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Icariin and icaritin ameliorated hippocampus neuroinflammation via inhibiting HMGB1-related pro-inflammatory signals in lipopolysaccharide-induced inflammation model in C57BL/6 J mice

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ABSTRACT

Inflammation is a defensive response of the body and is at the center of many diseases' process like depression. High mobility group protein box 1 (HMGB1), has been proved to function as a pro-inflammatory cytokine. We aim to explore the role of HMGB1 played in the neuroinflammation here. In this study, we used LPS to induce an acute inflammatory response, and to measure the anti-neuroinflammation effect of icariin (ICA) and icaritin (ICT). We found that LPS could increase the expression of HMGB1 in serum and hippocampus, along with a high expression of HMGB1 in the cytoplasm and a high expression of RAGE, which could be rescued by ICA and ICT, and ethyl pyruvate (EP) pretreatment showed similar effects here. We speculated that the translocation of HMGB1 from the nucleus to the cytoplasm played an important role in neuroinflammatory process, and HMGB1-RAGE signal was involved in this process. Furthermore, we found that ICA and ICT treatment activated TLR4-XBP1s related NF-κB signal, which we thought was relevant with the neuroprotective effect of ICA and ICT. However, EP pretreatment suppressed TLR4-XBP1s- endoplasmic reticulum stress related NF-κB signal to anti-inflammatory response, which was almost absolutely opposite with ICA and ICT treatment. We speculated that it might be caused by the duration of inflammation. We supposed that ICA and ICT could ameliorate neuroinflammation in hippocampus via suppressing HMGB1-RAGE signaling and might show a neuroprotective effect via activating TLR4-XBP1s related NF-κB signal at the same time, making it possible to act as an anti-neuroinflammatory drugs.

1. Introduction

Inflammation is a defensive response of the body to harmful stimuli, such as infection, jury, and stress, with the purpose of balancing the homeostatic equilibrium. Inflammation is a dynamic process under the strict control of regulatory mechanisms and is at the center of many diseases' process such as chronic obstructive pulmonary disease (COPD), metabolic diseases [1,2], and moreover, inflammation in the central nervous system (CNS) also plays an important role not only in normal brain functions, but also in pathological brain process [3], and is involved in the pathogenesis of depression [4]. A recent human imaging study showed a higher binding of translocator protein 18 kDa (TSPO), a putative biomarker of neuroinflammation, in the brains of major depressive disorder (MDD) patients versus healthy control, and exploratory analyses demonstrated that unmedicated MDD patients had the highest level of TSPO binding with inerleukin-5 in cerebrospinal fluid [5].

Evidence showed that high mobility group protein box 1 (HMGB1) was a key inflammatory mediator released by immune cells and necrotic tissue where it functions as a damage-associated molecular pattern (DAMP) [6,7]. HMGB1 is a nuclear protein which acts as a DNA chaperone and facilitates gene transcription [8,9]. However, HMGB1 can translocate to the cytoplasm and to the extracellular medium to alert nearby cells and the immune system to immediate danger, triggering inflammation, functioning as a DAMP [10]. Besides playing an important role in the periphery inflammatory response, HMGB1 can also trigger inflammation in the CNS [11]. Once translocated out from the nucleus, HMGB1 can upregulate cytokines and other pro-

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inflammatory molecules expression via binding to receptors like receptor for advanced glycation end products (RAGE), toll-like receptor 4 (TLR4), which expressed in the brain [12–14]. Moreover, intracerebroventricular HMGB1 induced an increased expression of TNF-α and IL-6 in the brain [15]. In a Parkinson's disease (PD) model, Gao et al. [16] revealed that HMGB1 stimulated microglia to release inflammatory factors and caused chronic dopaminergic neurodegeneration. Taking together, we can find that HMGB1 can function as a cytokine-like factor to mediate inflammation in the brain, and was involved in the pathogenesis some CNS disorders, like depression [17]. Studies revealed that HMGB1 was related to the lipopolysaccharide (LPS)-induced depressive-like behavior [12,18] and might induce depressive-like behavior in vivo mainly via neuroinflammatory response activation [19]. Likewise, Lian YJ et al. demonstrated that HMGB1 mediated chronic-stress-induced depressive behavior via activating the kynurenine pathway, accompanied with the high expression of TNF-α and IFN-γ [20]. Though the relationship between HMGB1 and neuroinflammation is certain, there still need more efforts on exploring the detailed mechanism between them to help us uncover the curtain on CNS disorders related to neuroinflammation.

Herba Epimedii is a medicinal herb in traditional Chinese medicine. Previous studies have showed that *Herba Epimedii* has multiple beneficial biological effects, including antioxidant, anti-depressant, anti-inflammatory and neuroprotective activities [21–23]. Icariin (ICA) and icaritin (ICT) (Fig. 1) are major active ingredient of *Herba Epimedii*. There exists amounts of evidence that ICA have a potent anti-inflammatory effects [24–26], and our group has also demonstrated the anti-inflammation effects of ICA [27–29]. As a metabolite of ICA, ICT showed an anti-inflammatory and neuroprotective effects as well [30–32]. In the present study, we have investigated the relationship between HMGB1 and neuroinflammation following stimulation by LPS. The results showed that the high cytoplasmic HMGB1 expression was closely related to neuroinflammation. Further, we studied the detailed mechanism involved in it, and extended the biological effects of ICA and ICT's anti-inflammation.

2. Experimental procedures

2.1. Materials and regents

Icariin (ICA, purity ≥98%) and icaritin (ICT, purity ≥98%) are obtained from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). LPS (*Escherichia coli* O111:B4) and ethyl pyruvate (EP, E47808-25G), a kind of HMGB1 antagonist, was obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). HMGB1 ELISA Kit (6010) was obtained from Chrondrex (Redmond, WA, USA). TNFα ELISA Kit (MEC1003) was obtained from Anogen (Mississauga, Ontario, Canada). IL-10 ELISA Kit (ELM-IL 10) was obtained from RayBiotech (GA, USA). Anti-MAP-2 (ab32454), anti-TLR4 (ab47093), anti-RAGE (ab3611) and anti-Iba-1 (ab5076) primary antibodies were obtained from Abcam (Cambridge, UK). Anti-β-Actin (3700S), anti-LaminB1 (13435), anti-HMGB1 (3935S), anti-p65 (4764S), anti-BIP (3177S), anti-XBP1s (12782S), anti-IκB (4814S) and anti-p-IκB (9246S) were obtained from Cell Signaling Technology (USA). EqiQuick Nuclear Extraction Kit (OP-0002) was obtained from Epigentek (NY, USA). RevertAid First strand cDNA Synthesis Kit (K1622) and Power SYBR™ Green PCR Master Mix (4367659) were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Animals and treatment

Male, 7-week-old C57BL/6 J mice were obtained from Vital River Laboratories (Beijing, China) and placed in a 12-h cycle, optimal temperature and humidity at School of Pharmacy, Fudan University Animal Center. All procedures related to animal care and experiment were carried out in accordance with the Experimental Animal Ethics Committee of Shanghai Medical College, Fudan University.

