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HMGB1 gene silencing inhibits neuroinflammation via down-regulation of NF-κB signaling in primary hippocampal neurons induced by $A\beta_{25–35}$

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ABSTRACT

High mobility group box 1 protein (HMGB1) is potentially triggered by Aβ oligomers and other sterile injuries, and is a non-histone DNA binding nuclear protein with roles in neural development and neurodegeneration, which contribute to memory impairment and chronic neuroinflammation in the brain. However, the exact molecular mechanisms of HMGB1 activation in Alzheimer's disease (AD) were previously unknown. The present study aimed to elucidate the effects of HMGB1 in $A\beta_{25–35}$-induced neuroinflammation in hippocampal neuron cultures. RNA interference (RNAi) HMGB1 treatment significantly reduced $A\beta_{25–35}$-induced HMGB1 expression by almost 70% in primary hippocampal neurons. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR), western blotting, and enzyme-linked immunosorbent assay (ELISA) demonstrated that short hairpin RNA (shRNA) for HMGB1 ameliorated $A\beta_{25–35}$-treated neuroinflammation, including activation of advanced glycosylation end product-specific receptor (RAGE), toll-like receptor 4 (TLR4), and nuclear factor-kappa B (NF-κB)-p65, as well as induced the release of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), IL-6, and HMGB1 in primary hippocampal neurons and the culture supernatant. In addition, pretreatment with HMGB1-shRNA dramatically reduced both the degree of nuclear-cytoplasmic HMGB1 translocation of HMGB1 and NF-κB DNA binding. Together, the data indicate that HMGB1 mediates the pathogenesis of AD by activating RAGE/TLR4 signaling and that shRNA targeting HMGB1 may be a promising therapeutic strategy for treating AD.

1. Introduction

Alzheimer's disease (AD) is one of the most common dementia subtypes or neurodegenerative disorders, and is pathologically characterized by excessive deposition of senile plaques and neurofibrillary tangles (NFT), which contribute to neuronal cell death [1]. The number of patients with AD increases each year, as a result of aging populations worldwide. Furthermore, the United States is experiencing challenges with medical care and with an overwhelming social burden resulting from AD [2].

Previous studies overwhelmingly support neuroinflammation as a key event in AD, especially in hippocampal neuronal cells [3]. Current knowledge indicates that amyloid-β (Aβ) is the most crucial component of senile plaques, mediating the response of sterile inflammation [4] [5]. Unfortunately, anti-Aβ drugs fail in clinical trials, and anti-inflammatory targets are therefore urgently required [6].

High mobility group box 1 protein (HMGB1) is a non-histone DNA binding nuclear protein with roles in nucleosome structure and regulation of gene transcription, including during neural development and neurodegeneration [7] [8]. Emerging evidence suggests that HMGB1 expression is elevated in the brains of patients with AD [9]. HMGB1 is activated by Aβ, with damage-associated molecular patterns (DAMPs) sustaining the inflammatory response. Furthermore, HMGB1 can be released into extracellular milieu from immune cells or non-immune cells via various stimuli, and HMGB1 translocation usually suggests activation [10] [11]. In addition, receptors for advanced glycation end

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products (RAGE) and toll-like receptor 4 (TLR4) are key receptors of HMGB1. RAGE, a member of the immunoglobulin superfamily, is a multi-ligand receptor with an important function in neurodegeneration, and TLR4 is an immune receptor controlling the innate immune response. These receptors share common signaling pathways following inflammation [12]. Activation of the nuclear factor-κB (NF-κB) family of transcription factors mediates neuroinflammation, such as in AD, by regulating pro-inflammatory cytokine expression [13]. However, the exact molecular mechanisms of HMGB1 activation in AD were previously unknown. Therefore, the present study investigated HMGB1 is a potential biomarker and therapeutic target for prevention and treatment of AD.

