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ORIGINAL ARTICLE

Disrupted Tryptophan Metabolism Induced Cognitive Impairment in a Mouse Model of Sepsis-associated Encephalopathy

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Abstract—Sepsis-associated encephalopathy (SAE) is a common complication in critically ill patients and is associated with a poor prognosis. However, the precise mechanisms underlying sepsis-induced cognitive impairment remain largely to be elucidated. The aim of the present study was to investigate whether indoleamine 2, 3-dioxygenase (IDO) activation-mediated neurotoxicity is involved in the pathophysiology of sepsis-induced cognitive impairment. Sepsis was induced by cecal ligation/perforation (CLP). The animals were randomly divided into the following five groups: Sham+vehicle group; Sham+1-methyl-D, L-tryptophan group; Sham+L-Kynurenine group; CLP+vehicle group; or CLP+1-methyl-D, L-tryptophan group. The survival rate was estimated by the Kaplan–Meier method. Behavioral tests were performed by the open field and fear conditioning tests at days 13 and 14 after operation. In the present study, we demonstrated that sepsis induced a deficit in hippocampus-dependent cognitive impairment in a mouse model of SAE. Furthermore, a single peripheral kynurenine administration, the metabolic product of IDO, induced a deficit in the cognitive impairment in the sham mice. However, mice treated with IDO inhibitor 1-methyl-D, L-tryptophan were protected from sepsis-induced cognitive impairment. In conclusion, our study implicates IDO-dependent neurotoxic kynurenine metabolism as a critical factor responsible for the sepsis-induced cognitive impairment and a potential novel target for the treatment of SAE.

KEY WORDS: sepsis; cognitive impairment; indoleamine 2, 3-dioxygenase; kynurenine.

INTRODUCTION

Sepsis-associated encephalopathy (SAE) remains a common complication in critically ill patients, which represents one brain dysfunction caused by a systemic inflammation in the absence of a direct brain infection [1–4].

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Clinical studies have demonstrated that the brain may be one of the first organs affected by sepsis, and septic patients often suffer from cognitive impairment after discharge from the hospital [1]. It is reported that severe sepsis is independently associated with new cognitive impairment, functional disability, and increased mortality among survivors [1, 4]. Although several mechanisms, including oxidative stress, inflammation, neurotransmission disturbance, and cell death have been proposed [2–4], the precise mechanisms responsible for sepsis-induced cognitive impairment remain largely to be elucidated.

The kynurenine pathway, a major route for tryptophan catabolism, is activated during neuroinflammation in several neurodegenerative diseases [5, 6]. Increased cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β can activate indoleamine 2, 3-dioxygenase (IDO) to generate several neuroactive

intermediates: quinolinic acid (QA), 3-hydroxykynurenine (3-HAA), and kynurenic acid (KA) [5, 6]. The first stable intermediate in the pathway is kynurenine, which can be converted to two other intermediates via two different pathways: the neurotoxic 3-HAA and QA, and the neuroprotective KA [5]. It has been shown that the level of QA is usually elevated while KA is decreased in the blood and brain of Alzheimer's disease (AD) [7, 8]. In addition, the alteration of QA and KA has also been implicated in the pathogenesis of depression [9, 10], schizophrenia [11], and other neuroinflammatory diseases [12]. These findings lead to the hypothesis that IDO inhibition may have a therapeutic efficacy for sepsis-induced cognitive impairment.

In the present study, we therefore hypothesize that sepsis-induced IDO activation mediated-disrupted tryptophan metabolism is involved in the pathophysiology of sepsis-induced cognitive impairment.

MATERIALS AND METHODS

Animals

One hundred eight male C57BL/6 mice (3–4 months) were obtained from the Animal Center of Nanjing University of Chinese Medicine, Nanjing, China. The study protocol was approved by the Ethics Committee of the Nanjing Integrated Traditional Chinese and Western Medicine Hospital, affiliated with the Nanjing University of Chinese Medicine and all procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. All experimental mice were housed under a 12-h light/dark cycle (lights on at 07:00–19:00 hours) in a temperature-controlled room at 24 ± 1 °C with free access to food and water.

Drug and Reagents

The IDO inhibitor, 1-methyl-D, L-tryptophan, was obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA) and was administered in the drinking water (5 mg/ml, pH 10.7) one day before operation and for another seven consecutive days. L-Kynurenine was purchased from Sigma (St. Louis, MO, USA). Mice received a single i.p. injection of 100 mg/kg L-kynurenine 30 min before the behavioral tests, while sham mice were injected with the equivalent volume (0.1 ml) of normal saline (vehicle). We chose this time point because it has been suggested that this interval is sufficient to allow for an increase in peripheral and central kynurenine metabolites by peripherally administered L-kynurenine [12].