2.3. LPS challenge and animal grouping

C57 BL/6 J mice were injected intraperitoneally (i.p.) with 0.3 mg/Kg LPS or phosphate buffered saline (PBS) for 24 h. Then, mice were divided into four groups: control group, LPS group, LPS + ICA group and LPS + ICT group. Except the LPS group was sacrificed after a 24-h injection of LPS, the other three groups were sacrificed after giving normal saline (NS) (200 μL/mouse/day), ICA (20 mg/Kg/day), ICT (20 mg/Kg/day) respectively by gavage for 4 weeks after a 24-h injection of PBS or LPS (Fig. 1A). Because of their bad water solubility, ICA and ICT were suspended in 0.5% carboxymethylcellulose Na (CMC-Na) and the doses we used here were based on our previous studies [33–35]. In another assay, 80 mg/Kg EP was injected i.p. for 5 days, then these mice were divided into EP group and EP + LPS group. EP group was

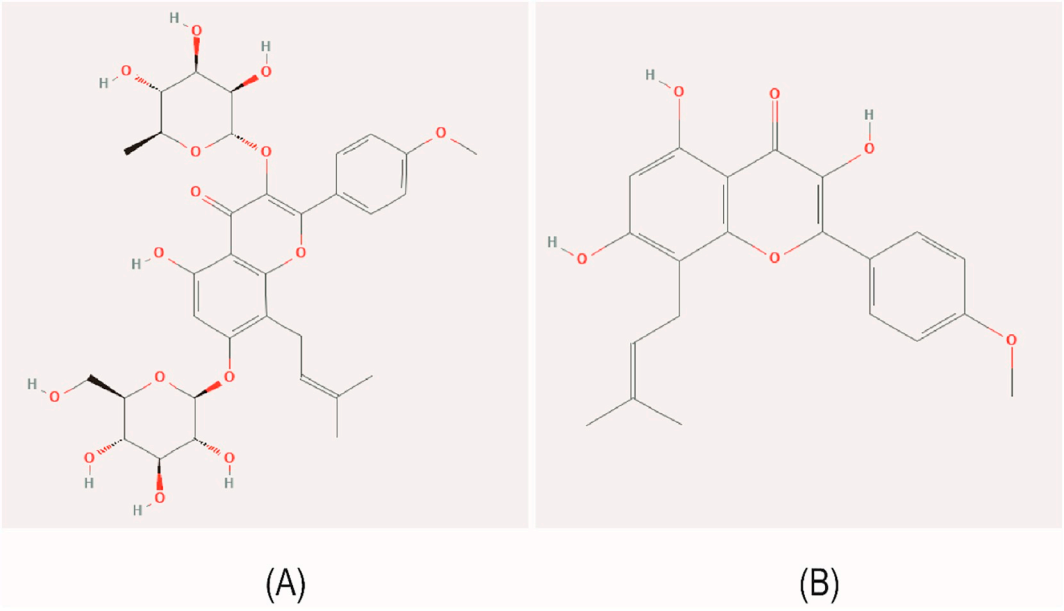

Fig. 1. Chemical structure of icariin (A) ($C_{33}H_{40}O_{15}$; molecular weight: 676.668 g/mol) and icaritin (B) ($C_{21}H_{20}O_6$; molecular weight: 358.386 g/mol).

sacrificed at the 5 day, and EP + LPS group received a 0.3 mg/Kg LPS injection i.p. for 24 h.

2.4. Measurement of serum TNFα, IL-10 and HMGB1

Blood samples were collected by removal of eyeball prior to sacrificing, and serum was separated by centrifugation at 5000 rpm at 4 °C for 30 min, and was stored at −80 °C. Serum TNFα, IL-10 and HMGB1 were measured by sandwich ELISA based methods according to the manufacture instructions.

2.5. Immunohistochemistry

Mice were transcardially perfused with NS followed by 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde for 24 h. Brain tissue was paraffin-embedded and cut into sections 4 μm thick. Sections were incubated with anti-MAP2 (1:100) and anti-Iba1 (1:100) overnight at 4 °C, followed by anti-rabbit and anti-goat secondary antibody for 30 min at room temperature (RT). The hippocampus was observed by light microscopy (BX43, Olympus, Japan) and analyzed with Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of hippocampus was isolated using Trizol reagent, and cDNAs were synthesized using RevertAid First strand cDNA Synthesis Kit according to the manufacture's protocol. Detection of mRNA was performed using Power SYBR™ Green PCR Master Mix and a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). Primers used for PCR amplification are listed in Table 1. The expression levels of mRNAs were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic extracts were prepared using EqiQuick Nuclear Extraction Kit. Hippocampus tissues were collected and stored at −80 °C. LaminB1 (nuclear protein) and β-actin (cytoplasmic protein) were used as loading controls.

Table 1
Primers used for qRT-PCR analysis.

Primer	Sequence
TNFα RT F	CATCTTCTCAAAATTCGAGTGAC
TNFα RT R	TGGGAGTAGACAAGGTACAACCC
IL-1β RT F	TGGAAAAGCGGTTTGTCTTC
IL-1β RT R	TACCAGTTGGGGAACTCTGC
IL-6 RT F	GAGGATACCACTCCCAACAGACC
IL-6 RT R	AAGTGCATCATCGTTGTTCATACA
IL-10 RT F	CAACATACTGCTAACCGACTC
IL-10 RT R	AACTGGATCATTTCCGATAAG
Arginase-1 RT F	GTGAAGAACCCACGGTCTGT
Arginase-1 RT R	GCCAGAGATGCTTCCAACTG
CD206 RT F	CTTCGGGCCTTTGGAATAAT
CD206 RT R	TAGAAGAGCCCTTGGGTTGA
HMGB1 RT F	CACCGTGGGACTATTAGGAT
HMGB1 RT R	GCTCACACTTTTGGGGATAC
RAGE RT F	GATCCTGCCTCTGAACTCAC
RAGE RT R	AGAGGACCAGAGAAGGAAGTG
TLR4 RT F	CAGTTTCAATCGCATAGAGAC
TLR4 RT R	CCAACACATAAGGAGGTARRCAT
GAPDH RT F	AAATGGTGAAGGTCGGTGTG
GAPDH RT R	AGGTCAATGAAGGGGTCGTT

