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# LncRNA Lethe protects sepsis-induced brain injury via regulating autophagy of cortical neurons

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**Abstract. – OBJECTIVE: To investigate the role of long non-coding RNA (lncRNA) Lethe in mediating autophagy of cortical neurons in mice with sepsis-induced brain injury (SIBI).**

**MATERIALS AND METHODS: A total of 60 wild-type C57BL/6 mice were divided into sham-operated wild-type (SWT) group and wild-type model (MWT) group. Sixty Lethe$^{-/-}$ mice were divided into sham-operated knockout (SKO) group and model knockout (MKO) group. Each group had 30 mice. Sepsis model in mice was established by cecal ligation and puncture (CLP). Neurobiological score was recorded at 6 h after CLP. Mice with lower than 6 scores of neurobehavioral tests were diagnosed with SIBI. Quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to determine mRNA levels of Lethe and interferon-γ (INF-γ) in cortical neurons of SIBI mice. Western blot was conducted to detect protein levels of LC3-II, LC3-I and SQSTM1 in mice. Neuronal impairment in mouse brain was evaluated by hematoxylin and eosin (HE) staining.**

**RESULTS: Expressions of LC3-I and LC3-II in cerebral cortex of MWT group began to increase at 6 h after CLP, and remained at high levels until 96 h. On the contrary, SQSTM1 expression in cerebral cortex of MWT group began to decrease at 6 h after CLP. Compared with SWT group, expressions of Lethe and IFN-γ were remarkably upregulated in cortex of MWT group at 12 h after CLP. Expression of LC3-II in MWT group was remarkably upregulated, while SQSTM1 was downregulated at 12 h after CLP, which were contrary to those in MKO group. At 12 h after CLP, the neurobiological scores of the MKO group (4.97±0.71) were markedly lower than those of the MWT group (5.43±0.86). HE staining showed worse damage in cerebral cortex and fewer neurons of MKO group relative to MWT group.**

**CONCLUSIONS: Lethe has a protective effect on SIBI mice by regulating autophagy in mouse cortical neurons.**

*Key Words:*
Sepsis-induced brain injury, Cerebral cortex, Autophagy, Lethe.

## Introduction

Sepsis is a common critical illness in children. More than 50% of sepsis patients are accompanied by different degrees of brain damage[1]. The pathogenesis of sepsis may be related to immune dysfunction secondary to systemic inflammatory response, including dysfunctions of monocytes[2] and lymphocytes[3], immune response to the blood-brain barrier[4,5] and etc. However, the specific mechanism of sepsis has not been completely clarified. Autophagy is a process in which eukaryotic cells are encapsulated, degraded and recycled, showing a crucial function in the development of infectious diseases[6]. Long non-coding RNAs (LncRNAs) are ubiquitous transcripts with more than 200 nt expressed in eukaryotic cells. They could not encode proteins by themselves, but are capable of regulating nuclear or cytoplasmic proteins at multiple levels. LncRNAs are closely related to pathological processes, such as tumor formation, viral replication, and inflammatory injury[7]. Therefore, searching for disease-related lncRNAs and exploring their biological functions have been widely concerned. Rapicavoli *et al*[8] found that lncRNA Lethe acts on p50, the nuclear factor-kappa B (NF-κB) subunit in mouse embryonic fibroblasts. Lethe mainly distributes in nuclear chromosome, which is involved in the folding of NF-κB subunits, TNF-α and IL-1β. Nuclear Lethe inhibits the NF-κB pathway by binding to p65 and thus inactivates it. Hence, a negative feedback loop formed by Lethe is able to inactivate genes regulated by NF-κB pathway. However, the existence of Lethe homologous genes and their potential functions still remain unclear. In this study, sepsis-induced brain injury (SIBI) model in mice was established by cecal ligation and puncture (CLP). We aim to study the role of Lethe in autophagy of cortical neurons in SIBI mice.



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## Materials and Methods

### Experimental Animals

Sixty wild-type C57BL/6 mice with 30 days old were provided by the Hangzhou Medical College Laboratory Animal Center. Sixty Lethe$^{-/-}$ mice were provided by Zhejiang University-Science B Biotechnology Company. Wild-type C57BL/6 mice were randomly divided into sham-operated wild-type (SWT) group and wild-type model (MWT) group. Lethe$^{-/-}$ mice were randomly divided into sham-operated knockout (SKO) group and model knockout (MKO) group. Each group had 30 mice. All mice were given to free access to food and water in avoidance of intense light and noise. This study was approved by the Animal Ethics Committee of Chongqing Medical University Animal Center.