Animal Model

Sepsis was induced by CLP as described previously [13, 14]. Briefly, male C57BL/6 mice were anesthetized with an intraperitoneal injection of 2 % sodium pentobarbital (60 mg/kg; Sigma Chemical Co, St. Louis, MO). Thereafter, the abdominal region was shaved and cleaned thoroughly with povidone iodine. The cecum was ligated with 4.0 silk below the ileocecal junction, approximately 0.8 cm from the distal end, then the cecum was then punctured twice on the anti-mesenteric side with a sterile 22-gauge needle and was gently squeezed to extrude the fecal contents into the peritoneal cavity. After that, the cecum was placed back into the abdomen and the incision was closed with sutures in layers. Animals in the sham groups underwent laparotomy without ligation or perforation. All mice were resuscitated with subcutaneous lactated Ringers 30 ml/kg and antibiotic therapy (ertapenem, 20 mg/kg; Merck Research Laboratory, USA) immediately after surgery then returned to their cages.

Experimental Grouping

The animals were randomly divided into the following five groups: Sham+vehicle group ($n=12$); Sham+1-methyl-D, L-tryptophan group ($n=12$); Sham+L-Kynurenine group ($n=12$); CLP+vehicle group ($n=16$); or CLP+1-methyl-D, L-tryptophan group ($n=16$). Separate groups of animals were used to determine the inflammatory mediators at 12 h after operation. The flow chart for the experimental protocol is shown in Fig 1a.

Open Field Tests

An investigator who was blind to the treatment condition performed all the behavioral experiments at 14:00–17:00 hours in a sound-isolated room as we previously described [15]. On the 13th day, mice were gently placed in the center of a white plastic chamber (40 cm×40 cm×40 cm) for 5 min and the exploratory behavior was automatically recorded by a video tracking system (XR-XZ301, Shanghai Soft maze Information Technology Co, Ltd, Shanghai, China). The total distance and total time traveled in the open field were recorded.

Fear Conditioning Test

Briefly, the conditioning (acquisition) trial consisted of a 3-min exploration period followed by one conditioned stimulus (CS)–unconditioned stimulus (US) pairing separated by 1 min each. A contextual test was performed in the conditioning chamber for 5 min without
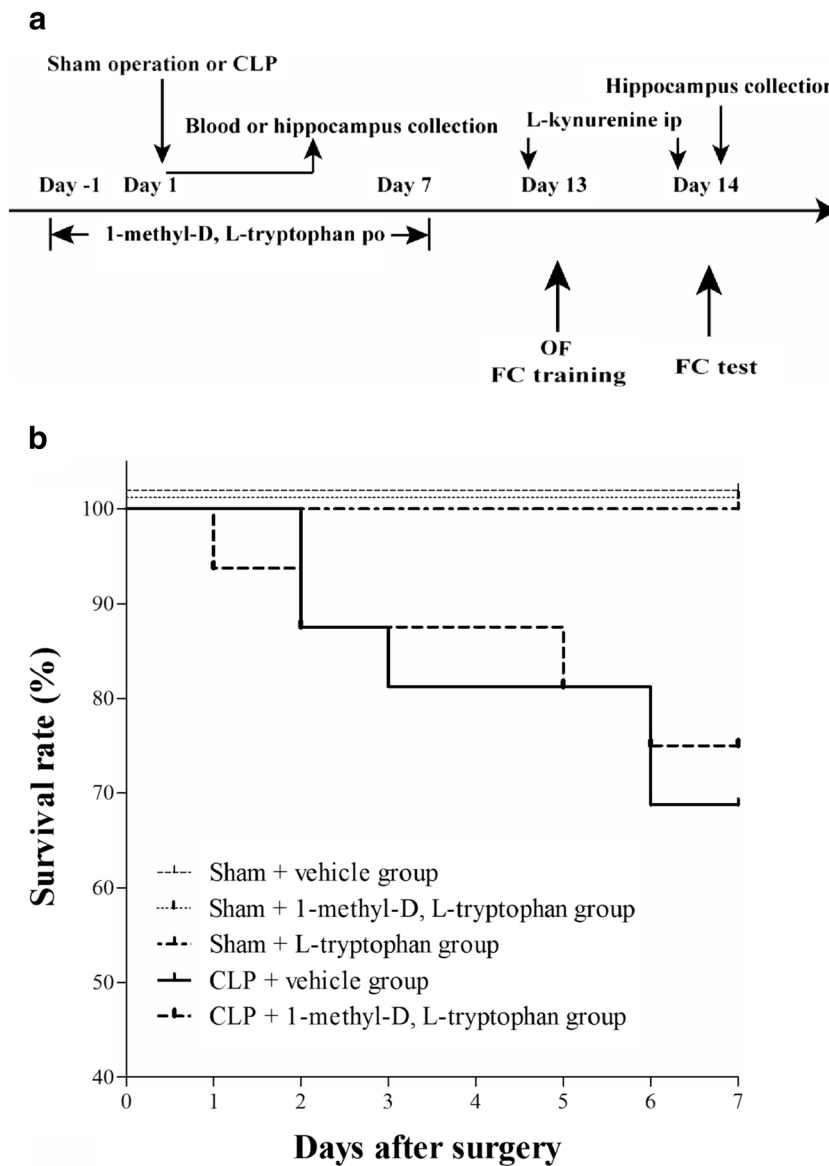
Fig. 1. Schematic timeline of the experimental protocol (**a**) and effects of 1-methyl-D, L-tryptophan treatment on the survival rate within 7 days after CLP. 1-methyl-D, L-tryptophan treatment did not affect the survival rate after CLP ($n = 12$–16 per group). *CLP* cecal ligation/perforation, *OF* open field, *FC* fear conditioning.