2.8. Western blot analysis

Hippocampus tissues were collected and lysed for protein expression analysis. The protein concentration was quantified using the BCA method. Around 30 μg of protein per sample was used for western blot analysis and was separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferred onto a polyvinylidene fluoride membrane (Millipore, MA, USA), it was blocked with 5% non-fat milk for 1 h at RT. Immunoblots were incubated with different primary antibodies overnight at 4 °C. After washing with TBST, the membranes were incubated with secondary antibody at RT. Immunopositive bands were detected with enhanced chemiluminescence (ECL) and measured by ImageQuant™ LAS4000 mini system. Band intensities were quantified using Image J analysis software.

2.9. Statistical analysis

Our data was expressed as mean ± SEM. Statistical analysis was performed using the IBM SPSS Statistics 21. Multiple comparisons were evaluated by Least Significant Difference (LSD) after a one-way analysis of variance (ANOVA). A p-value < 0.05 was considered to be statistically significant.

3. Results

ICA and ICT can significantly reduce the high expression of HMGB1, TNFα and IL-10 in serum induced by LPS.

After a 24-h-LPS treatment, the LPS group was sacrificed, and drug intervention groups were sacrificed after being given 20 mg/Kg/day ICA or ICT by gavage for 4 weeks, then we measured the expression of HMGB1, TNFα and IL-10 in serum using ELISA method. As shown in Fig. 2A, we can find that the expression of HMGB1, TNFα and IL-10 in LPS group showed a higher expression compared to control, while after giving ICA and ICT for 4 weeks, the higher expression of HMGB1, TNFα and IL-10 in serum induced by LPS were significantly reduced ($p < 0.05$). Next, we used EP which was reported as an anti-inflammatory and neuroprotective agent with the ability to inhibit the release of HMGB1 [36–38], to verify the mechanism of ICA and ICT's anti-inflammatory and neuroprotective effects. As shown in Fig. 2B, compared to LPS group, EP + LPS group showed a lower expression of HMGB1 and TNFα, and had no influence on IL-10 expression in serum.

LPS can increase the total and the cytoplasmic HMGB1 expression in hippocampus, while ICA and ICT could reduce the total HMGB1 expression and increase its nuclear expression with an increase of nuclear p65 expression.

We detected the total HMGB1 expression in hippocampus, and also detected HMGB1 expression in the cytoplasm and nucleus. Results showed that compared to the control, a 24-h-LPS treatment could significantly increase the total and the cytoplasmic HMGB1 expression ($p < 0.05$) (Fig. 3A–D). Compared to the LPS group, ICA + LPS group and ICT + LPS group showed a lower expression of the total HMGB1 and a higher expression of the nuclear HMGB1 expression ($p < 0.05$), with a lower cytoplasmic p65 expression ($p < 0.01$) and a higher nuclear p65 expression at the same time (Fig. 3A–F). Further, we found that EP alone could reduce the total HMGB1 and p65 expression in hippocampus and increase the nuclear HMGB1 expression compared to the control ($p < 0.05$). Compared to the LPS group, EP + LPS group also obviously lowered the total expression of HMGB1 and p65 in hippocampus, and increased the nuclear HMGB1 expression ($p < 0.01$) and nuclear p65 expression ($p < 0.05$) (Fig. 3G–L).

ICA and ICT could rescue the XBP1s expression lowered by LPS, while EP pretreatment showed a lower expression of BIP, XBP1s, IκB and p-IκB compared to LPS group.

Evidence showed that endoplasmic reticulum (ER) stress was induced under various inflammatory states [39,40], and was associated