### Establishment of Sepsis Model in Mice

Sepsis model in mice was established by CLP[5]. Mice were anesthetized with 10% chloral hydrate by intraperitoneal injection at a dose of 4 mL/kg. A 2-cm incision was cut in the midline of the abdomen to expose the cecum. Mesentery between the cecum was carefully cut in avoidance of blood vessels on the membrane. Contents of the cecum were gently squeezed to the distal cecum. A ligation was made at 4 cm away from the cecum, where 2 repeated punctures were performed using an 18 G needle. 1 mm excreta were squeezed from the puncture site. The incision was sutured using the 6-0 suture. After CLP procedures, abdominal injection of Ringer's solution (5 mL/100 g) was given to prevent shock. Clindamycin (150 mg/kg) and ceftriaxone (50 mg/kg) (Xian-Janssen Pharmaceutical Ltd., Xian, China) were intraperitoneally injected every 6 hours to prevent infection. Mice in SWT and SKO group received the same procedures except for CLP.

### Neurobiological Score and SIBI Diagnosis

Six hours after CLP procedures, mouse mental and activity status, wound healing status and presence of infection were recorded. Neurobehavioral tests including auricular reflex, corneal reflex, righting reflex, appendix reflex and escape reflex were recorded as well. Neurobehavioral scores were recorded (0, no reflection; 1, reflection reduction; 2, normal reflection). The highest score of neurobehavioral tests was 10. Mice with lower than 6 scores of neurobehavioral tests were diagnosed with SIBI, and were enrolled in MWT or MKO group.

### Hematoxylin and Eosin (HE) Staining of Cerebral Cortex

Mice were sacrificed at different time points. Brain tissues anterior to chiasma opticum and 1 cm posterior to it were resected in coronal section. The middle part of brain tissues was fixed in 4% paraformaldehyde for HE staining (Boster, Wuhan, China) and immunohistochemistry. Tissues were dewaxed, rehydrated and stained with hematoxylin/eosin. Subsequently, tissues were subjected to gradient dehydration, and they were sealed and observed using a microscope.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from mouse cerebral cortex was extracted at different time points according to the total RNA purification kit instructions. Complementary deoxyribose nucleic acid (cDNA) was synthesized using the TaqMan reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). Analysis was performed on a StepOnePlus™ PCR Thermal Cycler (Applied Biosystems, Foster City, CA, USA). QRT-PCR system was 20 μL in total, with 10 μL of mix, 0.2 μL (50 pmol/μL) of upstream and downstream primers, 7.6 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) and 2 μL of cDNA. QRT-PCR conditions were pre-denaturation at 93°C for 2 min; denaturation at 93°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 30 s, for a total of 40 cycles. The relative expression was calculated by $2^{-\Delta\Delta Ct}$. Lethe, F: 5'-ACAATGAAGCCAAACTGCCG-3', R: 5'-AGTTTGTCCAAGGGACCCCA-3'; INF-γ, F: 5'-TGAACGCTACACACTGCATCTTGG-3', R: 5'-CGACTCCTTTTCCGCTTCCTGAG-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5'-CGGCCGCATCTTCTTGTGCA-3', R: 5'-GCCGTGAGTGGAGTCATACT-3'.

### Western Blot

Total protein in mouse brain cortex tissues was extracted for determining protein expression. Protein sample was quantified by bicinchoninic acid (BCA) (Abcam, Cambridge, MA, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (ECL).

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was uti-

lized for statistical analysis. Normally distributed measurement data were represented as mean ± standard deviation ($\bar{x}$±s). Comparison between groups was analyzed using one-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). Intergroup differences were analyzed by SNK-q test. $p<0.05$ was considered as statistically significant.

## Results

### Neuronal Autophagy in Cortex of SIBI Mice

Western blot analysis showed that protein expressions of LC3-I and LC3-II in cerebral cortex of MWT group began to increase at 6 h after CLP, and remained at high levels until 96 h. Relative intensity of LC3-II/LC3-I reached the peak at 24 h ($p<0.05$, Figure 1). On the contrary, SQSTM1 expression in cerebral cortex of MWT group began to decrease at 6 h after CLP ($p<0.05$, Figure 2).