any stimulation at 24 h after the conditioning trial. A cued test was performed by presentation of the cue (80-dB noise, 3-min duration) in an alternative context with distinct visual and tactile cues.

Qualitative and Quantitative Analyses of Kynurenine and Tryptophan

Samples of plasma and brains were performed using the high-performance liquid chromatography (HPLC) analysis by a well-trained investigator who is employed in the First People's Hospital of Nanjing Medical University. L-Tryptophan and L-kynurenine were quantified using absorbance detection spectrometry. The processing of the data was conducted using a HPLC-1260 Chromatograph workstation (Agilent). Also, the kynurenine/tryptophan ratio, an estimate of IDO activity, was determined by dividing the plasma kynurenine concentration by the plasma tryptophan concentration.
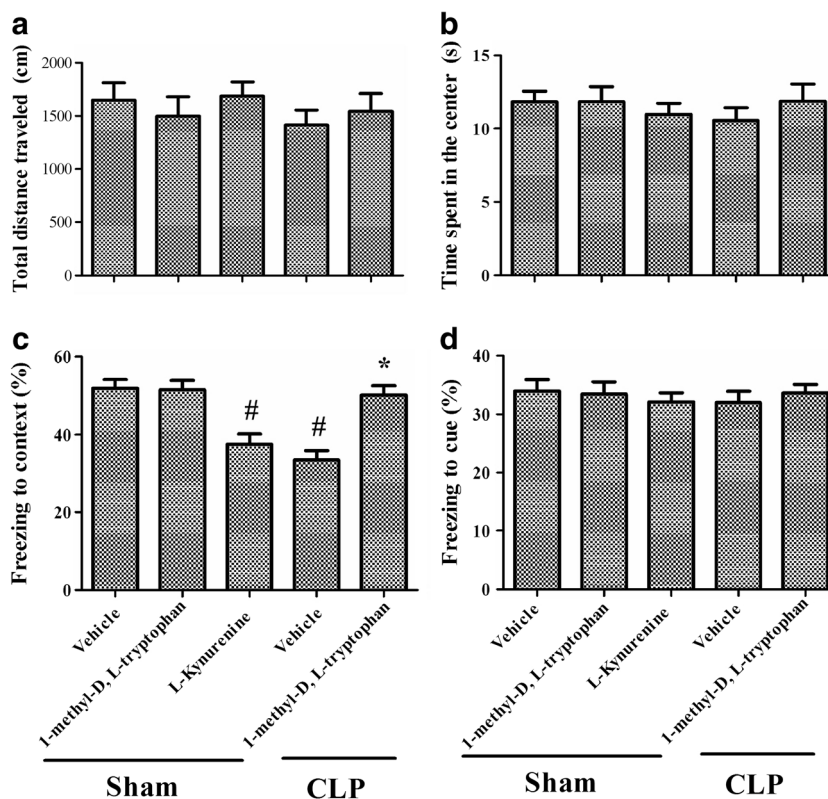
Fig. 2. CLP-induced cognitive impairment was attenuated in mice treated with 1-methyl-D, L-tryptophan. CLP mice treated with vehicle did not show the abnormal locomotor activity and anxiety-like behavior (**a**, **b**). The freezing time to context was decreased in the CLP + vehicle group compared with the sham groups, whereas 1-methyl-D, L-tryptophan treatment increased the freezing time to context in the CLP + 1-methyl-D, L-tryptophan group compared with the CLP + vehicle group (**c**). However, no difference was observed in the cued fear conditioning test among the four groups (**d**). A single peripheral L-kynurenine administration induced cognitive impairment in the sham mice (**c**). Data are shown as mean ± SEM ($n = 10–12$ per group). [#]$P < 0.05$ versus the sham groups; [*]$P < 0.05$ versus the CLP + vehicle group.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of TNF-α, IL-1β, IL-6, and IDO were determined at 12 h after sham operation or CLP, while the brain-derived neurotrophic factor (BDNF) level was measured at 14 days after sham operation or CLP. The mice were killed by decapitation and then the hippocampus was collected, then separated, and placed in a homogenizer. The tissue was homogenized with 1 ml of ice-cold physiological saline per 100 mg of brain tissue. Hypothermal centrifugation was performed at 10,000×g for 10 min and the supernatant was obtained. The quantifications of TNF-α, IL-1β, IL-6, and BDNF were done by the instructions (North China Institute of Biotechnology, Beijing, China). IDO concentrations were determined using a commercially available ELISA (R&D, Minneapolis, MN, USA) following the manufacturer's instructions.