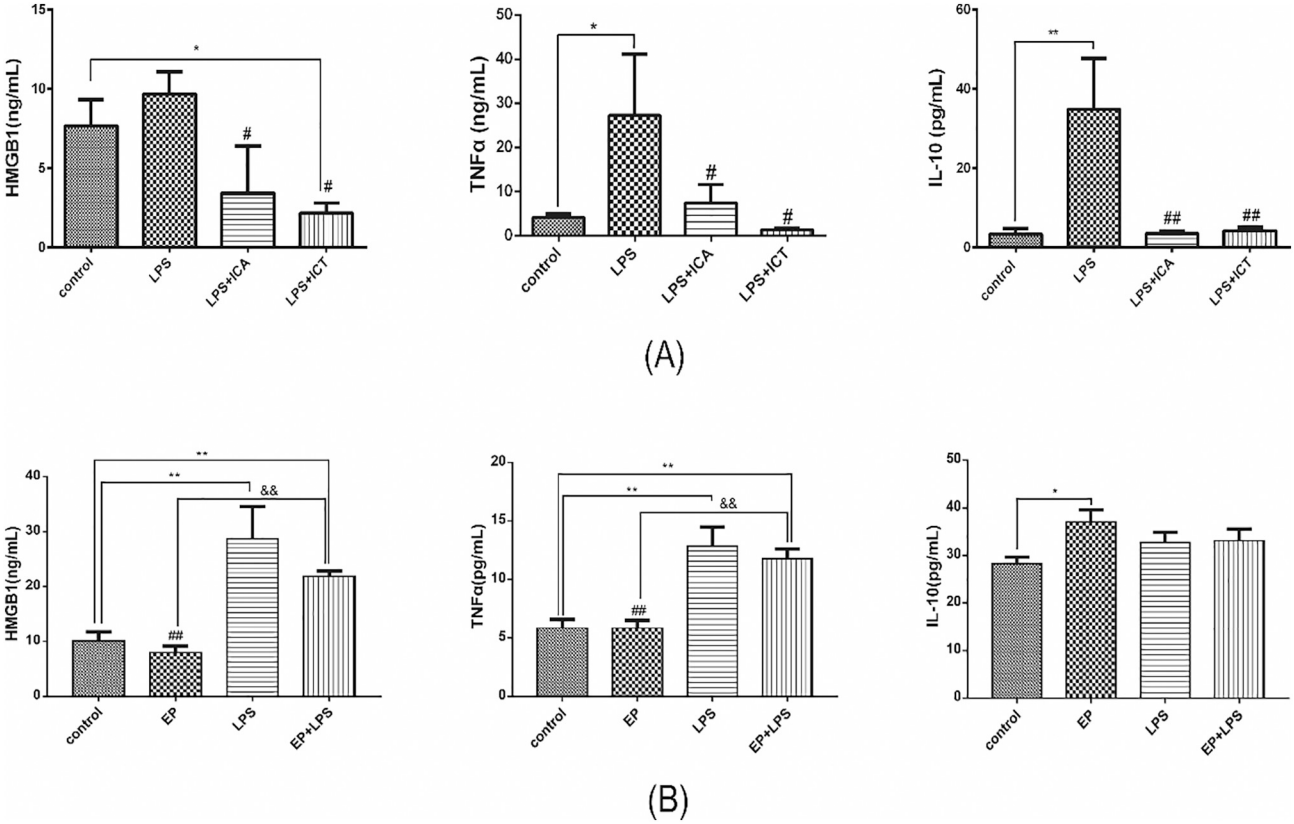

Fig. 2. The HMGB1, TNFα and IL-10 expression in mice serum (n = 8–10 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs LPS group; &&p < 0.01, each group vs EP group).

with NF-κB signaling [41]. Here, we further detected the expression of BIP (a marker protein of ER stress) and XBP1s and IκB, and we found that LPS injection showed no effect on BIP expression and IκB activation, nor did ICA and ICT treatment for 4 weeks, while XBP1s showed a significant lower expression in LPS group compared to the control, and ICA and ICT treatment could increase XBP1s expression significantly (Fig. 4A). However, compared to LPS group, EP + LPS group could reduce the expression of BIP, XBP1s, IκB and p-IκB in hippocampus, obviously suppressed the IκB activation ($p < 0.05$) (Fig. 4B).

LPS could increase RAGE expression and reduce TLR4 expression in hippocampus, while ICA and ICA treatment rescued these effects of LPS.

We further explored the expression of the classic receptors of HMGB1, RAGE and TLR4. Results showed that on the one hand, LPS increased RAGE expression ($p < 0.01$), on the other hand, LPS reduced TLR4 expression ($p < 0.05$). However, after ICA and ICT treated for 4 weeks, RAGE expression decreased and TLR4 expression increased in hippocampus compared to LPS group ($p < 0.01$) (Fig. 5A). Whereas, compared to LPS group, EP + LPS group showed a decrease expression of both RAGE and TLR4 ($p < 0.05$) (Fig. 5B).

ICA and ICT could ameliorate the high expression of TNFα, IL-1β and IL-6 mRNA induced by LPS in hippocampus.

We next used PCR method to detect the pro-inflammatory and anti-inflammatory cytokines mRNA expression in hippocampus. As shown in Fig. 6A, we can find that LPS could significantly increase pro-inflammatory cytokines, TNFα, IL-1β and IL-6 mRNA expression ($p < 0.01$), and for anti-inflammatory cytokines, the IL-10 mRNA expression was decreased ($p < 0.01$) while Arginase-1 mRNA expression was increased ($p < 0.01$), and CD206 mRNA expression wasn't changed. After 4 weeks' ICA and ICT treatment, compared to LPS group, the TNFα, IL-1β and IL-6 mRNA expression were decreased ($p < 0.01$), and ICA treatment showed a higher expression of IL-10 mRNA with lower expression of Arginase-1, CD206 mRNA ($p < 0.01$), while ICT treatment only showed a higher expression of CD206 mRNA ($p < 0.01$) (Fig. 6A). Interestingly, pretreated with EP i.p. for 5 days followed a 24-h LPS injection not only increased the TNFα, IL-1β and IL-6 mRNA expression, but also increased IL-10, Arginase-1 and CD206 mRNA expression ($p < 0.05$) (Fig. 6B).

ICA and ICT could suppress the microglia activation induced by LPS.

As we can see in Fig. 7A and B, a 24-h-LPS treatment could significantly increase the expression of Iba-1 and decrease the expression of MAP-2 in hippocampus ($p < 0.01$), while ICA + LPS group and ICT + LPS group showed a significant decrease of Iba-1 expression ($p < 0.05$) and an increase of MAP-2 expression in hippocampus (data showed no difference). However, compared to LPS group, EP + LPS group showed a significant decrease of Iba-1 expression ($p < 0.05$) with no difference in MAP-2 expression (Fig. 7C and D).

4. Discussion

"Danger Theory" proposed that our body evolved mechanisms to detect pathogens through the release of DAMPs [42], triggering the immune system against stimuli in physiological or pathological conditions [43–45], and this theory remained a theoretical model for many years until HMGB1 was recognized as DAMP [10]. HMGB1 is a mobile chromatin protein playing a role in the formation of nucleosome, transcription, replication and DNA repair [46]. With development of the research, HMGB1 was found to act as a cytokine-like factor, mediating the inflammatory response [47]. Mice treated with 0.3 mg/Kg LPS i.p. for 24 h showed a high expression of TNFα and IL-10 in serum and a high expression of TNFα, IL-1β and IL-6 mRNA in hippocampus, along with the activation of microglia in hippocampus, indicating that peripheral inflammatory reaction could active neuroinflammation. Consistent with "Danger Theory", we found that HMGB1 remained a high expression both in serum and in hippocampus. Further, we

