD1 group, while in the other groups, the same volume of normal saline was administered to the mice. At 24 hours after the operation, the blood was collected by intracardiac puncture and kept at 4°C for 3 hours. Then the blood samples were centrifuged at 4000 rpm for 10 minutes. The serum was isolated and kept at -80°C for further use. The lungs were harvested for hematoxylin and eosin (H&E) staining and the hippocampal tissues of the brain were harvested for Iba-1 immunohistochemistry and cytokines detection.

RvD1 (10 nM) or PBS was pre-added into BV2 cells 30 minutes before the stimulation of lipopolysaccharide (LPS, 100 ng/ml). The production of cytokines was detected 24 hours after the simulation of LPS by ELISA or quantitative real-time PCR (Q-PCR).

Survival analysis

Animals from the Sham group, the CLP group and the CLP+RvD1 group (n=10 for each group) were kept under observation for 7 days after grouping. The survival curves were plotted.

Morris water maze

To evaluate the learning and cognitive ability of the mice, we performed the experiment of Morris water maze [32]. The cylindrical tank (150 cm in diameter and 60 cm high) was divided into four parts and marked as 1st, 2nd, 3rd and 4th quadrant. Different geometric figures in visible areas were used as signs to help the mice memorize the locations and swimming routes. The pool was covered before experiment to decrease the disturbance of environment. Non-transparent water containing titanium dioxide at 25±2°C was poured into the tank at the height of 40 cm.

The escape platform was hidden below the water to ensure being invisible to the mice and settled in the 2nd quadrant. The position of the platform was set at 2 cm under the water to be high enough for the mice to stay. The activities of the mice and the related data were recorded by the image acquisition equipment and analyzed to evaluate their learning and cognitive ability.

All the mice were trained with 3 trials per day for 5 days in Morris water maze before grouping. During the training phase, the mice were expected to find the platform within 60 s and then stay on it for 30 s in one trial. If the mice were not able to get the platform, we would guide them there and kept them stayed on the platform for 15 s. The time of reaching the platform was defined as latency and it was recorded by the computer system. At the 1st day to 5th day after operation, we put the mice into the water randomly and the data of their latency

and average swimming speed was recorded. The probe trail was conducted at the last day and the platform was removed. We monitored the mice for 60 s to observe the tracking of their swimming. The swimming time of the mice in the target quadrant was also recorded.

ELISA

Inflammatory cytokines in the supernatants of the cell culture or in the serum of the mice from different groups were analyzed using mouse IL-6, TNF-α and IL-1β ELISA kits following the manufacture instructions.

Western blot

The cells were added with cell lysis buffer (Cell Signaling Technology) with 1% protease inhibitor cocktail (Calbiochem) and then centrifuged. The protein was extracted, and the concentration of the protein was measured using BCA assay (Thermo fisher). Protein of equal amount (30-50 μg) was fractionated with SDS-PAGE gels (10%). The protein on the gels was transferred to PVDF membrane (Millipore), which was then blocked for 2 h by Tris-buffered saline with 0.05% Tween 20 (TBST) and 5% fat-free milk. Then the membrane was incubated with primary antibodies (1:1000) at 4°C overnight. We used TBST to wash the membrane for 10 min, three times. Then the horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) was used to incubate the membrane for 2 h at room temperature. Images of the blots were observed and captured by the enhanced chemiluminescence system (Millipore).

RNA qualification

RNA in the cells or tissues was extracted and reverse-transcribed. Then we used SYBR RT-PCR kit for quantitative real-time PCR under the instruction. The reaction system was as following: SYBR-green 10 μl, deionized water 4.6 μl, Primer F/R 0.2 μl/0.2 μl, cDNA 5 μl. The primers are shown below: TNF-α (forward: 5'-AAT GGC CTC CCT CTC ATC AG-3', reverse: 5'-CCC TTG AAG AGA ACC TGG GA-3'), IL-6 (forward: 5'-TAG TCC TTC CTA CCC CAA TTT CC-3', reverse: 5'-TTG GTC CTT AGC CAC TCC TTC-3'), IL-1β (forward: 5'-CTT CAG GCA GGA AGT ATC AC-3', reverse: 5'-TTG TTG TTC ATC TCG GAG CC-3'), GAPDH (forward: 5'-AGC AGT CCC GTA CAC TGG CAA -3', reverse: 5'-TCT GTG GTG ATG TAA ATG TCC-3'). The reaction program is shown as below: 95°C for 10 s; 95°C for 5 s, 56°C for 15 s and 72°C for 10 s, repeated 2-4 for 40 cycles; 72°C for 15 s; 76°C for 1 s; 65°C and 95°C for Melt Curve (6 and 7). All the data were normalized by the expression of GAPDH.

Statistical analysis

Data were expressed in the form of mean ± standard deviation (SD). The differences between two groups were analyzed by Student's t-test. The data among three different groups were analyzed by one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA followed by Bonferroni multiple comparison tests in the behavioral experiments. The analysis of survival was conducted by Kaplan-Meier method and analyzed by Log-rank (Mantel-Cox) test. The statistical analyses of the data were performed using GraphPad Prism, and $P<0.05$ (two-sided) was considered to have statistically significant difference.