### Lethe Expression in Cortex Neurons of SIBI Mice

Compared with SWT group, the expression of Lethe was remarkably upregulated in cortex of MWT group at 12 h after CLP ($p<0.05$, Figure 3A). Meanwhile, the mRNA expression of IFN-γ also increased after CLP ($p<0.05$, Figure 3B). It is indicated that inflammatory response after SIBI upregulated Lethe expression in cortical neurons, which may be related to IFN-γ upregulation in brain tissues.

### Lethe Knockout Regulated Expressions of LC3 and SQSTM1 in Cortex Neurons of SIBI Mice

The baseline level of autophagy activity in cortical neurons of the SWT and SKO groups was quite low, manifesting as little expression of LC3-II and a great abundance of SQSTM1. However, the expression of LC3-II in MWT group remarkably increased, while SQSTM1 expression was downregulated at 12 h after CLP (Figure 4A). Lethe$^{-/-}$ mice in MKO group barely showed LC3-II expression, while SQSTM1 remained a high level in cortical neurons (Figure 4B, 4C). The above results demonstrated the protective effect of Lethe on reducing autophagy of SIBI mice.

### Protective Effect of Lethe on Cortical Neurons of SIBI Mice

At 6 h after CLP, the prevalence of SIBI was 90% (27/30) in the MWT group and 96.67% (29/30) in the MKO group ($\chi^2=1.05$, $p=0.031$). At 12 h after CLP, the neurobiological scores of the MKO group (4.97±0.71) were markedly lower than those of the MWT group (5.43±0.86) (n=22, $t=2.284$, $p=0.026$) (Table I and Table II). HE staining of mouse cortex showed worse damage in cerebral cortex and fewer neurons of MKO group relative to MWT group (Figure 5).

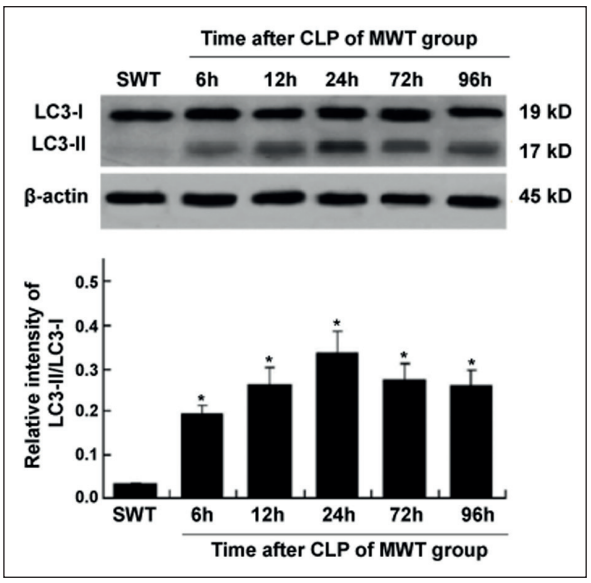


**Figure 1.** LC3-I and LC3-II in the cortical neurons of mice in SWT and MWT groups. Protein expression of LC3-I and LC3-II in the cerebral cortex of mice by Western blot (n=5, $\bar{x}$±s). *$p<0.05$ *vs.* the SWT group.

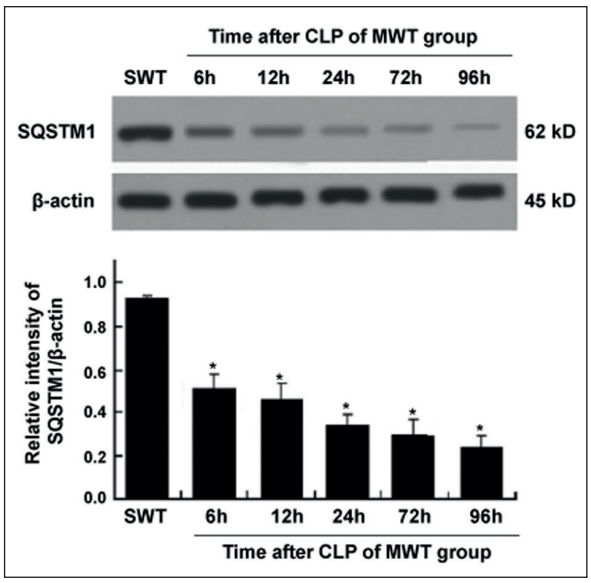


**Figure 2.** SQSTM1 in the cortical neurons of mice in SWT and MWT groups. Protein expression of SQSTM1 in the cerebral cortex of mice by Western blot (n=5, x±s). *$p<0.05$ *vs.* the SWT group.