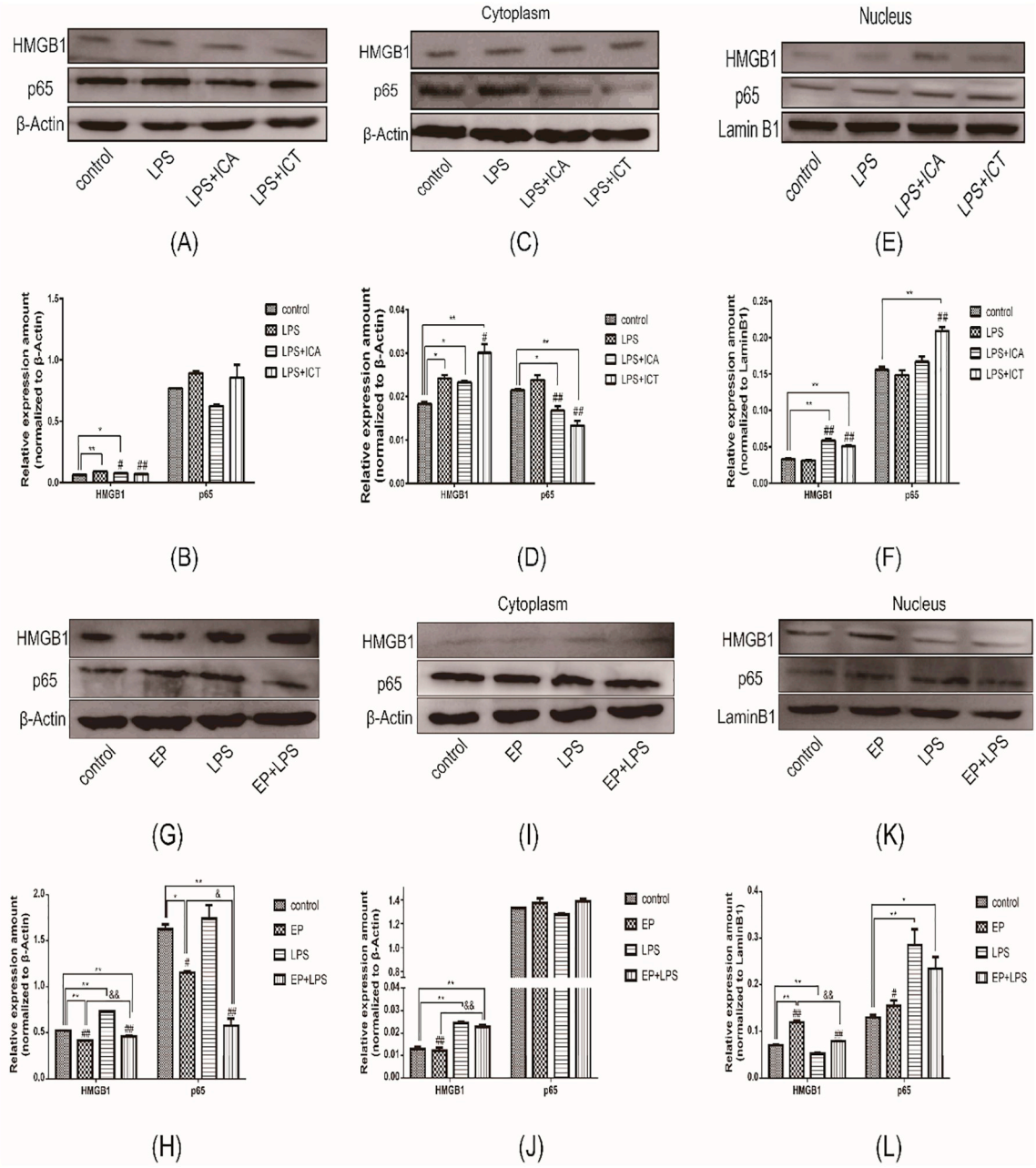

Fig. 3. The HMGB1 and p65 expression in mice hippocampus. After different treatments, the total HMGB1 and p65 expression (A and B, G and H), the cytoplasmic HMGB1 and p65 expression (C and D, I and J) and the nuclear HMGB1 and p65 expression (E and F, K and L) of each group were measured by western blotting method (n = 6 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs LPS group; &p < 0.05, &&p < 0.01, each group vs EP group).

detected the cytoplasmic and the nuclear HMGB1 expression in hippocampus, and we found that LPS injection significantly increased the cytoplasmic HMGB1 expression. This result was consistent with previous studies that the release of HMGB1 from the nucleus could alert body about danger [7,42]. We next used EP, the HMGB1 antagonist, to further explore the relationship between the anti-inflammatory effect and the translocation into the nucleus of HMGB1. In accord with previous studies [48], we found that pretreatment with EP could reduce HMGB1 expression in serum and in hippocampus, and increase its translocation into the nucleus, and reduce TNFα in serum slightly, and effectively suppress the microglia activation in hippocampus. Interestingly, in the EP + LPS group, TNFα, IL-1β and IL-6 mRNA expression significantly increased, and in the meantime, anti-inflammatory factors IL-10, Arginase-1 and CD206 mRNA expression also increased obviously. All these told us that HMGB1, especially the cytoplasmic HMGB1 was closely related to neuroinflammation. Furthermore, we found that mice treated with ICA and ICT showed a low expression of TNFα and IL-10 in serum and a low expression of TNFα, IL-1β and IL-6 mRNA in hippocampus, along with a suppression of microglia activation, indicating that ICA and ICT could help to relieve the inflammatory response, showing a well anti-inflammatory effect. Meanwhile, ICA and ICT decreased the expression of HMGB1 in hippocampus and helped HMGB1 to translocate from the cytoplasm to the nucleus, telling us that these effects might be relative with their anti-inflammatory effect.

HMGB1 usually functions as a DAMP through interacting with its receptors like RAGE, TLR4 [49]. Our results showed that LPS injection significantly increased the RAGE expression in hippocampus, while EP pretreatment and ICA and ICT treatment both decreased the RAGE