Results

RvD1 treatment improved the learning and cognitive ability of the mice with SAE

To determine whether RvD1 administration could improve the outcomes of the mice with SAE, the survival of the mice was evaluated for 7 days after grouping. All the mice of the Sham group survived during the observation, while the mortality was significantly increased in the CLP group. However, with the treatment of RvD1, the survival was significantly improved in the CLP+RvD1 group comparing with the CLP group (**Figure 1A**).

In order to investigate the protective function of RvD1 on SAE mice, we evaluated the motor ability and the learning and cognitive function of the mice from different groups in the experiment of Morris water maze. The data of average swimming speed of the mice from different groups revealed no significant statistical difference ($F(2, 12) = 2.947$, $P=0.0909$), which indicated that there was no change of motor ability of the mice after operations (**Figure 1B**). So, we considered that the changes of the mice's activity were resulted from the impairment of their cognition induced by sepsis, which could be defined as SAE. However, there were significant differences in the latency among three

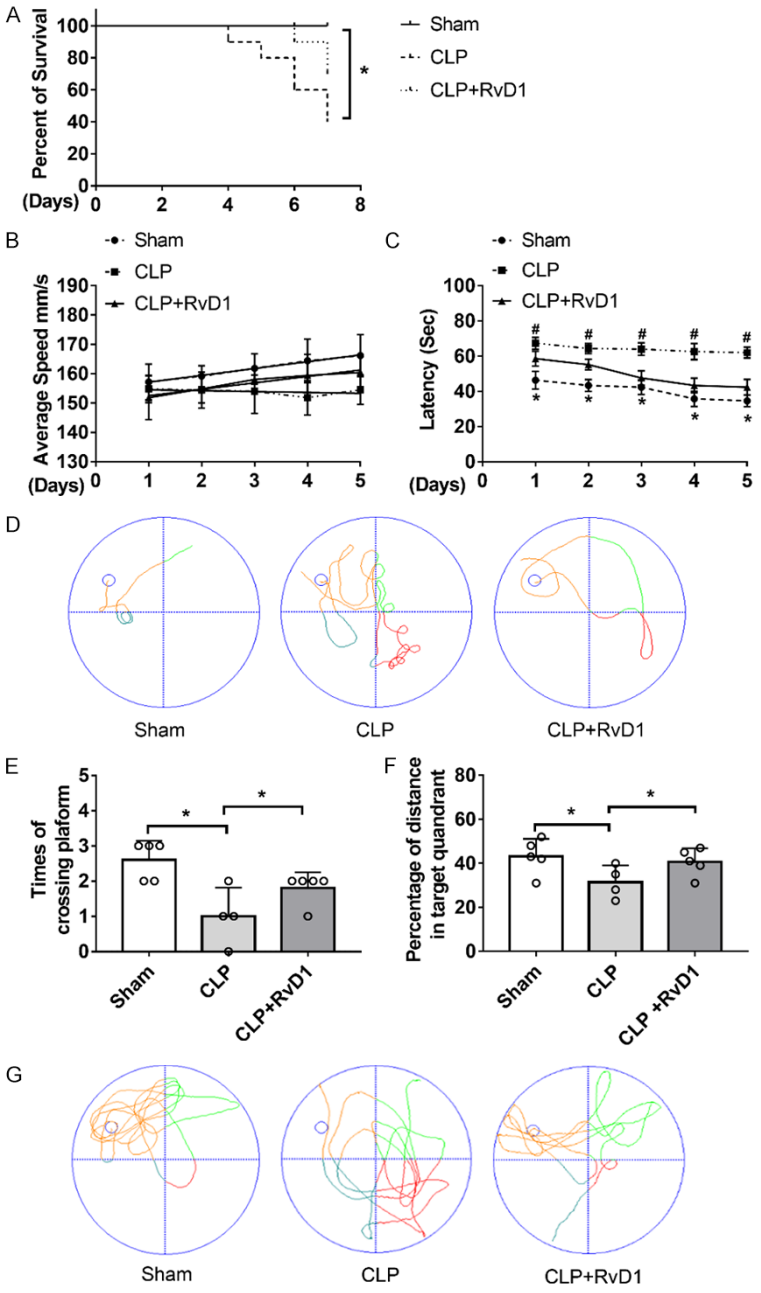

Figure 1. RvD1 treatment improved the learning and cognitive function of the mice with SAE. A. The survival curve of the mice from the Sham group, the CLP group and the CLP+RvD1 group. Comparing with the CLP group, RvD1 treatment significantly improved the survival of the SAE mice during observation. (*P<0.05) (n=10 per group). B. The average swimming speed of the mice from the Sham group, the CLP group and the CLP+RvD1 group from 1st day to 5th day after operations. There was no significant difference among three groups (F (2, 12) =2.947, P=0.0909). (n=5 per group). C. The escape latency of the mice from the Sham group, the CLP group and the CLP+RvD1 group (RvD1 5 mg/kg) from 1st day to 5th day after operations. There were significant differences among three groups (F (2, 8) =77.39, P<0.0001). Comparing with the Sham group, the latency was significantly increased in the CLP group from 1st day to 5th day (the CLP group versus the Sham group, *P<0.05), while RvD1 treatment significantly decreased the latency from 1st day to 5th day in the CLP+RvD1 group (the CLP+RvD1 group versus the CLP group, #P<0.05). D. The typical escape passages of the mice from the Sham group, the CLP group and the CLP+RvD1 group in the last time of hidden platform experiment. E. The times of crossing the platform of the mice from the Sham group, the CLP group and the CLP+RvD1 group in the probe trail. Comparing with the Sham group, the times of crossing the platform were significantly decreased in the CLP group (*P<0.05), while RvD1 treatment significantly increased the times in the CLP+RvD1 group (*P<0.05). F. The percentage of the activity distance in target quadrant of the mice from the Sham group, the CLP group and the CLP+RvD1 group in the probe trail. Comparing with the Sham group, the percentage of the activity distance in target quadrant was significantly decreased in the CLP group (*P<0.05), while RvD1 treatment significantly increased the percentage in the CLP+RvD1 group (*P<0.05). G. The typical escape passages of the mice from the Sham group, the CLP group and the CLP+RvD1 group in the probe trail.