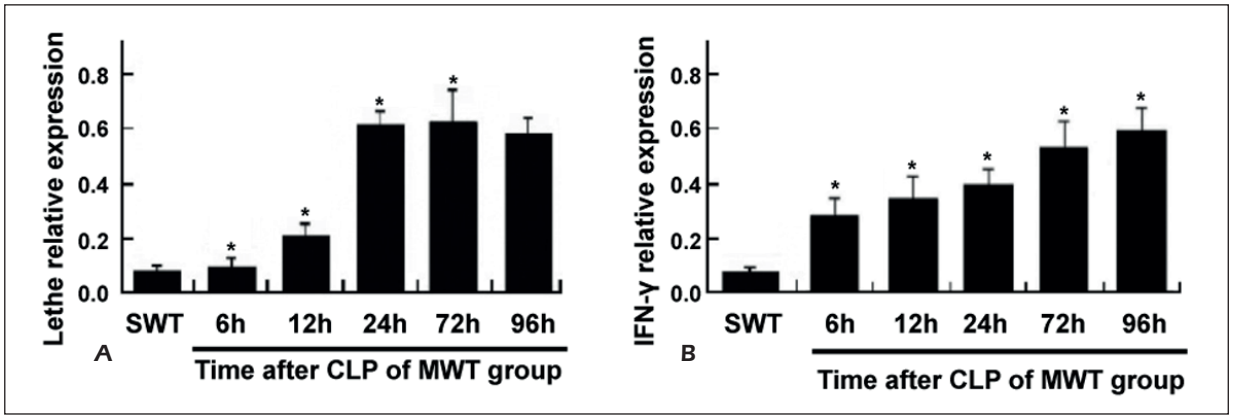


**Figure 3.** Expressions of Lethe and IFN-γ in the cerebral cortex and neurons of mice in SWT and MWT groups. **A**, Lethe relative expression in the cerebral cortex of mice by Real-time quantitative PCR (n=5, $\bar{x}\pm s$). *$p<0.05$ *vs.* the SWT group. **B**, Relative abundance of IFN-γ mRNA in the cerebral cortex of mice by Real-time quantitative PCR (n=5, $\bar{x}\pm s$). *$p<0.05$ *vs.* the SWT group.

## Discussion

Autophagy, also known as type II programmed cell death, exerts an essential role in growth and development, cell differentiation, and disease development[9]. On the one hand, cells can degrade macromolecular substances to maintain the normal cellular biology through the process of autophagy. Autophagy also encapsulates and degrades certain toxins, pathogens and denatured cytoplasmic components, so as to prevent further impair. Therefore, appropriate activation of autophagy in a certain degree contributes to prevent the progression of lung cancer[10], breast cancer[11]