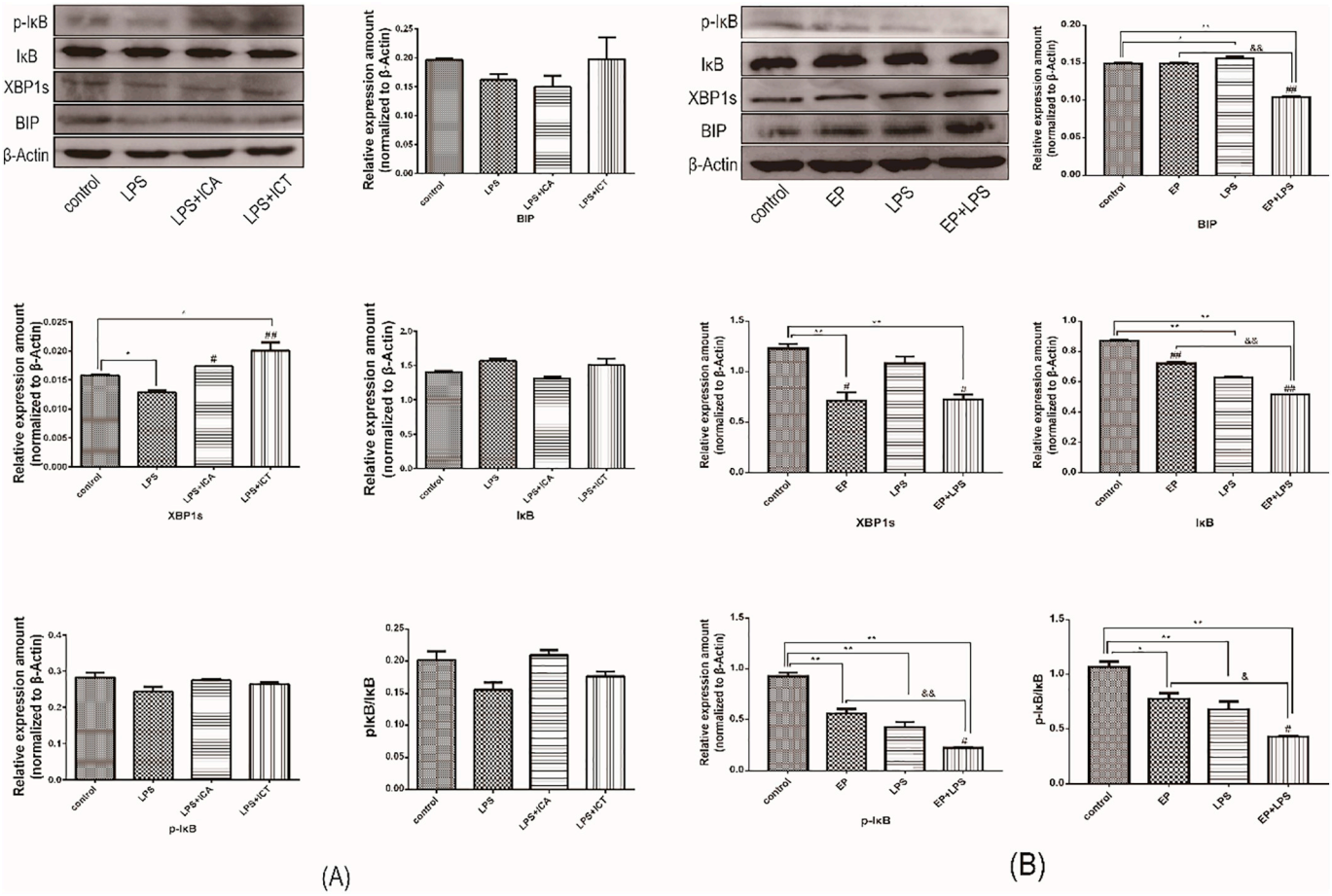

Fig. 4. The expression of BIP, XBP1s, IκB and p-IκB expression in mice hippocampus (n = 6 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs LPS group; &p < 0.05, &&p < 0.01, each group vs EP group).

expression, indicating that HMGB1-RAGE signal might play an important role in inflammatory response by reference to the HMGB1 expression in hippocampus. Previous studies reported that LPS could activate HMGB1-TLR4 axis [50,51], while our study here showed that LPS decreased TLR4 expression in hippocampus. Otherwise, EP pretreatment showed a lower TLR4 expression compared to LPS group, thus we speculated that it was not HMGB1-TLR4 axis but other signals which played roles in this model.

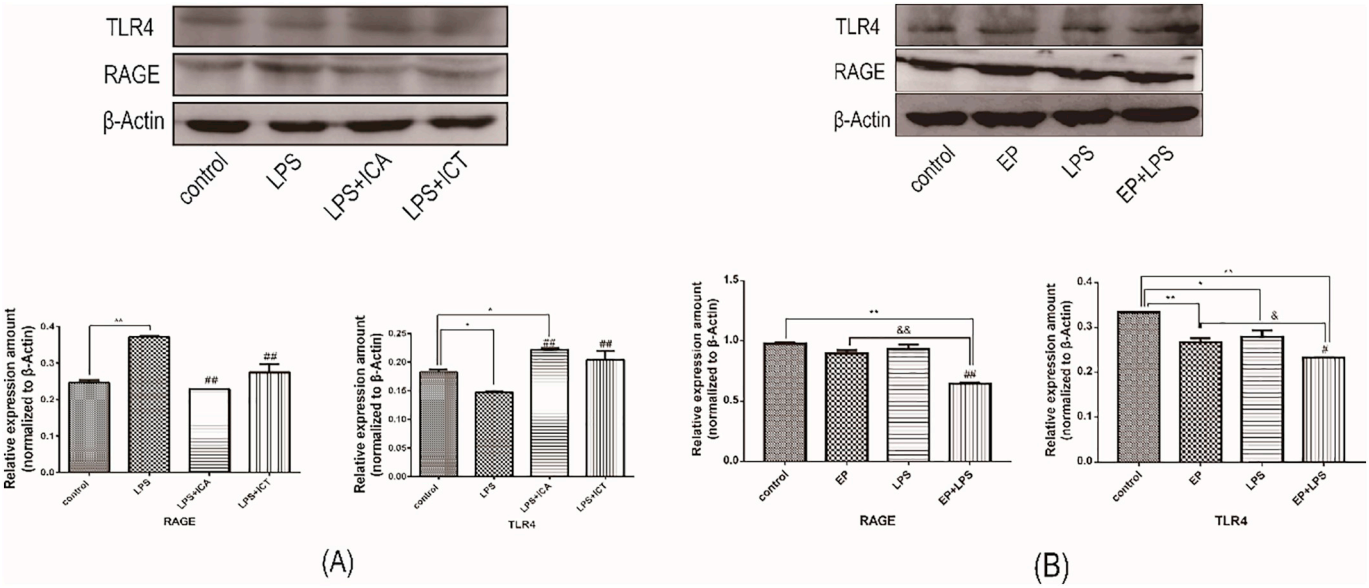

Fig. 5. The expression of RAGE and TLR4 expression in mice hippocampus (n = 6 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs LPS group; &p < 0.05, &&p < 0.01, each group vs EP group).