groups (F (2, 8) =77.39, P< 0.0001). The escaping latency was significantly increased from 1st day to 5th day in the CLP group, suggesting that the spatial learning and memory ability of the mice was impaired due to SAE. With the treatment of RvD1, the latency in the CLP+RvD1 group was significantly decreased from 1st day to 5th day after operations, indicating that the cognitive impairment induced by SAE was improved by RvD1 (**Figure 1C**). In the last hidden platform experiment, the mice in the CLP+RvD1 group spent less time to get the hidden platform with shorter distance comparing with the mice in the CLP group (**Figure 1D**). Moreover, in the probe trail, the times of crossing the hidden platform were increased in the CLP+RvD1 group (**Figure 1E**), with longer activity distance in target quadrant

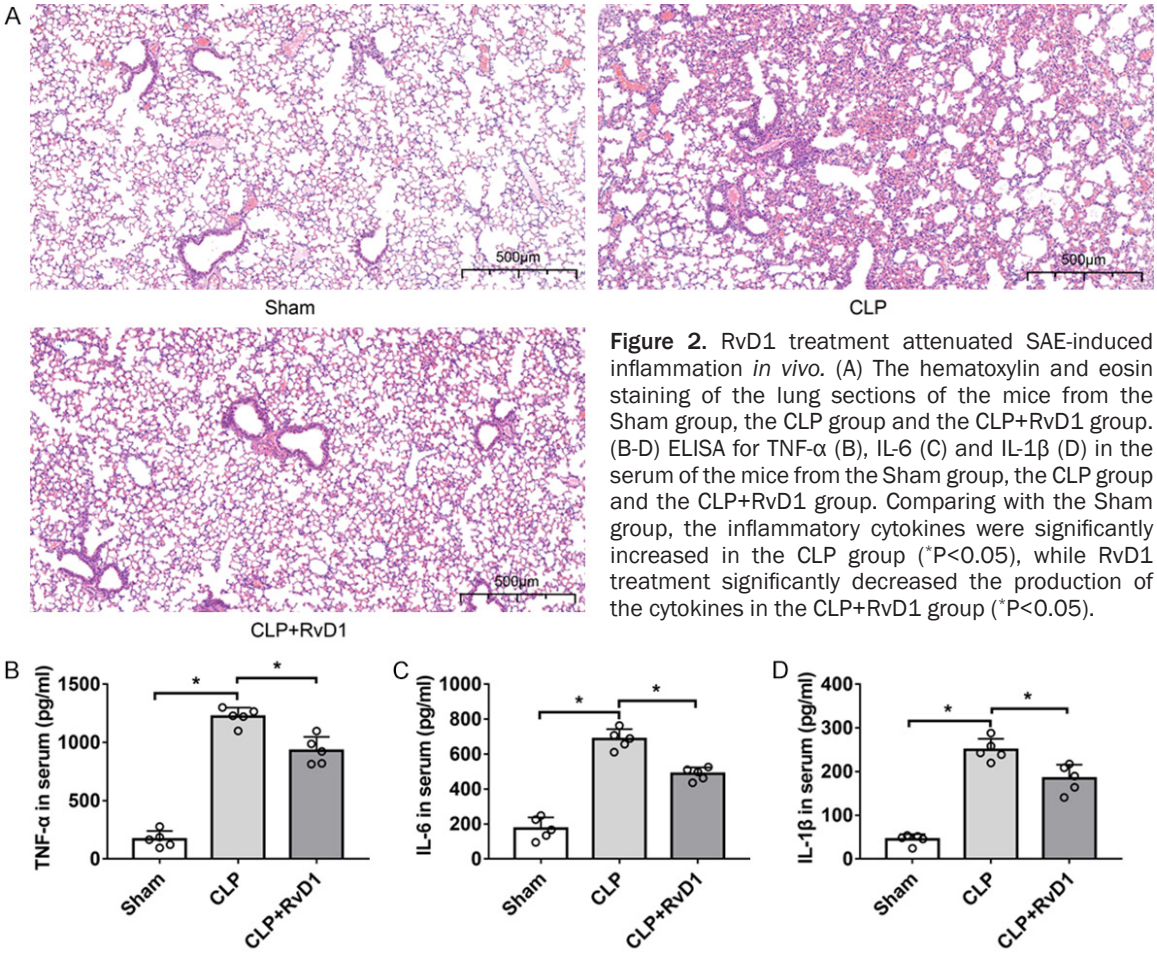

Figure 2. RvD1 treatment attenuated SAE-induced inflammation *in vivo*. (A) The hematoxylin and eosin staining of the lung sections of the mice from the Sham group, the CLP group and the CLP+RvD1 group. (B-D) ELISA for TNF-α (B), IL-6 (C) and IL-1β (D) in the serum of the mice from the Sham group, the CLP group and the CLP+RvD1 group. Comparing with the Sham group, the inflammatory cytokines were significantly increased in the CLP group (*P<0.05), while RvD1 treatment significantly decreased the production of the cytokines in the CLP+RvD1 group (*P<0.05).