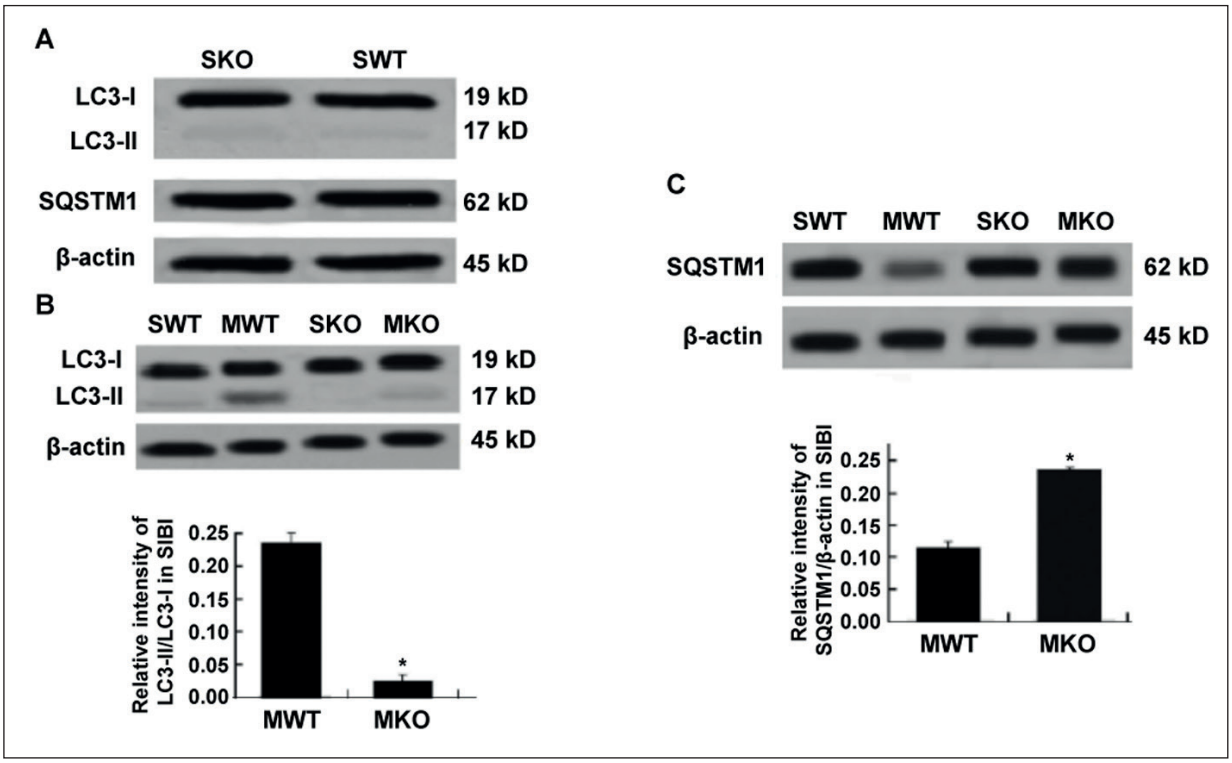


**Figure 4.** Effects of Lethe knockout on the expressions of LC3 and SQSTM1 in cerebral cortex of mice with SIBI. **A**, Expressions of LC3-I and LC3-II in the cerebral cortex of mice at baseline by Western blot. **B**, Expressions of LC3-I and LC3-II in the cerebral cortex of mice by Western blot at 12 h after CLP (n=5, $\bar{x}\pm s$). *$p<0.05$ *vs.* the MWT group. **C**, Expression of SQSTM1 in the cerebral cortex of mice by Western blot at 12 h after CLP (n=5, $\bar{x}\pm s$). *$p<0.05$ *vs.* the MWT group.