Immunofluorescence

At 12 h after sham operation or CLP, the mice were deeply anesthetized with 2 % sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with 30 mL of saline followed by 4 % paraformaldehyde in phosphate-buffered saline (pH 7.4). The brains were immediately removed, postfixed in the same 4 % paraformaldehyde for 2 h, and dehydrated in 30 % sucrose at 4 °C overnight. The brains were embedded in optimum cutting temperature compound, cut in 10-μm-thick sections on a freezing microtome, and mounted on glass slides. Slices were blocked with 1 % bovine serum albumin for 1 h at room
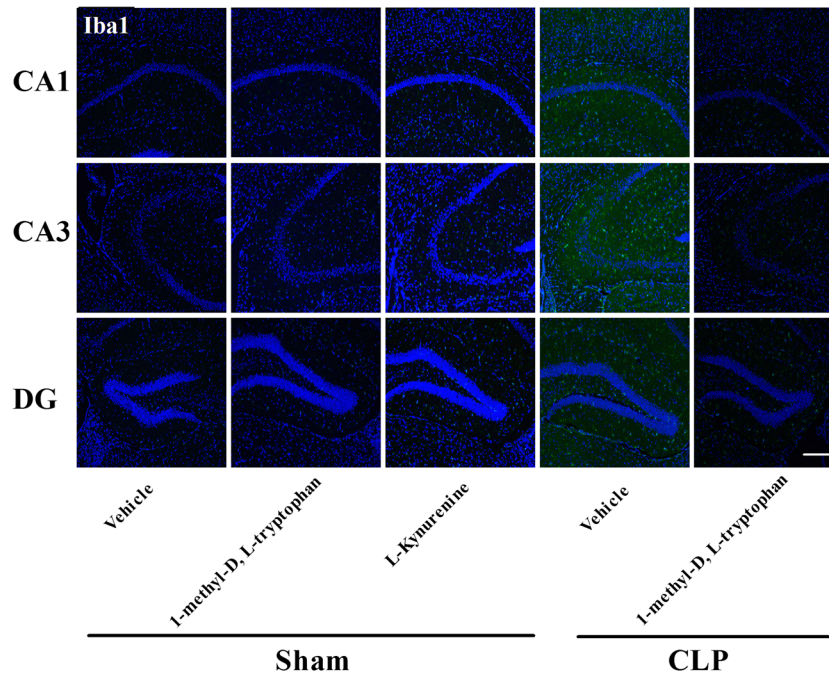
Fig. 3. 1-methyl-D, L-tryptophan treatment downregulated marker of microglia activation induced by CLP. CLP significantly increased the expression of microglial Iba1 in the CLP+vehicle group, while 1-methyl-D, L-tryptophan treatment partly decreased the expression of Iba1 in the CLP+1-methyl-D, L-tryptophan group. Representative images of Iba1 in the DG, CA1, and CA3 regions of the hippocampus at 12 h after operation (nuclei counterstained with DAPI; scale bar, 100 μm).

temperature followed by incubating the following primary antibodies: rabbit anti-Iba1 (1:600; Abcam) in 1 % bovine serum albumin at 4 °C overnight. After three washes with phosphate-buffered saline, sections were incubated with the secondary antibodies, including goat anti-rabbit IgG-FITC (1:300; Santa Cruz Biotechnologies), and goat anti-mouse IgG-cy3 (1:600; Bioworld) for 1 h at room temperature. After washing out the secondary antibody, sections were incubated with 4′, 6-diamidino-2-pheny-lindole (DAPI) for nuclear staining. The fluorescent images were captured by a confocal microscope (Olympus, Melville, NY, USA).

Data Analysis

Statistical analyses were performed by the Statistical Product for Social Sciences (SPSS; version 16.0, IL, USA). Data are expressed as mean±SEM (standard error of the mean). Normal distribution of data was analyzed using the Kolmogorov–Smirnov test. Differences among groups were evaluated by one-way of analysis of variance (ANOVA) followed by a Tukey test as appropriate. The survival rate was estimated by Kaplan–Meier method and compared by the log-rank test. Bivariate relationship was evaluated by Pearson correlation coefficients. A result of $P<0.05$ was regarded as statistically significant.