(**Figure 1F**). By analyzing the typical trails, it was found that the trajectory in target quadrant of the mice was reduced in the CLP group but increased in the CLP+RvD1 group (**Figure 1G**).

These data revealed that the SAE mice with RvD1 treatment in the CLP+RvD1 group were easier to find the hidden platform with more activity in target quadrant, indicating that RvD1 significantly improved the learning and cognitive function of the mice and functioned as a protective role on the SAE mice.

RvD1 treatment attenuated SAE-induced inflammation in vivo

To further find out how RvD1 functioned on the mice with SAE, we evaluated the severity of inflammatory responses in three different groups. Comparing with the Sham group, more inflammatory cells infiltrated in the lung tissues were observed in the CLP group, while the inflammatory cells were significantly reduced with the addition of RvD1 in the CLP+RvD1 group (**Figure 2A**). In the meantime, cytokines induced by the inflammation during sepsis, including IL-6, TNF-α and IL-1β, were significantly increased in the CLP group, while these cytokines were significantly decreased in the serum of the mice from the CLP+RvD1 group (**Figure 2B-D**). These results revealed that RvD1 could attenuate SAE-induced inflammation *in vivo*.

RvD1 treatment inhibited the activation of microglia in the hippocampus

The hippocampal region of the brain is reported to be responsible for the ability of studying, memory and cognition [33]. In previous study, it has been reported that RvD1 combines with the ALX/FPR2 receptor of microglia and inhibits the inflammation induced by LPS in microglia [34], which exist massively in the hippocampal region and participate in the pathogenesis of SAE. Thus, it is conceivable that the anti-inflammatory function of RvD1 on the SAE mice might be induced by its function on microglia.

To confirm this hypothesis, the hippocampal tissues of the mice from different groups were harvested. After Iba-1 immunohistochemically staining, the tissues were observed under microscope of different magnification (100×, 200× and 400×). It was found that comparing with the Sham group, the microglia from the CLP group were significantly increased and appeared as obviously active state with extended cell processes and bigger cell bodies. However, with the treatment of RvD1, the amount of microglia was significantly decreased and appeared as unapparent activation state (**Figure 3A-C**).

To further explore whether the change of microglia influenced the inflammatory responses, we detected the concentration as well as their mRNA levels of the inflammatory cytokines in the hippocampus. It was found that comparing with the Sham group, the concentration and mRNA levels of IL-6, TNF-α and IL-1β were significantly increased in the SAE mice from the CLP group (**Figure 3D-F**), however, these inflammatory cytokines and their mRNA levels were significantly decreased with the treatment of RvD1 in the CLP+RvD1 group (**Figure 3G-I**).

These data demonstrated that RvD1 could inhibit the activation of microglia in the hippocampal region of the SAE mice and attenuated the production of inflammatory cytokines in the hippocampus.

RvD1 treatment attenuated SAE-induced inflammation in vitro

To further investigate the function of RvD1 on microglia, we established the experiment model of SAE *in vitro* by treating BV2 cells with LPS. After 24 hours' stimulation of LPS with or without the treatment of RvD1, we detected the production of inflammatory cytokines. We found that, with the treatment of RvD1, the production of IL-6, TNF-α and IL-1β in the supernatant of BV2 cells was significantly decreased (**Figure 4A-C**). Accordingly, the mRNA levels of them were also decreased in the cells (**Figure 4E**, **4F**).

To further evaluate the anti-inflammatory function of RvD1, we treated BV2 cells with different doses of RvD1 (1 nM, 10 nM and 100 nM). It was found that the production of the cytokines in the supernatant of BV2 cells was significantly reduced with the addition of RvD1 at the dose of 10 nM and 100 nM (**Figure 4G-I**).

These results revealed that RvD1 significantly inhibited LPS-induced inflammation of microglia *in vitro* and the function of anti-inflammation might be dose-dependent.

RvD1 treatment inhibited the phosphorylation of signaling pathways

The nuclear factor-κB (NF-κB), the mitogen-activated protein kinases (MAPKs) and the signal transducer and activator of transcription (STAT) pathways play vital roles in inflammation during sepsis. To investigate the mechanism through which RvD1 impaired inflammatory responses in microglia, we analyzed the expression and their phosphorylation of some molecules in these signaling pathways, including p65, JNK, ERK, p38, STAT1 and STAT3. It was found that the treatment of RvD1 significantly decreased the phosphorylation of p65, JNK, ERK, STAT1 and STAT3 induced by the stimulation of LPS (**Figure 5**). The phosphorylation of these factors led to the activation of NF-κB, MAPKs and STAT signaling pathways and induced inflammatory responses. These findings demonstrated that RvD1 treatment could restrain the activation of p65, JNK, ERK, STAT1 and STAT3 and inhibit the inflammatory signaling pathways, which might also explain how RvD1 attenuated the inflammation of microglia, as well as how it functioned as a protective role on the SAE mice.