2. Material and methods

2.1. Reagents and kits

Dulbecco's Modified Eagle medium (DMEM)/F12, fetal bovine serum (FBS), neurobasal medium, 100× GlutaMAX, 50× B27 supplement, trypsin-EDTA and penicillin-streptomycin solution were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) were obtained from Hyclone (Logan, UT, USA). Primary antibodies used for immunofluorescence staining and western blotting analysis, including rabbit polyclonal antibody against Neu-N, HMGB1, and RAGE, as well as mouse monoclonal antibodies against TLR4 and β-actin, were purchased from Abcam (Cambridge, UK) or Santa Cruz Biotechnology (Dallas, TX, USA). Secondary antibodies, including DyLight-488 AffiniPure donkey anti-rabbit IgG, horseradish peroxidase (HRP)-labeled goat anti-rabbit, and HRP-labeled goat anti-mouse, were obtained from Earthox (Millbrae, USA) or Fdbio Science (Hangzhou, China). The TRIzol reagent, RevertAid RT kit and NE-PER nuclear cytoplasmic extraction reagent kit were purchased from Thermo Fisher Scientific. SYBR green real-time PCR master mix-plus was purchased from TOYOBO (Osaka, Japan). The DIG gel shift 2nd generation kit was purchased from Roche Diagnostics (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for rat HMGB1, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α were purchased from R& D Systems (Minnesota, USA).

2.2. Primary hippocampal neuron cultures

Primary hippocampal neurons were cultured as described previously [14]. In brief, the hippocampal tissue was obtained from Sprague Dawley rat embryos (E18) provided by Laboratory Animal Centre of Wenzhou Medical University, and transported on ice (license: SYXK 2010–0150 to ZHE). The study design was approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University (license: wydw2013-0025). The tissue was dissociated in HBSS containing 10 mg/mL DNase I (Sigma-Aldrich) and 0.025% trypsin-EDTA (20 min, 37 °C). The mixture was inactivated with DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and centrifuged at 1000*g* for 5 min. The cells were re-suspended in DMEM/F12 supplemented with 10% FBS and 1% penicillin-streptomycin solution. The hippocampal cell suspension was plated directly onto 6-well plates pretreated with 0.5 mg/mL poly-L-lysine (Sigma-Aldrich) at a density of 1.5×10^6 per well and incubated at 37 °C and 5% CO_2. The medium was replaced with serum-free neurobasal medium supplemented with 2% B27 supplement and 2 mM GlutaMAX 4 h later. Half the volume of the medium was exchanged with fresh medium twice-weekly.

2.3. Immunofluorescence staining

The purity of hippocampal neurons and the nuclear translocation of HMGB1 were assessed by immunofluorescence staining, as described previously [11] [15]. Briefly, cells were seeded on 24-well chamber slides pre-coated with 0.5 mg/mL poly-L-lysine. After 7 d of cultivation, cells were rinsed with PBS 3 times for 5 min each, fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized using 0.3% Triton X-100 for 20 min, and blocked with 10% BSA for 60 min. Rabbit polyclonal antibody against Neu-N (1:100 in PBS-T containing 1% BSA) and HMGB1 (1:200 in PBS-T containing 1% BSA) was used as the primary antibody, and was incubated at room temperature (RT) for 1 h and then overnight at 4 °C. Primary rabbit polyclonal antibodies against Neu-N (1:100 in PBS-T containing 1% BSA) and HMGB1 (1:200 in PBS-T containing 1% BSA) were used (60 min at RT and then overnight at 4 °C). DyLight-488 AffiniPure donkey anti-rabbit IgG (1:200) was used as a fluorescent secondary antibody (60 min, RT). Nuclei were subsequently stained with DAPI (1:5000, Abcam). Images were then captured using a fluorescence microscope (Olympus, Japan), using a minimum of 3 randomly selected microscopic fields ($n = 3$ per group). Neu-N is a marker for hippocampal neurons, and the findings indicated that the hippocampal neurons cultures were > 95% pure. Data are not shown. The HMGB1 antibody was used to assess nuclear translocation of HMGB1.