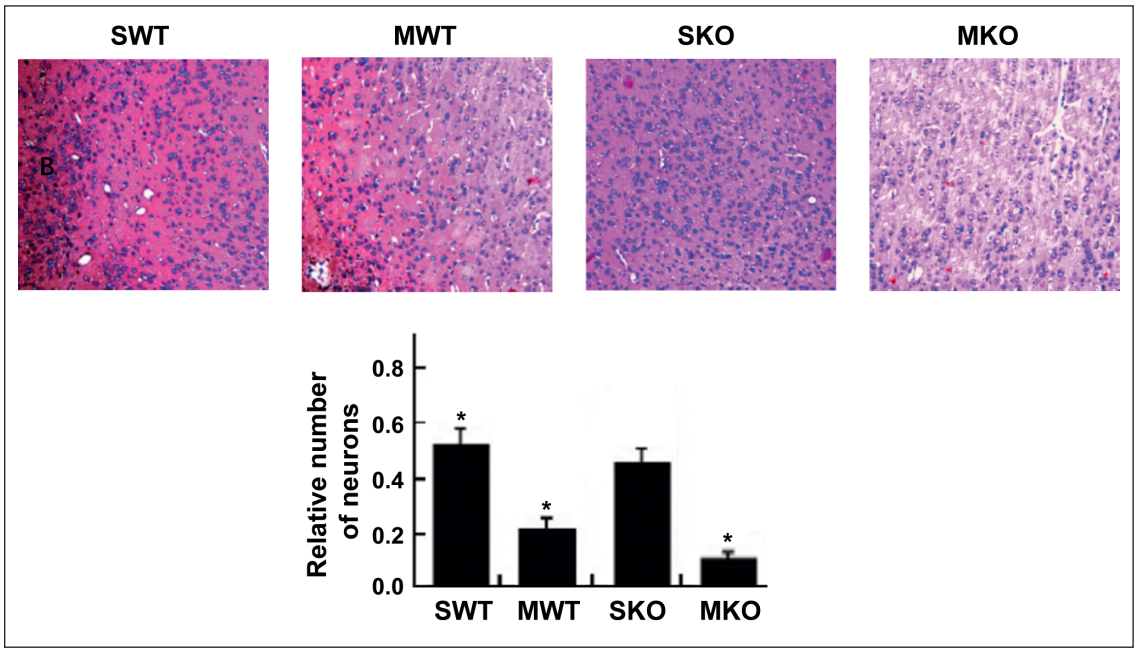


**Figure 5.** Pathological changes of cerebral cortex in mice (HE, ×200).

and bacterial infectious diseases[12]. Excessive autophagy, on the other hand, destroys the normal structure of cells and leads to apoptosis and necrosis. As a consequence, disease conditions of tumors, myopathy, neurodegenerative diseases and infections are aggravated under the circumstance of uncontrolled autophagy. Autophagy has been considered as a therapeutic target for some diseases, such as cardiac hypertrophy[13] and tumors[14]. Sepsis is resulted from the unbalanced systemic inflammatory reaction due to the release of a large number of inflammatory factors into the blood. Severe sepsis cases are prone to progress into multiple organ dysfunction syndrome. Autophagy regulated by T cells or endoplasmic reticulum has been identified to participate in the development of sepsis[15, 16]. Previous study has demonstrated the neuronal autophagy in hippocampus of rats with sepsis-associated encephalopathy[17]. This study showed that LC3-II, a marker of autophagy activity, was highly expressed in the cerebral cortex of SIBI mice in MWT group compared with those in SWT group. On the contrary, the expression of the ubiquitin-binding protein SQSTM1, which was negatively correlated with autophagy activity, was markedly downregulated. Electron microscopy images also revealed endoplasmic reticulum expansion, mitochondrial swelling, and increased number of autophagosomes in cortical neurons of SIBI mice in MWT group relative to controls. Hence, we confirmed the impairment and autophagy of cortical neurons in wild-type SIBI mice. However, the specific pathogenesis of SIBI remains unclear. Lethe, activated by NF-κB (a complex of multiple proteins) or glucocorticoid receptor agonist, regulates inflammatory signals and suppresses inflammatory responses. It is speculated that Lethe exerts a crucial role in the development of severe sepsis/septic shock[18]. However, potential regulatory effect of Lethe on sepsis brain injury is rarely reported. In this study, we found expressions of Lethe and IFN-γ were upregulated in cortex tissues of wild-type SIBI mice than controls. To further clarify the function of Lethe in SIBI-induced neuronal autophagy, expression levels of LC3, SQSTM1 and Lethe in cortex tissues of wild-type SIBI mice (SKO group) and Lethe$^{-/-}$ SIBI mice (MKO

**Table I.** The prevalence of SIBI at 6 h after CLP.

| Group | SIBI incidence | $\chi^2$ | *p* |
|---|---|---|---|
| MWT<br>MKO | 27/30<br>29/30 | 1.05 | 0.031 |

**Table II.** The mouse neurobiological score at 12 h after CLP.

| Group | Neurobiological score | n | *t* | *p* |
|---|---|---|---|---|
| MWT<br>MKO | 5.43±0.86<br>4.97±0.71 | 22 | 2.284 | 0.026 |

group) were determined. Lethe$^{-/-}$ SIBI mice barely expressed LC3-II in the cerebral cortex, while SQSTM1 expression was greatly upregulated. SIBI-induced inflammatory response upregulated Lethe in mouse cortical neurons, which may be related to the increased expression of IFN-γ. Lethe overexpression markedly promoted neuronal autophagy in SIBI mice. To investigate the neuroprotective effect of Lethe on SIBI-induced autophagy, we detected neurobiological scores and cerebral cortical pathology of mice in each group. Mice in MKO group showed lower neurobiological scores and fewer neurons in cortex relative to MWT group, suggesting a pronounced cerebral cortical damage. We believed that Lethe-induced neuronal autophagy exerted a protective effect on SIBI mice. To sum up, systemic inflammatory response can lead to SIBI under the circumstance of severe sepsis. During the inflammatory period, IFN-γ expression was upregulated by immunoregulation and activation of mononuclear macrophages, thus activating Lethe to induce neuronal autophagy. Subsequently, certain toxins and denatured cytoplasmic components in the neurons were encapsulated and degraded, thereafter preventing further neuronal damage.

## Conclusions

We revealed that lethe has a protective effect on SIBI mice by regulating autophagy in mouse cortical neurons.

### Conflict of Interests

The authors declared no conflict of interest.

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