Discussion

The endogenous lipid mediator RvD1 has been reported by its therapeutic function in many organ injuries induced by sepsis, however, its role on SAE has not been found yet. In our study, we found that RvD1 improved the ability of learning, memory and cognition of the mice with SAE by inhibiting the activation of microglia in hippocampus and attenuated the inflammation of microglia both *in vitro* and *in vivo*. RvD1 treatment significantly inhibited the production of TNF-α, IL-6 and IL-1β by attenuating the activation of molecules from NF-κB, MAPKs and STAT signaling pathways of microglia. Our findings of the protective function of RvD1 on the SAE mice provided a novel therapeutic method to the treatment of SAE.

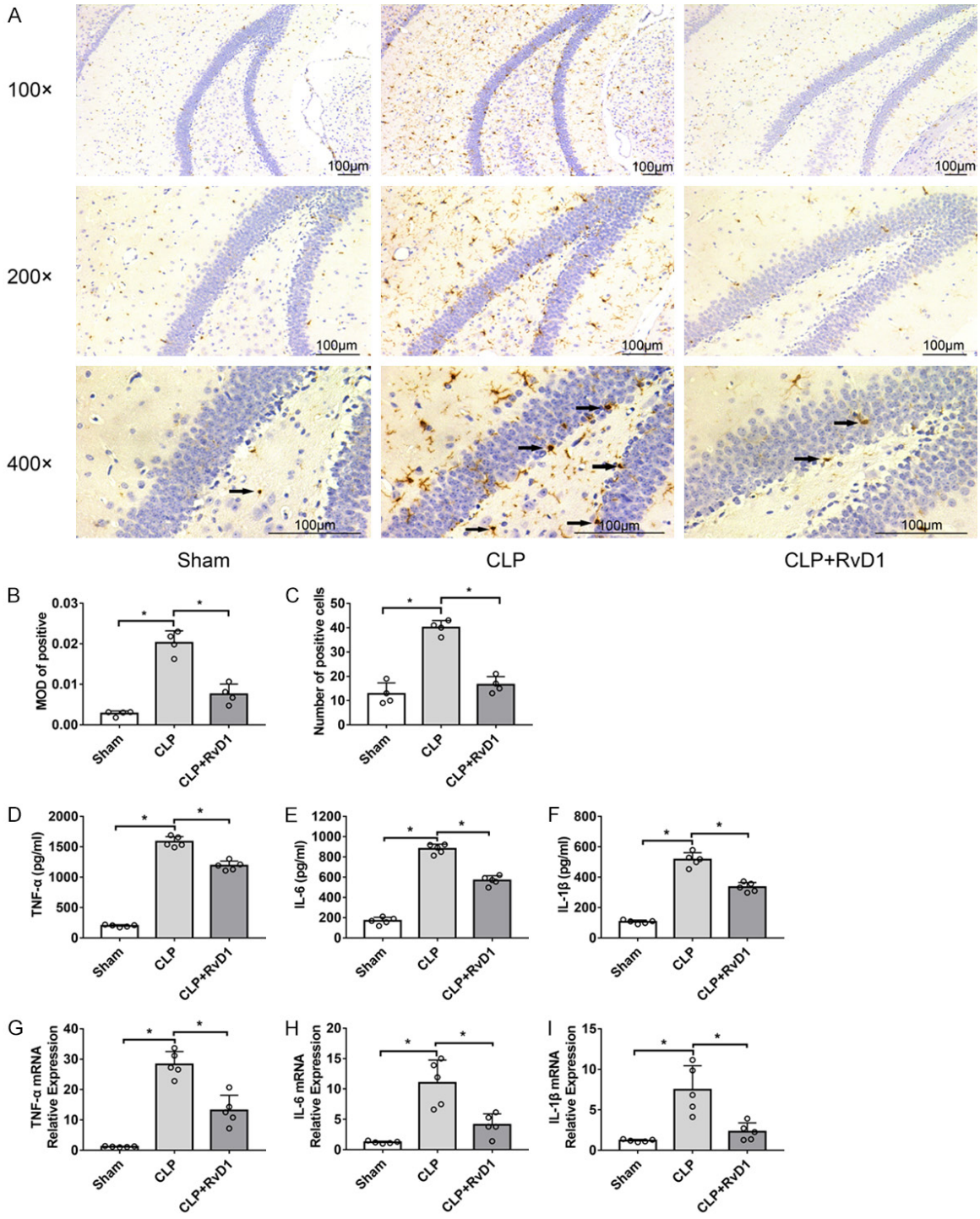

Figure 3. RvD1 treatment inhibited the activation of microglia in the hippocampus. (A) Iba-1 immunohistochemically staining of the hippocampus tissues observed under the microscope of different magnifications (100×, 200× and 400×) of the mice from the Sham group, the CLP group and the CLP+RvD1 group. (B, C) The immunohistochemical quantification of the staining by Image J (B). The number of the positive cells in hippocampus tissues of the mice from the Sham group, the CLP group and the CLP+RvD1 group were counted (C). Comparing with the Sham group, the positive cells were significantly increased in the CLP group (*P<0.05), while RvD1 treatment significantly reduced these cells in the CLP+RvD1 group (*P<0.05). (D-F) ELISA for TNF-α (D), IL-6 (E) and IL-1β (F) in the hippocampus tissues of the mice from the Sham group, the CLP group and the CLP+RvD1 group. Comparing with the Sham group, the inflammatory cytokines were significantly increased in the CLP group (*P<0.05), while RvD1 treatment significantly reduced these cytokines in the CLP+RvD1 group (*P<0.05). (G-I) Q-PCR analysis of TNF-α (G), IL-6