2.4. Construction and identification of a lentivirus encoding HMGB1 short hairpin RNA (shRNA)

To prevent off-target effects of RNAi and identify its effectiveness, 3 pairs of potential sequences targeting the rat HMGB1 gene (GenBank accession number: NM_012963) were designed. The RNAi candidate target and the negative control sequences are shown in Table 1. The lentivirus vector was constructed by GeneChem Co., Ltd., Shanghai, China. All constructs were identified by sequence analysis. The most effective lentivirus shRNA fragment was selected by PCR and western blotting for subsequent experiments.

2.5. Lentivirus transduction and detection assay

Hippocampal neurons were plated onto 6-well plates for 24 h, followed by transduction with lentivirus vectors at a multiplicity of infection (MOI) of 5 at 37 °C and 5% CO_2 for 24 h. The medium was then completely replaced with fresh neurobasal medium.

2.6. Cell treatment

Hippocampal neuronal cells (1.5×10^6) were pretreated with RNAi using non-target or HMGB1 shRNA (see Table 1) and were then incubated with $A\beta_{25–35}$ (Sigma-Aldrich) for 24 h on day 6. All groups were harvested on day 7 for further use in the study. Hippocampal cells were isolated from 1 animal and replicated at least 3 times in independent experiments.

2.7. MTT assay

Cell inhibition was performed in a 96-well plate, using an MTT assay (Sigma-Aldrich) according to the manufacturer's instructions. After drug treatment, MTT reagents were added to cells and incubated at 37 °C and 5% CO_2 for 4 h. The medium was replaced by dimethyl sulfoxide (DMSO) and the optical density at 570 nm was measured for each well using a microplate reader (Thermo Fisher Scientific). The

Table 1
Short hairpin RNA (shRNA) target and negative control sequences.

Name	Sequences (5′ → 3′)
HMGB1-shRNA1(51059-1)	TCTGTAATTTGAGGAGGAATA
HMGB1-shRNA2(51060-1)	CCCTACTAAAGACCTGAGAAT
HMGB1-shRNA3(51061-1)	AAACTAATAATTGCAGAGGTT
Negative control	TTCTCCGAACGTGTCACGT

Table 2
Primer sequences for real-time quantitative PCR (RT-qPCR).

Name		Sequences (5′ → 3′)
HMGB1	Forward primer	TATCTAAATACGGATTGCTCAGGAA
	Reverse primer	AGGGACAAACCACAATATAGGAAAA
RAGE	Forward primer	GGACTCGGTAGTTGGACTTGAC
	Reverse primer	GCCACTTATGCTGAGCTGTAA
TLR4	Forward primer	ATCAGAGGAAGAACAAGAAGCAA
	Reverse primer	AGAAACCCAGATGAACTGTAGCA
GAPDH	Forward primer	TCTCTGCTCCTCCCTGTTC
	Reverse primer	ACACCGACCTTCACCATCT

inhibitory rate was calculated using the following formula:

Inhibitory rate (%) = $(A_{control} - A_{sample}) / A_{control} * 100\%$.

2.8. Real-time quantitative PCR (RT-qPCR)

Briefly, total RNA was isolated from neurons using TRIzol reagent and used to generate cDNA using RevertAid RT kit (Thermo Fisher scientific, USA) according to the manufacturer's instructions. RT-qPCR analysis was conducted using SYBR Green. The amplification PCR consisted of a 1-min denaturation step at 95 °C followed by 40 cycles of 15 s at 95 °C and 20 s at 60 °C on an iCycler real-time PCR detection system (Bio-Rad Laboratories, CA, USA). The relative expression levels of target values were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Ct}$ method [16]. The primers are shown in Table 2. All samples were analyzed in triplicate.

2.9. ELISA

ELISA kits were used to measure the levels of TNF-α, IL-1β, IL-6, and HMGB1 in culture supernatants, according to the manufacturer's instructions.