RESULTS

1-Methyl-D, L-Tryptophan Treatment did not Affect the Survival Rate After CLP

As shown in Fig. 1b, 1-methyl-D, L-tryptophan treatment did not significantly increase the survival rate in the CLP+1-methyl-D, L-tryptophan group as compared with that in the CLP+vehicle group (75 % versus 68.75 %, $P=0.727$).

CLP-Induced Cognitive Impairment was Attenuated in Mice Treated with 1-Methyl-D, L-Tryptophan

To assess the locomotor activity and anxiety-like behavior, mice underwent the open field test. As compared with the sham groups, CLP mice treated with the vehicle did not show the abnormal
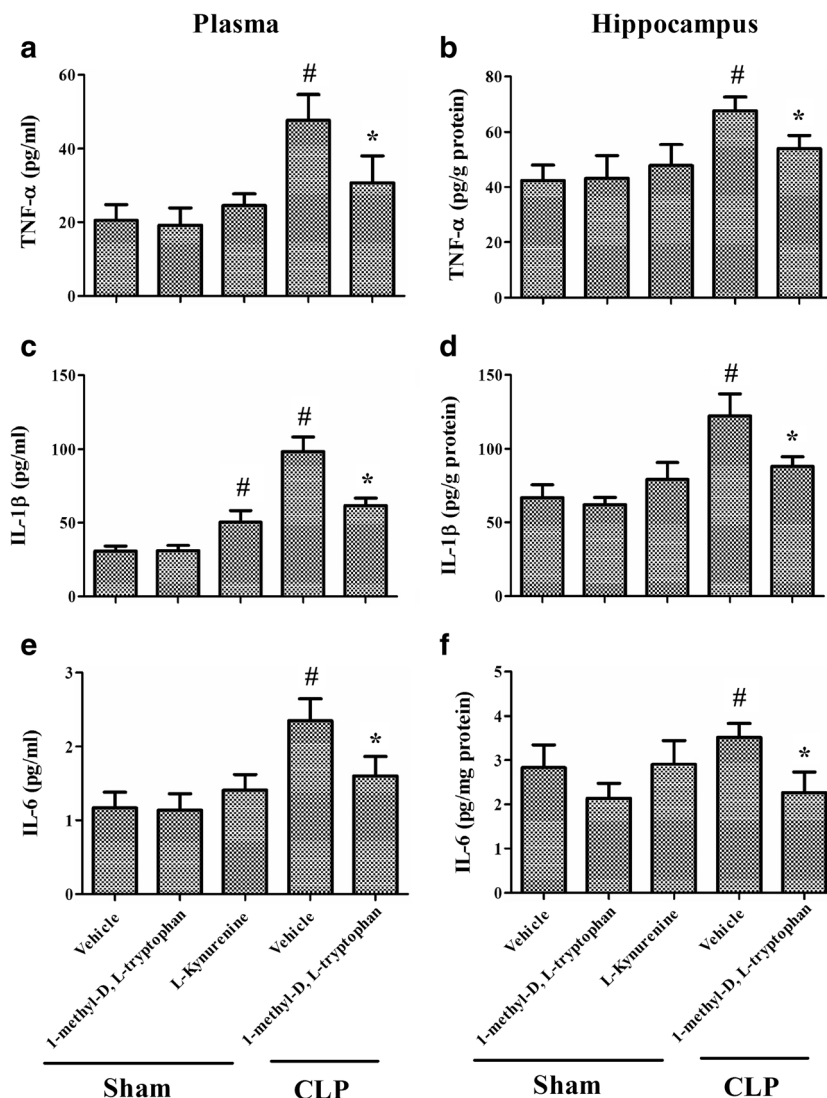
Fig. 4. 1-methyl-D, L-tryptophan treatment downregulated the increased proinflammatory cytokines induced by CLP. CLP significantly increased plasma and hippocampal levels of TNF-a, IL-1β, and IL-6 as compared with the sham groups (**a–f**). However, L-Kynurenine administration only significantly increased IL-1β level when compared with the sham groups (**c**). 1-methyl-D, L-tryptophan treatment downregulated the increased TNF-a, IL-1β, and IL-6 levels in the CLP+1-methyl-D, L-tryptophan group compared with the CLP+vehicle group (**a–f**).

locomotor activity and anxiety-like behavior as assessed by the ambulatory distance and time spent in the center during the open field tests (all $P>0.05$, Fig. 2a, b). To test whether CLP leads to long-term cognitive impairment, mice were subjected to contextual/cued fear conditioning tests. The freezing time to context was significantly decreased in the CLP+vehicle group compared with the sham groups, whereas 1-methyl-D, L-tryptophan treatment increased the freezing time to context in the CLP+1-

methyl-D, L-tryptophan group compared with the CLP+vehicle group ($P<0.05$, Fig. 2c). However, no difference was observed in the cued fear conditioning test among the four groups ($P>0.05$, Fig. 2d).