(H) and IL-1β (I) mRNA levels in the hippocampus tissues of the mice from the Sham group, the CLP group and the CLP+RvD1 group. Comparing with the Sham group, the mRNA levels of these inflammatory cytokines were significantly increased in the CLP group (*P<0.05), while RvD1 treatment significantly decreased the mRNA levels of them in the CLP+RvD1 group (*P<0.05). The results were normalized to GAPDH expression.

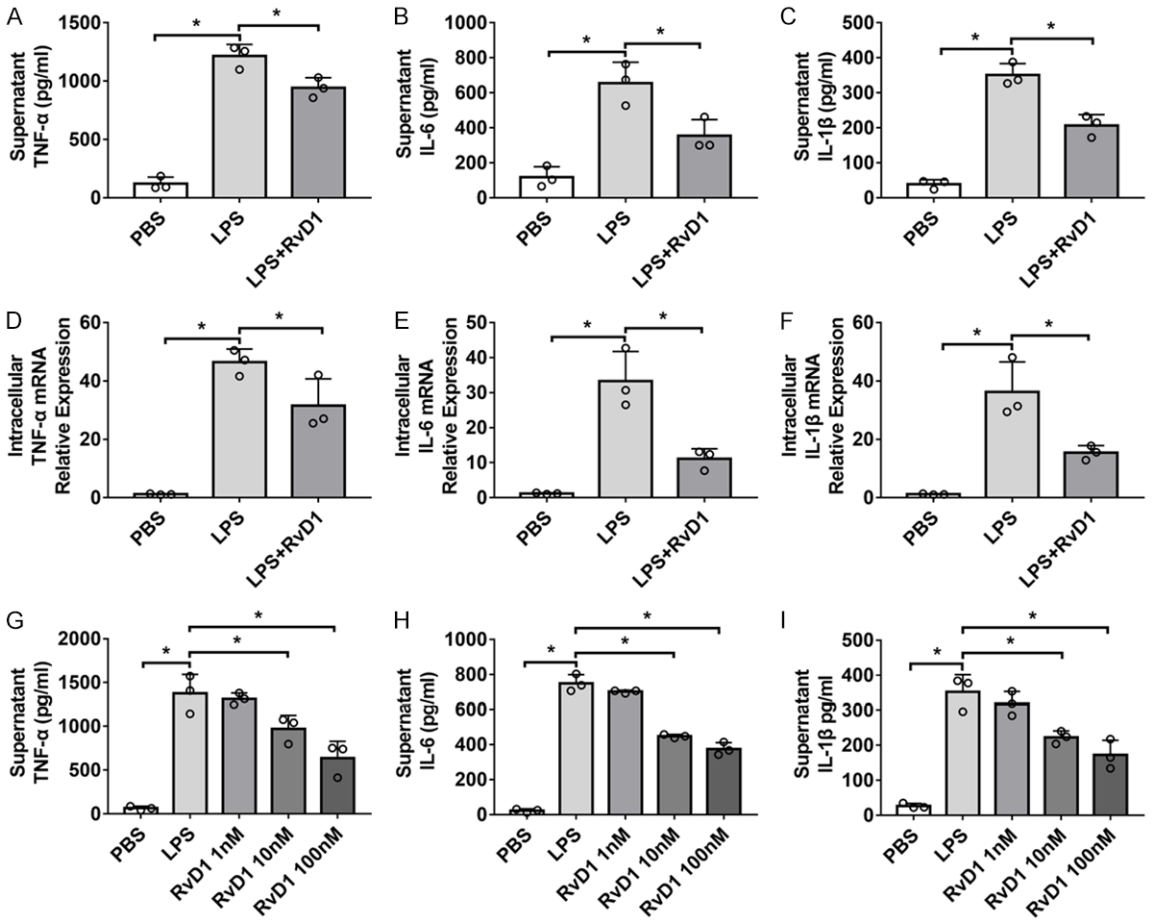

Figure 4. RvD1 treatment attenuated SAE-induced inflammation *in vitro*. (A-C) ELISA for TNF-α (A), IL-6 (B) and IL-1β (C) in the supernatant of the BV2 cells from the PBS group, the LPS (100 ng/ml) group and the LPS+RvD1 (10 nM) group. Comparing with the PBS group, the inflammatory cytokines were significantly increased in the LPS group (*P<0.05), while RvD1 treatment significantly decreased the cytokines in the LPS+RvD1 group (*P<0.05). (D-F) Q-PCR analysis of TNF-α (D), IL-6 (E) and IL-1β (F) mRNA levels in the BV2 cells from the PBS group, the LPS group and the LPS+RvD1 group. Comparing with the PBS group, the mRNA levels of the inflammatory cytokines were significantly increased in the LPS group (*P<0.05), while RvD1 treatment significantly decreased the mRNA levels of them in the LPS+RvD1 group (*P<0.05). The results are normalized to GAPDH expression. (G-I) ELISA for TNF-α (G), IL-6 (H) and IL-1β (I) in the supernatant of the BV2 cells from the PBS group, the LPS group and the RvD1 1 nM group, the RvD1 10 nM group, the RvD1 100 nM group. Comparing with the PBS group, the inflammatory cytokines were significantly increased in the LPS group (*P<0.05), while RvD1 treatment (10 nM and 100 nM) significantly decreased the production of the cytokines (*P<0.05).