2.10. Preparation of total protein and nuclear extracts

Total protein was extracted from hippocampal neurons for use in western blot analyses. Nuclear extraction was conducted using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit, according to the manufacturer's instructions for the subsequent electrophoretic mobility shift assay (EMSA).

2.11. Western blotting

Protein expressions of HMGB1, RAGE, and TLR4 were separated from total protein on 12% sodium dodecyl sulfate (SDS) polyacrylamide gels by electrophoresis and electro-transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 10% non-fat milk for 1 h, the membranes were incubated at 4 °C overnight with specific primary antibodies, including for HMGB1 (1:1000), RAGE (1:1000), TLR4 (1:500) and β-actin (1:3000), followed by HRP-labeled secondary antibodies (1:2000) for 2 h. Finally, the images were digitized from the membranes using a Super Signal West Femto assay kit (Thermo Fisher Scientific) according to manufacturer's instructions.

2.12. EMSA

The NF-κB activity was determined using the DIG gel shift 2nd generation kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Briefly, a NF-κB probe primer (sense sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′) was synthesized and labeled with DIG-11-ddUTP at the 3′ end. Nuclear extractions were incubated for 20 min at RT for the binding assay. The binding complex was separated on a 6% non-denatured polyacrylamide gel at 80 V for 2 h by electrophoresis and electro-transferred to a nylon membrane at 400 mA for 30 min on ice. The DNA protein was fixed by ultra-violet cross-linking for 5 min. After blocking for 30 min, the membrane was incubated with anti-DIG antibody for 30 min, followed by detection buffer for 5 min. A chemiluminescent substrate (CSD, ready-to-use) was then applied and results were visualized by X-ray exposure for 25 min.

2.13. Statistical analyses

The data are expressed as means ± standard deviations. Western blot band densities were quantified using Quantity One software (Bio-Rad, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) or Tamhane's T2 post-hoc tests, using SPSS software 19.0 (IBM, Armonk, NJ, USA). The results are presented using GraphPad Prism software (GraphPad, San Diego, CA, USA 5.0 and statistical significance was indicated by $P < 0.05$.

3. Results

3.1. Cytotoxicity of $A\beta_{25-35}$ in hippocampal neuronal cells

To establish the optimal concentration of $A\beta_{25-35}$ for subsequent experiments, an MTT assay was conducted to investigate the cytotoxicity of $A\beta_{25-35}$ in hippocampal neuronal cells. Hippocampal neuronal cells were exposed to $A\beta_{25-35}$ at different concentrations (0, 2.5, 5, 10, 20, 40 μmol/L) for 24 h, and the inhibitory activity was assessed. As shown in Fig. 1, the data established 25 μmol/L as the optimal concentration for subsequent experiments, based on the IC_{50} value.

3.2. HMGB1 shRNA reduces HMGB1 expression in hippocampal neuronal cells

RT-qPCR and western blot assays were used to evaluate whether our HMGB1 shRNA could effectively downregulate HMGB1 expression in hippocampal neuronal cells. Fig. 2 shows data from cells pretreated for 24 h with HMGB1-target shRNA and the negative control sequences. HMGB1 mRNA and protein expression were significantly downregulated by shRNA1 and shRNA2. However, shRNA3 silenced mRNA expression of HMGB1 without effects on its protein expression. The results suggested that HMGB1 shRNA1 was the optimal target sequence for subsequent experiments.