Administration of L-Kynurenine-Induced Cognitive Impairment in Sham Mice

Although no locomotor activity and anxiety-like behavior was affected in the open field test, a single
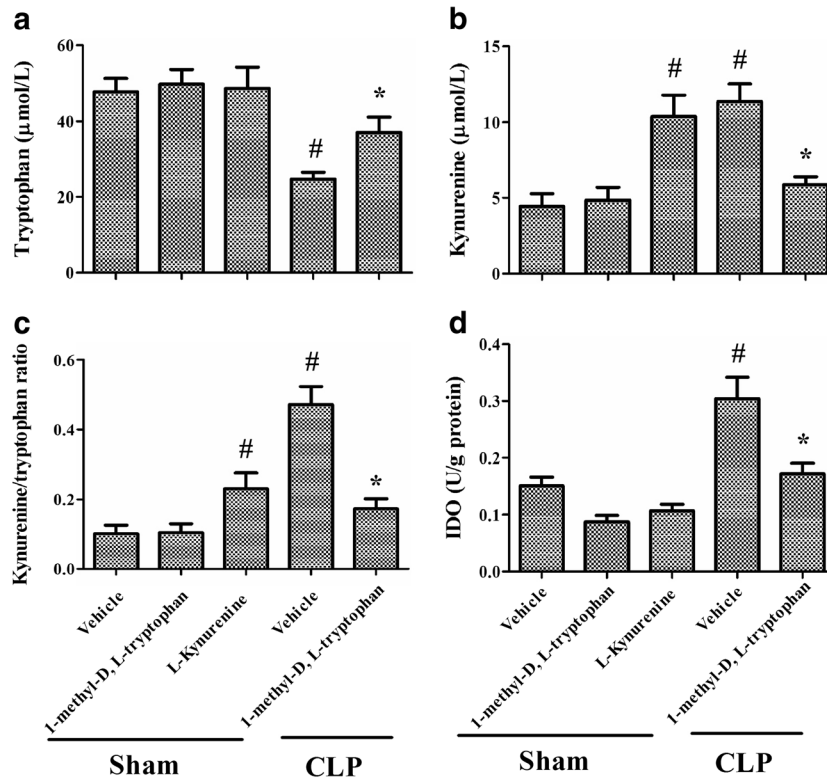
Fig. 5. 1-methyl-D, L-tryptophan treatment downregulated the increased expressions of kynurenine, kynurenine/tryptophan ratio, and IDO activity but upregulated the decreased tryptophan level induced by CLP. CLP significantly increased the levels of kynurenine, kynurenine/tryptophan ratio, and IDO activity but upregulated the decreased tryptophan level in the CLP+vehicle group compared with the sham groups (**a–d**). However, 1-methyl-D, L-tryptophan treatment downregulated the increased expressions of kynurenine, kynurenine/tryptophan ratio, and IDO activity but upregulated the decreased tryptophan level in the CLP+1-methyl-D, L-tryptophan group when compared with the CLP+vehicle group. Data are shown as mean±SEM ($n=6$ per group). $^{\#}P<0.05$ versus the sham groups; $^{*}P<0.05$ versus the CLP+vehicle group. IDO, indoleamine 2, 3-dioxygenase.

peripheral L-kynurenine administration, the metabolic product of IDO, induced a deficit in the cognitive impairment in sham mice ($P<0.05$, Fig. 2).

1-Methyl-D, L-Tryptophan Treatment Downregulated the Increased Proinflammatory Cytokines and Markers of Microglia Activation Induced by CLP

CLP significantly increased the expression of microglial Iba1 (Fig. 3), which was accompanied by the increased expressions of TNF-a, IL-1β, and IL-6 in the CLP+vehicle group as compared with the sham groups (all $P<0.05$, Fig. 4). However, 1-methyl-D, L-tryptophan treatment downregulated the increased TNF-a, IL-1β, and IL-6 levels in the CLP+1-methyl-D, L-tryptophan group when compared with the CLP+vehicle group. Interestingly, L-kynurenine administration only significantly increased IL-1β expression when compared with the sham groups ($P<0.05$, Fig. 4).