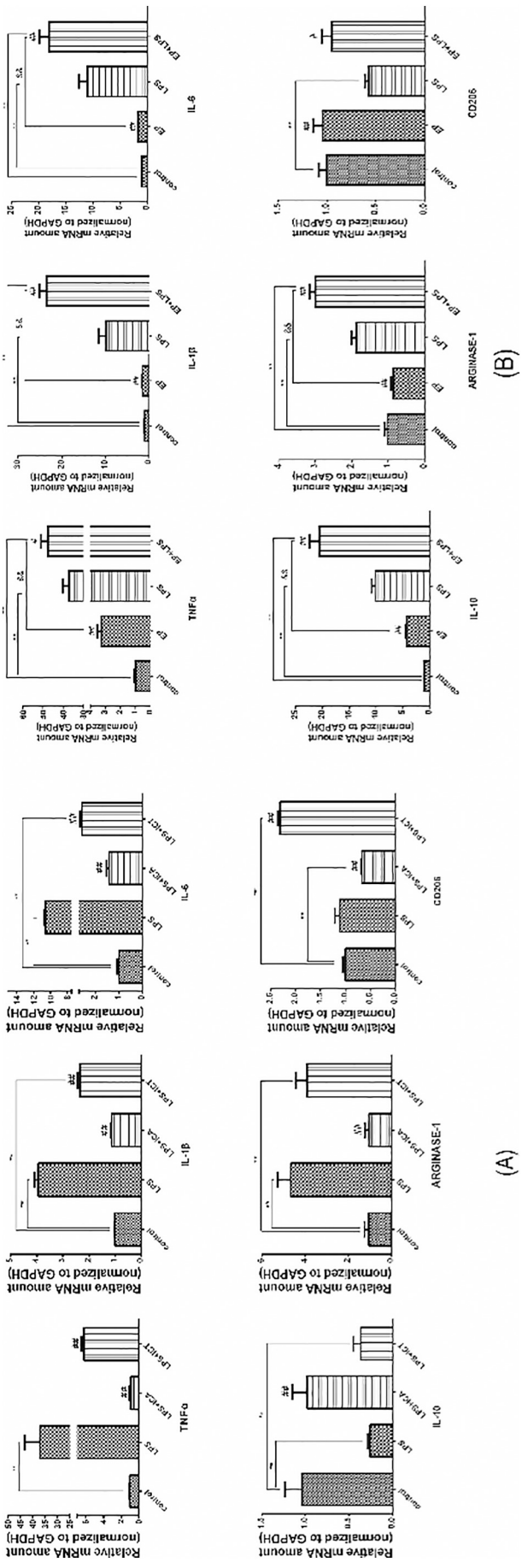

Fig. 6. The TNFα, IL-1β, IL-6, IL-10, Arginase-1 and CD206 mRNA expression in mice hippocampus (n = 6 each group). (**p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs LPS group; && p < 0.01, each group vs EP group).

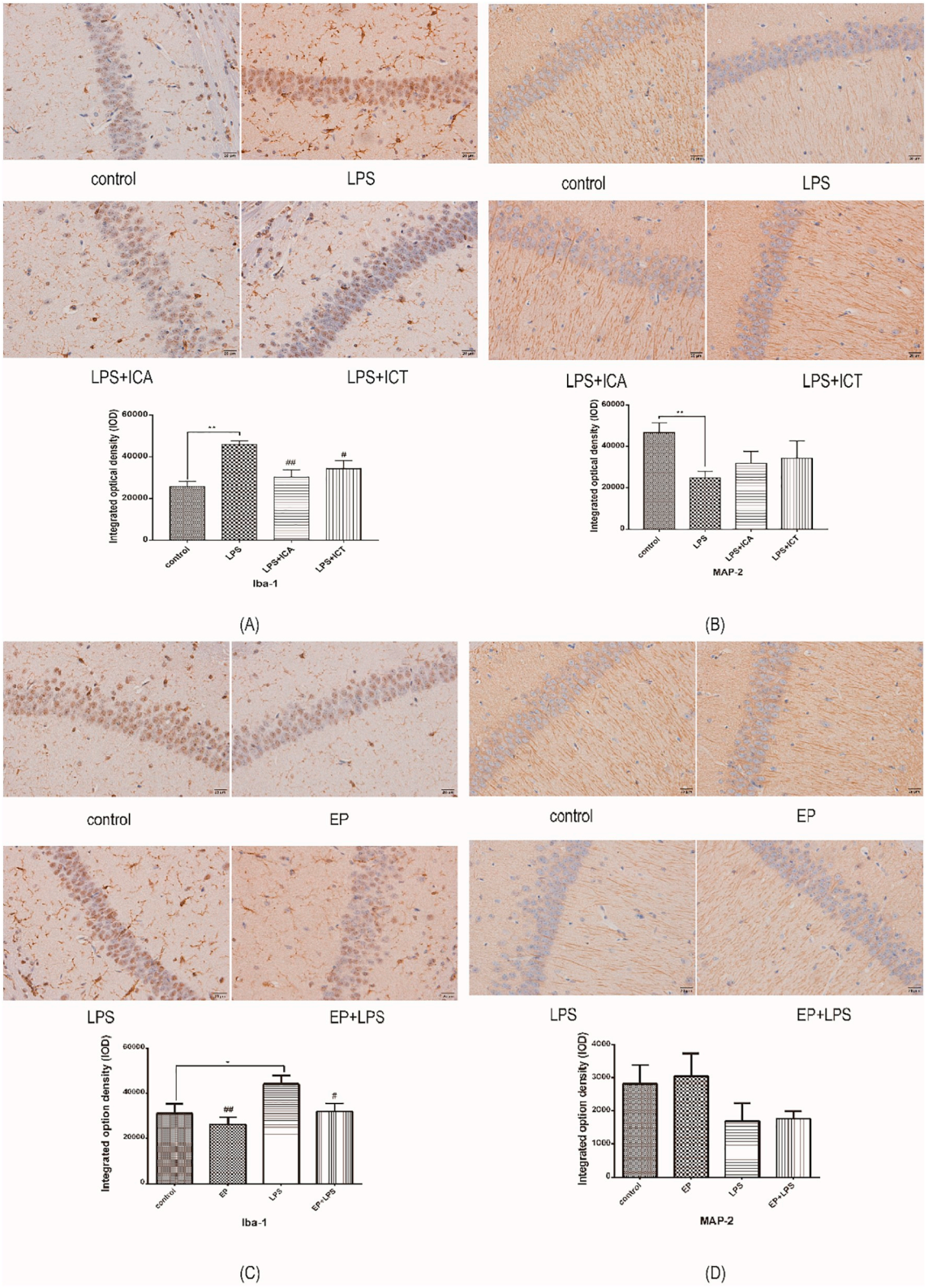

Fig. 7. The expression of Iba-1 and MAP-2 in hippocampus (n = 4 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs LPS group).