3.3. HMGB1 shRNA inhibits translocation of HMGB1 from nucleus to cytoplasm after $A\beta_{25-35}$ treatment

Immunofluorescence staining was performed to detect changes in the localization of HMGB1 in hippocampal neuronal cells. HMGB1 is

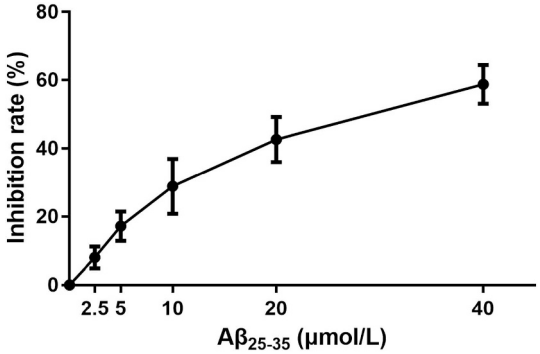

Fig. 1. Cytotoxicity of $A\beta_{25-35}$ in hippocampal neuronal cells. The optimal concentration for $A\beta_{25-35}$-induced cytotoxicity in cultured hippocampal neuronal cells was assessed. Growth inhibition is shown for hippocampal neuronal cells treated with $A\beta_{25-35}$ for 24 h at different concentrations, using an MTT assay. Means ± standard deviations (n = 9) are presented.

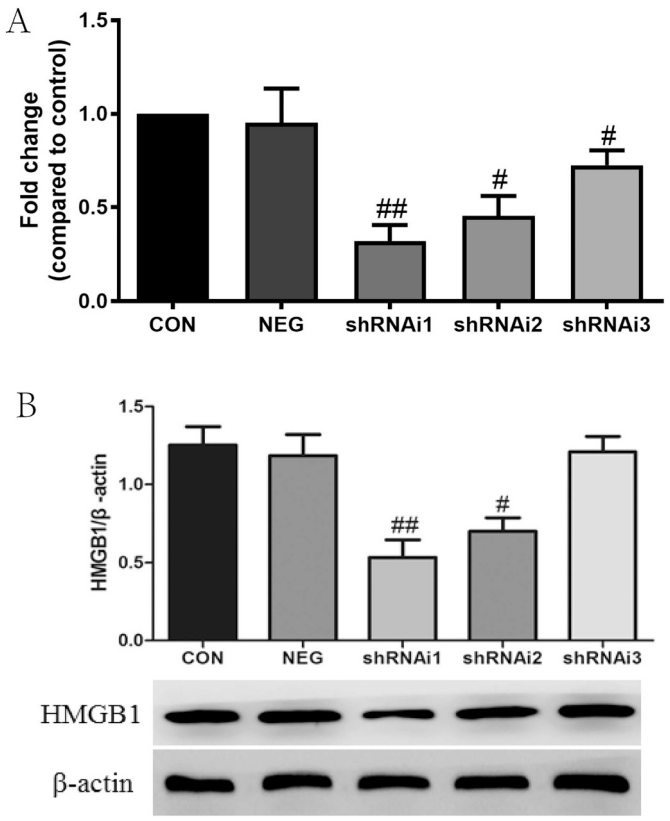

Fig. 2. High mobility group box 1 protein (HMGB1) short hairpin RNA (shRNA) reduces HMGB1 expression in hippocampal neuronal cells. Expression of HMGB1 in hippocampal neuronal cells pre-treated with different HMGB1 shRNA was determined using real-time qualitative PCR (A) and western blotting (B). CON = control, NEG = negative control, shRNAi1 = HMGB1-shRNA1 (51059-1), shRNAi2 = HMGB1-shRNA2 (51060-1), shRNAi3 = HMGB1-shRNA3 (51061-1). The data are expressed as means ± standard deviations (n = 6). $^{*}P < 0.05$ vs. CON. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. NEG.

normally predominantly expressed in the nucleus. The present results demonstrated that DyLight-488-labeled HMGB1 staining overlapped with stained nuclei. After $A\beta_{25–35}$ treatment, HMGB1 was observed in both the nucleus and the cytoplasm. Cytoplasmic accumulation of HMGB1 suggests that $A\beta_{25–35}$ induced HMGB1 translocation. To further determine whether secretion of HMGB1 was affected by $A\beta_{25–35}$ treatment, HMGB1 shRNA pretreatment was performed. Expression of HMGB1 in the cytoplasm decreased significantly, as shown in Fig. 3. Together, these results indicate that HMGB1 shRNA markedly reduces the degree of HMGB1 translocation from nucleus to cytoplasm after $A\beta_{25–35}$ treatment in hippocampal neuronal cells.