1-Methyl-D, L-Tryptophan Treatment Downregulated the Increased Expressions of Kynurenine, Kynurenine/Tryptophan Ratio, and IDO Activity but Upregulated the Decreased Tryptophan Level Induced by CLP

CLP significantly increased the levels of kynurenine and kynurenine/tryptophan ratio but deceased tryptophan expression in the CLP+vehicle group compared with the sham groups ($P<0.05$, Fig. 5). However, 1-methyl-D, L-tryptophan treatment downregulated the increased expressions of kynurenine, kynurenine/tryptophan ratio, and IDO activity but upregulated the decreased tryptophan level in the CLP+1-methyl-D, L-tryptophan group when compared with the CLP+vehicle group ($P<0.05$, Fig. 5). Since an examination of the IDO expression in different regions showed a significant increase in the hippocampus, compared with the thalamus, midbrain, and prefrontal cortex [16], thus, we only measured these parameters in the hippocampus in the present study.

1-Methyl-D, L-Tryptophan Treatment Prevented CLP-Induced Increase in Kynurenine Level and Decrease in BDNF Level

As shown in Fig 6, CLP significantly increased the kynurenine level but decreased the BDNF level in the hippocampus when compared with the sham groups ($P<0.05$). Notably, 1-methyl-D, L-tryptophan treatment prevented a CLP-induced increase in kynurenine level and decrease in BDNF level. Furthermore, an inverse correlation was observed between the BDNF and kynurenine ($r=-0.4396$, $P<0.0151$; Fig. 6c).

DISCUSSION

The results of the present study demonstrate that sepsis significantly impaired the hippocampus-dependent cognitive impairment in a mouse model of SAE. A single L-kynurenine administration, the metabolic product of IDO, was able to induce a cognitive deficit in a pattern similar to the CLP-induced cognitive impairment. Intriguingly, the IDO inhibitor 1-methyl-D, L-tryptophan, attenuated neuroinflammation and thus ameliorated sepsis-induced cognitive impairment.

There is a growing body of evidence supporting the role of inflammation in the development of SAE and long-term cognitive impairment [17–19]. Tryptophan is an essential amino acid that is central to cellular respiration and neurotransmission, and is also a key immune mediator [5, 6]. However, during inflammatory conditions, tryptophan is metabolized by extra-hepatic enzyme IDO to the toxic metabolite kynurenine [5]. Kynurenine can subsequently be metabolized via two distinct routes: via the kynurenine aminotransferases yielding KA or via kynurenine monooxygenase (KMO), kynureninase, and 3-hydroxyanthranilic acid 3,4-dioxygenase to produce QA [5, 6]. QA acts at N-methyl-D-aspartate receptors as an agonist in the brain, KMO activity in the microglia is the rate limiting step for the generation of QA [5, 6]. Several lines of evidence have suggested that the development of mood symptoms in patients with inflammatory conditions is often associated with reduced circulating tryptophan levels and concomitant increased serum or cerebrospinal fluid concentrations of one of its main metabolites,
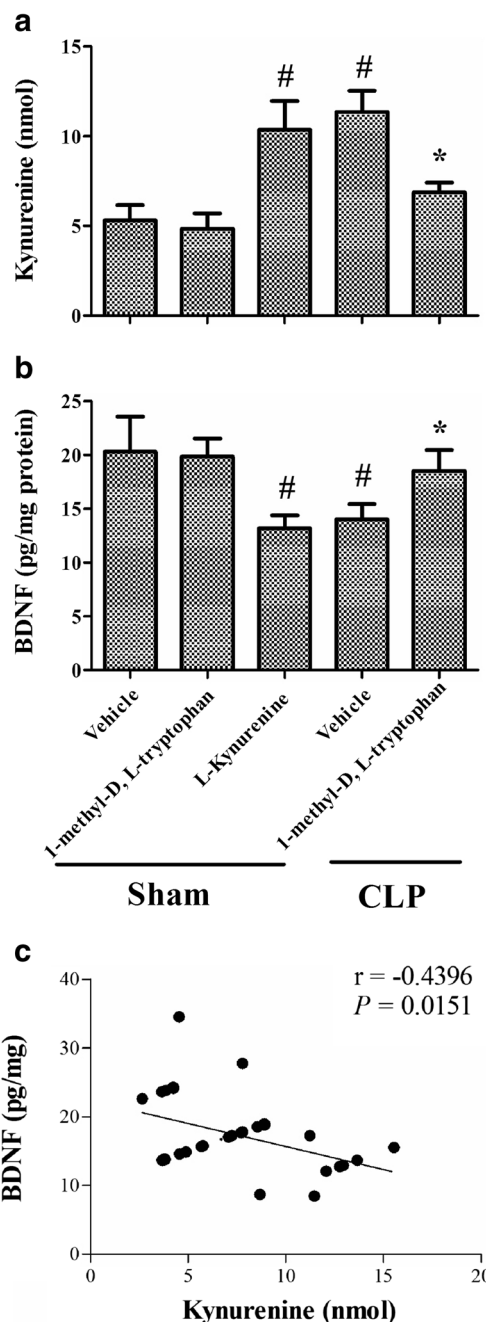