Sepsis and sepsis-associated organ dysfunctions have always been the severe public health problems that need to be solved. The brain injuries induced by sepsis is defined as SAE, which may take place at the early stage of sepsis or appear as one of the symptoms of multiple organ dysfunctions resulted from advanced sepsis. It is mainly characterized and diagnosed by its clinical manifestations of neurological symptoms, which differs from other organ injuries induced by sepsis. SAE is mainly diagnosed by the clinical features of the patients [5], so the diagnostic criteria is undefined and it's hard to evaluate the brain func-

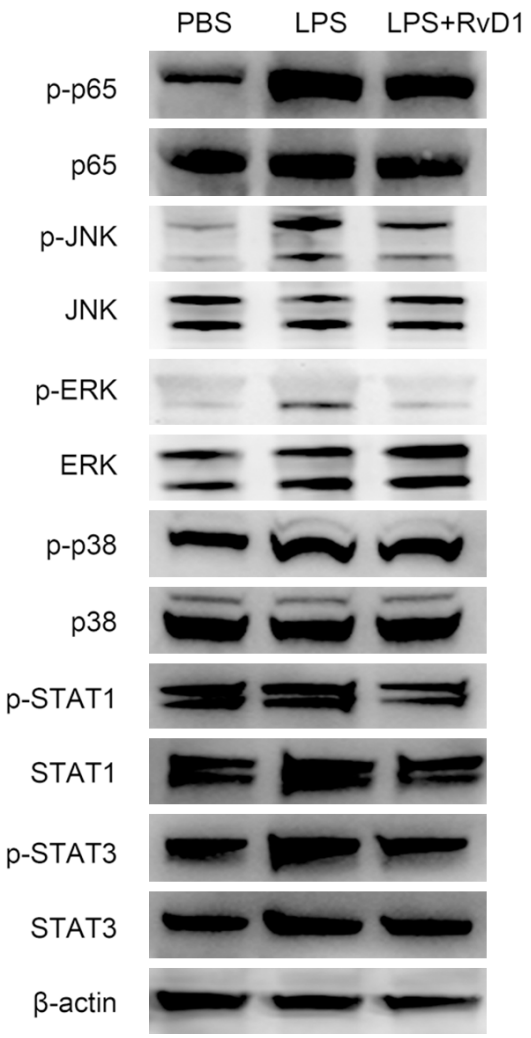

Figure 5. RvD1 treatment inhibited the phosphorylation of signaling pathways. The protein expression of p-p65, p65, p-JNK, JNK, p-ERK, ERK, p-p38, p38, p-STAT1, STAT1, p-STAT3, STAT3 and β-actin of the BV2 cells from the PBS group, the LPS group and the LPS+RvD1 group. The phosphorylation of p65, JNK, ERK, STAT1 and STAT3 was significantly decreased with the treatment of RvD1 in the LPS+RvD1 group.

tion of the SAE patients. Thus, the researches on SAE are mostly limited to animal experiments at present [35]. In our study, we established the SAE animal model by CLP method, which was reported as the classic and stably repeatable way to get septic mice [31], and evaluated the learning and cognitive ability of the mice by Morris water maze [32] to confirm the cognition impairment. We found that comparing with the Sham group, the mice from the CLP group spent longer time finding the hidden platform in the water, indicating that their cognitive ability was impaired during sepsis, which demonstrated that the SAE mice model could be successfully constructed by the method of CLP according to the definition and diagnosis of SAE.

Previous investigations have revealed that RvD1 is beneficial to the therapy of different organ dysfunctions. It has been found that RvD1 could activate NF-κB p50/p50-mediated cyclooxygenase-2 expression and improve the resolution of inflammation in acute respiratory distress syndrome [30]. It has also been reported that RvD1 have protective function against sepsis-induced cardiac injury and lung injury in mice and attenuate inflammatory responses [29, 36]. The dosage of RvD1 used in vivo was reported ranging from 1 ng to 1000 ng per mouse in different studies [37-39]. Others also reported that 100 ng RvD1 injected via caudal vein could improve the survival of the mice with sepsis [40]. In order to confirm the dosage of RvD1 used in this study, we first set the concentration gradient of it and treated the CLP mice with different dosages of RvD1 (1 ng, 10 ng, 100 ng per mouse) in our pre-experiment. By detecting the concentration of TNF-α, IL-1β and IL-6 in the serum of the mice, we found that these cytokines were significantly decreased with the treatment of 100 ng RvD1. The average weight of the mice was about 20 g, so we used 5 μg/kg as the dosage of RvD1 in our studies. Consistent with the researches above, our study found that RvD1 had protective function on the mice with SAE. The treatment of RvD1 could significantly improve the learning and cognitive ability of the SAE mice. Moreover, RvD1 could attenuate inflammatory responses *in vivo*.