3.4. HMGB1 shRNA reduces RAGE and TLR4 expression after $A\beta_{25–35}$ treatment in hippocampal neuronal cells

Previous studies have supported that RAGE and TLR4 are receptors of HMGB1. Therefore, RT-qPCR and western blotting were used to detect mRNA and protein expressions, respectively, of RAGE and TLR4 in hippocampal neuronal cells. These experiments investigated whether RAGE/TLR4 inflammatory signaling pathways are activated by $A\beta_{25–35}$, using the HMGB1 shRNA. The results demonstrated relatively higher expressions of RAGE and TLR4 after $A\beta_{25–35}$ treatment. However, downregulation of HMGB1 by shRNA markedly reduced the increases in expression of these receptors, as shown in Fig. 4.

3.5. HMGB1 shRNA decreased NF-κB activity after $A\beta_{25–35}$ treatment in hippocampal neuronal cells

NF-κB is one of the most critical nuclear transcription factors, regulating the process of inflammation and nervous system diseases. Therefore, EMSA with a DIG-labeled NF-κB probe was conducted to further explore potential effects of HMGB1 on the DNA binding activity of NF-κB. The results demonstrate a significant increase in NF-κB activation following $A\beta_{25–35}$ treatment in hippocampal neuronal cells (Fig. 5). In contrast, treatment with HMGB1 shRNA decreased NF-κB activation. Together, these results support that HMGB1 shRNA decreased NF-κB activity and the inflammatory response after $A\beta_{25–35}$ treatment in hippocampal neuronal cells.

3.6. HMGB1 shRNA reduces inflammatory cytokine secretion after $A\beta_{25–35}$ treatment

To identify the effects of HMGB1 shRNA during inflammation and the innate immune response after $A\beta_{25–35}$ treatment, we assessed effects on pro-inflammatory cytokines, such as HMGB1, IL-1β, IL-6, and TNF-α. Expressions were significantly up-regulated, as indicated by ELISA, in the culture supernatant after treatment with the optimal concentration of $A\beta_{25–35}$. Furthermore, HMGB1 shRNA pretreatment prevented these $A\beta_{25–35}$-induced increases in secretion of these cytokines in hippocampal neuronal cells, as shown in Fig. 6.

4. Discussion

The present study aimed to elucidate the role of HMGB1 signaling in $A\beta_{25–35}$-induced hippocampal neuroinflammation. We also explored the effects of RAGE/TLR4 pathway and NF-κB activation. We found that the $A\beta_{25–35}$ fragment is the ideal inducer to establish an AD-related model of neuroinflammation. Hippocampal cells were exposed to different concentrations of $A\beta_{25–35}$, and we selected the optimal dose of 25 μmol/L to induce the desired pathological process. AD is one of the most common neurodegenerative brain diseases, primarily characterized by the deposition of Aβ plaques. Approximately 5.5 million Americans have AD, which is a serious clinical problem affecting a person's cognitive ability to perform daily activities [2].The hippocampus is a crucial area in learning, memory, and emotion; nerve cells, especially hippocampal neurons, are vulnerable to damage or destruction in the brains of patients with AD [17]. The sustained formation and deposition of sterile Aβ aggregates produce chronic activation of the innate brain immune system [18]. Hippocampal neuronal cells are significant contributors to limbic system functioning, and neurons cultured in vitro are a powerful tool to analyze the cellular mechanisms and underlying morphology of hippocampal processes [14]. Aβ induced oxidative stress, apoptosis, and neuroinflammation are core determinants in the etiology of neuronal death. Furthermore, the $A\beta_{25–35}$ domain in the hydrophobic region of Aβ plaques has independent neurotoxic effects [19].