It has been proved that the cytokine-like effect of HMGB1 was related to the activation of NF-κB signal [52]. We found that LPS showed no influence on p65 expression in hippocampus, while ICA and ICT treatment reduced the cytoplasmic p65 expression and increased the nuclear p65 expression significantly in hippocampus. Besides, we found that in EP + LPS group, the total p65 and the nuclear p65 expression was reduced. This indicated that inflammation induced by LPS might be irrelevant with p65 activation here. Evidence showed that NF-κB signal took part in mediating gene transcription related to cell proliferation, inflammation and cell apoptosis [53], and the translocation into the

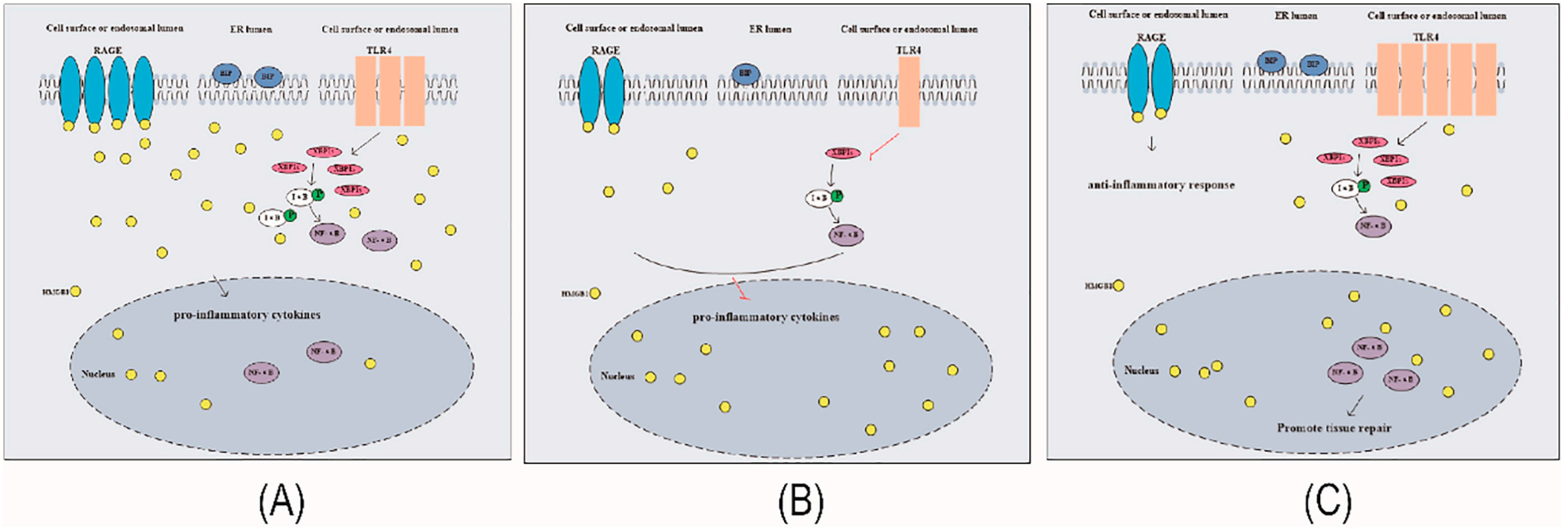

Fig. 8. The schematic diagram of the role HMGB1 played in neuroinflammation. (A) LPS could increase the expression of HMGB1 in the cytoplasm, activating HMGB1-RAGE signal, resulted in pro-inflammatory cytokines release in hippocampus. (B) EP pretreatment could decrease HMGB1 expression in the cytoplasm, suppressing HMGB1-RAGE signal and TLR4-XBP1s-ER stress related NF-κB signal, inhibiting pro-inflammatory cytokines release in hippocampus. (C) ICA and ICT could ameliorate neuroinflammation via suppressing HMGB1-RAGE signaling and might promote neuroregeneration via activating TLR4-XBP1s related NF-κB signal.

nucleus of NF-κB p65 also played a role in the process of tissue repair and regeneration [54,55]. And it was clarified that TLR4 could activate NF-κB signal [56,57]. Basing on these facts, we speculated that ICA and ICT might activate NF-κB signal via increasing TLR4 expression, thus making the number of neurons slightly increased, while the anti-inflammatory effect of EP pretreatment might be relevant with inactivation of NF-κB p65 caused by reducing TLR4 expression.

For the next step, we detected the IκB activation in hippocampus, and found that ICA and ICT had no difference on it, indicating that the activation of NF-κB signal via TLR4 was related to other ways. There existed mounts of evidence that ER stress was involved in the activation of NF-κB signal via TLR4 [58–61]. We found that in hippocampus, the expression of ER stress marker protein, BIP, had little changes compared to the control, while XBP1s expression had a significant decrease in LPS group and an obvious increase in ICA and ICT treatment group. XBP1s could interact with ER stress response elements to activate ER stress, and in the end activated NF-κB signal [62]. TLR4 also could activate XBP1s directly [63,64], participating in the immune response. So, we postulated that the TLR4 expression increased by ICA and ICT treatment might activate NF-κB signal via XBP1s here. Unlike the ICA and ICT treatment, EP pretreatment reduced the expression of BIP, XBP1s and suppressed IκB activation, and we speculated that this phenomenon might be related to the TLR4-XBP1s-ER stress signal which needs a further investigation.

In a word, ICA and ICT treatment and EP pretreatment both showed an effect on the total and the nuclear HMGB1 expression in hippocampus, and ICA and ICT treatment had a better anti-neuroinflammatory effect. Besides, EP pretreatment and ICA and ICT treatment both decreased RAGE expression, but showed absolutely opposite effects on TLR4-XBP1s related NF-κB signal. We thought that it might be caused by the different duration of inflammation. As shown in Fig. 8A, LPS injection could increase the total and the cytoplasmic HMGB1 expression to activate HMGB1-RAGE signal, inducing inflammatory response, and on the other hand, the suppression of TLR4-XBP1s axis might be relevant with the neuron damage. However, when HMGB1 expression was suppressed by EP, the inflammatory response caused by LPS challenge was ameliorated via suppressing the HMGB1-RAGE signal, and partly via suppressing TLR4-XBP1s-ER stress signal (Fig. 8B).When the inflammation lasts, notable is that tissue reconstruction and inflammation occur simultaneously [65]. As seen in Fig. 8C, ICA and ICT treatment for 4 weeks also ameliorated inflammatory response via suppressing HMGB1-RAGE signal, and showed a slight increase of neuron numbers. We postulated that it might be relevant with the activation of TLR4-XBP1s related NF-κB signal. ICA and ICT showed a good anti-neuroinflammation via mediating HMGB1 expression here, and its potential neuro-protective effects making it possible to be a therapeutic drugs for CNS disorders caused by inflammation.

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