Fig. 6. 1-methyl-D, L-tryptophan treatment prevented CLP-induced increase in kynurenine level and decrease in BDNF level. CLP significantly increased the kynurenine level and decreased the BDNF level in the hippocampus when compared with the sham groups (**a, b**). However, 1-methyl-D, L-tryptophan treatment prevented CLP-induced increase in kynurenine level and decrease in BDNF level. Furthermore, an inverse correlation was observed between BDNF and kynurenine (**c**).
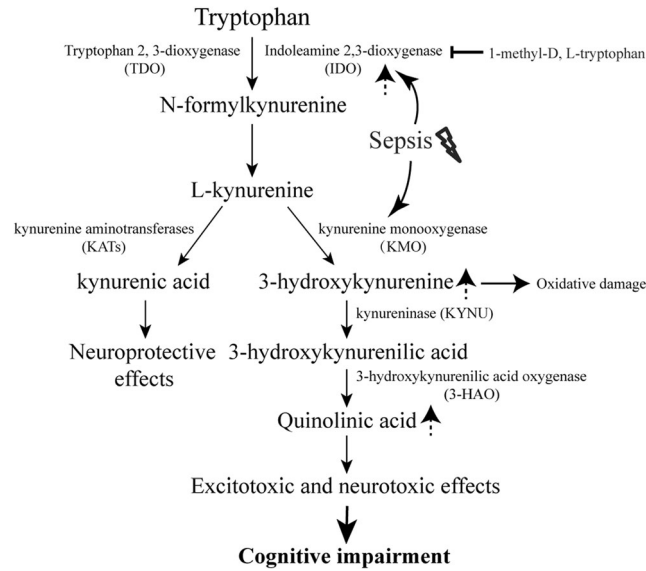
Fig. 7. Summary of the major findings of the present study. Sepsis induced IDO activation and increased inflammatory mediators and several neuroactive intermediates, which subsequently contributed to sepsis-induced cognitive impairment.

kynurenine [12, 20, 21]. Furthermore, there was a positive relationship between kynurenine/tryptophan ratio and inflammatory mediators, which were supported by the findings that mice treated with IDO inhibitors have lower plasma IL-6 concentrations [12]. In addition, the activation of brain IDO is sufficient to induce depression-like behaviors of mice in response to central lipopolysaccharide administration [22], whereas inhibition of IDO by 1-methyl-tryptophan prevented neuroinflammation-induced depressive-like behavior [23, 24].

Post-septic encephalopathy is a poorly understood condition that is characterized by cognitive and affective impairments in sepsis survivors [1]. One recent study has suggested that LPS-induced sepsis induces depressive-like and anxiety-like behaviors but no cognitive impairment [17]. However, our results showed that sepsis induced by CLP resulted in significantly impaired hippocampus-dependent cognitive impairment, which was accompanied by increased inflammatory mediators and kynurenine, and deceased BDNF concentrations. This discrepancy might be explained by the different animal model used and the time to perform these behavioral tests. Indeed, it has been demonstrated that sepsis induced gradual behavioral deficits during follow-up in a rat model of SAE [25]. However, we only performed the

open field and fear conditioning tests to reflect some of the behavioral alternations in sepsis survivors. Thus, further studies are required to assess other behavioral changes caused by sepsis, including the spatial working memory and depressive-like behavior.

Although physiological levels of various cytokines, including IL-1, TNF-a, IL-6, and IFN-γ, are required for synaptic plasticity and neurogenesis [26], pathophysiologically elevated levels of these cytokines have opposite effects [26, 27]. At high levels, IL-1β reduced hippocampal neurogenesis and BDNF expression in a nuclear factor (NF)-κB dependent way, inducing depressive-like behavioral symptoms in rodents [28]. Since substantial evidence suggests that neurotrophic factors play a key role in learning and memory [29], the sepsis-induced reduction in hippocampal BDNF could have deleterious effects on the cognitive function during sepsis development. In our study, we showed that 1-methyl-D, L-tryptophan treatment prevented synaptic loss by increasing BDNF levels induced by CLP. These results implicated IDO-dependent neurotoxic kynurenine metabolism and subsequent synaptic loss as pathogenic factors responsible for the sepsis-induced cognitive impairment.

In conclusion, the results of the present study implicate hippocampal IDO activation in sepsis-

associated cognitive impairment, suggesting a key role for IDO and subsequent kynurenine metabolism in mediating inflammation-induced cognitive impairment (summarized in Fig. 7).

ACKNOWLEDGMENTS

COMPLIANCE WITH ETHICAL STANDARDS

The study protocol was approved by the Ethics Committee of the Nanjing Integrated Traditional Chinese and Western Medicine Hospital, affiliated with the Nanjing University of Chinese Medicine and all procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals from the National Institutes of Health, USA.

Conflict of Interest. The authors declare that they have no competing interests.

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