DAMPs contribute to neuroinflammation in AD and pro-inflammatory mediators are thought to have a crucial role in the pathogenesis of AD [20]. HMGB1, a late inflammatory factor, can effectively identify the pattern recognition receptor that contributes to triggering and sustaining the development of inflammation [10]. Furthermore, neuroinflammatory mechanisms have essential roles in the progression of Aβ-induced secondary brain injury [21], which was confirmed in the present study. Thus, $A\beta_{25–35}$-induced increases in HMGB1 secretion may promote neuronal cell death. Extracellular HMGB1 initiates and sustains the inflammatory response by $A\beta_{25–35}$ treatment [9]. Under quiescent conditions, HMGB1 is generally localized to the nucleus. Once activated, HMGB1 is released from the nucleus into the cytoplasm. Accumulating evidence obtained by high resolution liquid chromatography–tandem mass spectrometric analysis (LC-MS/MS) demonstrates that biological HMGB1 requires acetylation, the post-

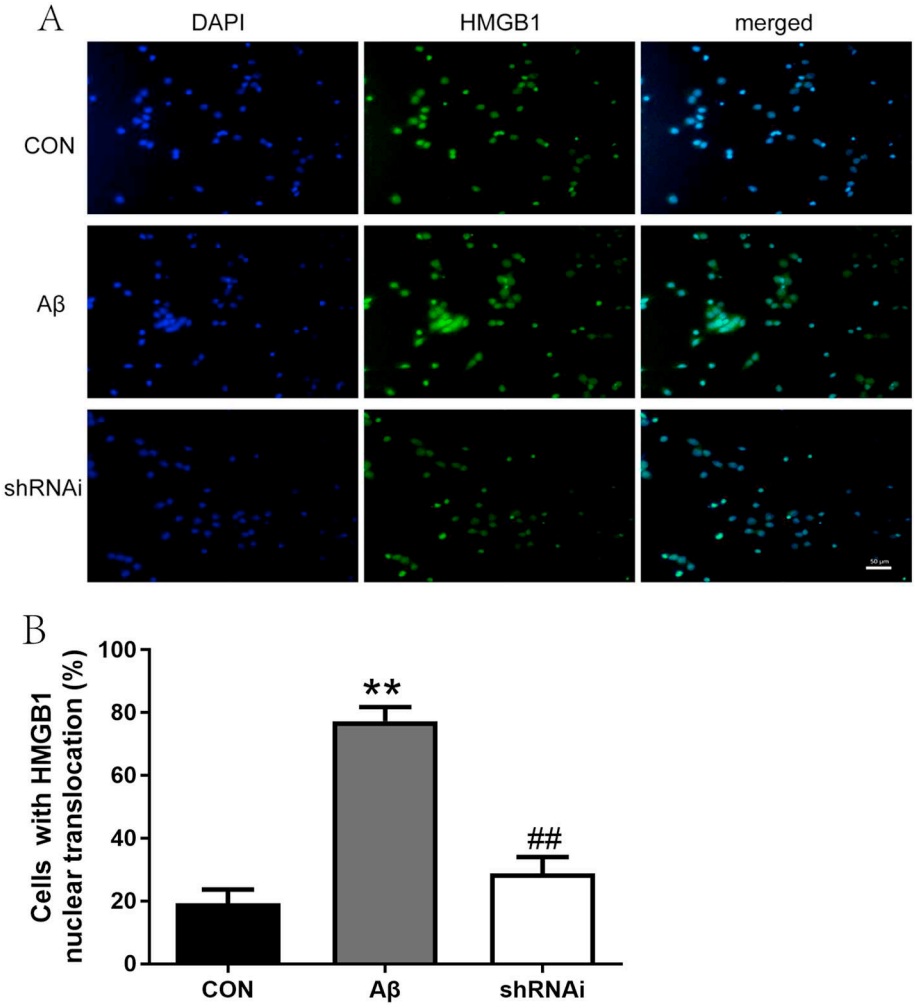

Fig. 3. High mobility group box 1 protein (HMGB1) short hairpin RNA (shRNA) inhibits translocation of HMGB1 from nucleus to cytoplasm after $A\beta_{25-35}$ treatment. (A) The changes in the localization of HMGB1 in hippocampal neuronal cells induced by HMGB1 shRNA pretreatment and $A\beta_{25-35}$ treatment were detected using fluorescent immune staining (scale bars, 50 μm). (B) Means ± standard deviations (n = 3). CON = control, Aβ = $A\beta_{25-35}$ treatment, shRNAi = HMGB1-shRNA interference. $^{**}P < 0.001$ vs. CON, $^{\#\#}P < 0.001$ vs. Aβ.

translational modifications dependent on 3 cysteine residues at positions 23, 45, and 106 [11] [22]. Calcium/calmodulin-dependent protein kinase (CaMK) IV is also activated by $A\beta_{25-35}$, and also promotes HMGB1 nucleocytoplasmic transport [23]. In the present study, cytoplasmic accumulation of HMGB1 occurred after $A\beta_{25-35}$ induced the HMGB1 translocation.

The evidence suggests that HMGB1 expression is correlated with AD progression. These results are consistent with studies demonstrating that $A\beta_{1-42}$ is a neurotoxic agent in SH-SY5Y cells [24]. Previous studies revealed that RAGE, a multi-ligand pattern recognition receptor, regulates chronic inflammation, and TLRs are type I transmembrane proteins involving in ligand binding. HMGB1 interacts