The pathogenesis of SAE is multifactorial with several mechanisms, including directed bacterial invasion, endotoxins, inflammatory mediators, the change of neurotransmitters and oxidative stress, have been reported as the possible causes of the brain damage induced by sepsis [41]. Accumulating evidence have shown that the neuro-inflammation could be a vital process during SAE, which leads to the exaggerated secretion of inflammatory cytokines and the dysfunction or apoptosis of neurons and endothelial cells [42]. The activation of the microglia may result in local and peripheral inflammation. Previous research has proved

that activated microglia release various inflammatory mediators [43], which could further improve the function of neuron-toxic A1 astrocytes and intensify neuro-inflammation [44]. Therefore, early control of the inflammation induced by microglia is critical for the treatment of SAE.

RvD1 is one of the Resolvin family members with anti-inflammatory function on many kinds of diseases. RvD1 was reported to combine with ALX/FPR2, a receptor of mice which also expressed in microglia and astrocytes. Considering the important role of microglia in SAE, it's reasonable to infer that the protective function of RvD1 may be related to them. Notably, the hippocampal region was closely associated with the impairment of cognition, so we chose the hippocampus as our researching target. As demonstrated above, we found that with the treatment of RvD1, the activation of microglia was significantly inhibited in the hippocampus with decreasing production of IL-6, TNF-α and IL-1β, indicating that RvD1 attenuated inflammatory responses induced by microglia during SAE. To further find out the mechanism, we analyzed the signaling pathways in BV2 cells stimulated by LPS, and found that the phosphorylation of p65, JNK, ERK, STAT1 and STAT3 was decreased with the treatment of RvD1.

The innate immune system is the first line to fight against infections when pathogens invade the host cells during sepsis. The pattern-recognition receptors (PRRs) of the host, such as Toll-like receptors (TLRs), recognize pathogen-associated molecular patterns (PAMPs), like LPS, and then the innate immune responses are triggered. The inflammatory cytokines released by the innate immune system, such as TNF-α and IL-6, generally leads to the activation of NF-κB, MAPK/ERK and JAK/STAT signaling pathways, which participate and regulate multiple gene expression in inflammation [45]. The subsequent activation of these signaling pathways then further induces more production of the cytokines, including TNF-α, IL-6 and IL-1β. However, the over-reaction of the immune system and the excessive production of the cytokines then conversely induces cell damages. The signaling pathway molecules can be activated by multiple post-translational modifications (PTMs) such as phosphorylation, which will lead to the promotion of innate immune responses. Thus, inhibiting the activation of these factors contributes to the control of the inflammation of many diseases. In our study, RvD1 treatment was found to decrease the phosphorylation of p65 from NF-κB signaling pathway, JNK and ERK from MAPKs signaling pathway, and STAT1 and STAT3 from STAT signaling pathway in microglia and reduced the production of TNF-α, IL-6 and IL-1β. These results can partly explain how RvD1 functions as a protective regulator on the SAE mice.

However, there are some limitations in our present study. In the study, we mainly evaluated the cognitive and learning ability of the mice with SAE by the hidden platform trial and the probe trail of Morris water maze. Although the motor ability of the mice from different groups was assessed by analyzing their swimming speed, there were still some other influential factors needed to be excluded, such as sedation or the emotion change of the mice. Evaluation of the sedation degree and the rotating rod test could be adopted in the further study.

Although we found the protective role of RvD1 on the SAE mice, the researches on the mechanism were not adequate and more studies on it are required. At present, we found that RvD1 could decrease the activation of several inflammatory signaling pathways, which might be more like a "phenomenon" rather than a "mechanism". It was found that the phosphorylation of p65, JNK, ERK, STAT1 and STAT3 was inhibited by RvD1, further investigations of the concrete mechanism and the specific interactions between RvD1 and the molecules from these signaling pathways are still needed. In consideration of the function of RvD1 on NF-κB signaling pathway, we plan to find out whether RvD1 could influence the translocation of NF-κB to the nucleus of microglia by immunofluorescent staining method with confocal laser scanning fluorescence microscope. Besides, it was reported that RvD1 could also attenuate the NLRP3 inflammasome and STAT3 signaling [43] and the function of RvD1 might be related to the deacetylase named SIRT1 [46]. Whether these factors are involved in the function of RvD1 on the SAE mice requires more investigations.

Moreover, in consideration of the complex mechanism of SAE, the inhibition of inflammation in microglia could not be the only reason of

the protective function of RvD1. In recent studies, researchers found that the activation of mitophagy or alleviating A1 astrocyte by blocking HMGB1/RAGE signaling could also attenuate SAE [44]. Since the receptor ALX/FPR2 also exists in astrocytes, whether RvD1 is related to these processes remains to be explored.

In summary, sepsis and sepsis-induced multiple organ dysfunctions remain to be great challenges for clinicians and scientists because of the versatile and complex pathology and mechanism. Our findings that RvD1 improved the learning and cognitive ability of the SAE mice by inhibiting the local and systemic inflammation could be helpful to better understand the pathophysiology processes of SAE and provided a novel therapeutic method in clinic.

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Disclosure of conflict of interest

None.

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