with RAGE, TLRs, including TLR2, -4, and -9, and with CD24 [25] [26] [27]. RAGE activates various signaling pathways, including mitogen-activated protein kinases (MAPK) p38, TLRs associated with a TIR domain-containing adaptor (TIRAP), and myeloid differentiation primary response protein 88 (MyD88). Both inflammatory signals result in downstream activation of NF-κB [28] [29]. Mazarati [30] also demonstrated that overexpression of HMGB1 in the brain induced memory defects, which may be regulated by both RAGE and TLR4.

In the present study, mRNA and protein expressions of RAGE and TLR4 in hippocampal neuronal cells were elevated after $A\beta_{25-35}$-induced HMGB1 nuclear translocation; these findings are consistent with those of previous studies. HMGB1 itself can induce C-X-C motif chemokine ligand 12 (CXCL12) secretion via RAGE engagement. However, CXCL12 is also secreted by immune cells after NF-kB activation [31]. Activation of NF-κB regulates expression of some target genes, including pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α), and chemokines (MIP-1, and MCP-1) [32]. In the central nervous system, NF-κB acts as a regulator of hippocampal neurogenesis [33] and neurodegeneration [34]. Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, inhibit hippocampal neuronal functions, including long term potentiation (LTP) and dendritic branching, which are cellular substrates of learning and memory [35]. The present research demonstrates that $A\beta_{25-35}$-induced HMGB1 nuclear translocation activates NF-κB, which regulates IL-1β, IL-6, and TNF-α gene expression, consistent with previous studies. These data suggest that neuroinflammation is an important contributor to AD and anti-neuroinflammatory agents may therefore be potential therapeutic targets.

Previous studies demonstrating that extracellular HMGB1 has an important role in the pathogenesis of chronic inflammation indicate that HMGB1 inhibitors may reduce the risk of neurodegeneration. Strategies for blocking HMGB1 include anti-HMGB1 monoclonal antibody (mAb), specific HMGB1 inhibitors (glycyrrhizin), and HMGB1 interference (siRNA and shRNA). Anti-HMGB1 monoclonal antibody strongly inhibits neurite degeneration in a mouse model of AD [36]. Glycyrrhizin, known for its anti-inflammatory effect as an HMGB1 inhibitor, alleviated AD-like symptoms in a mouse model of AD [37].

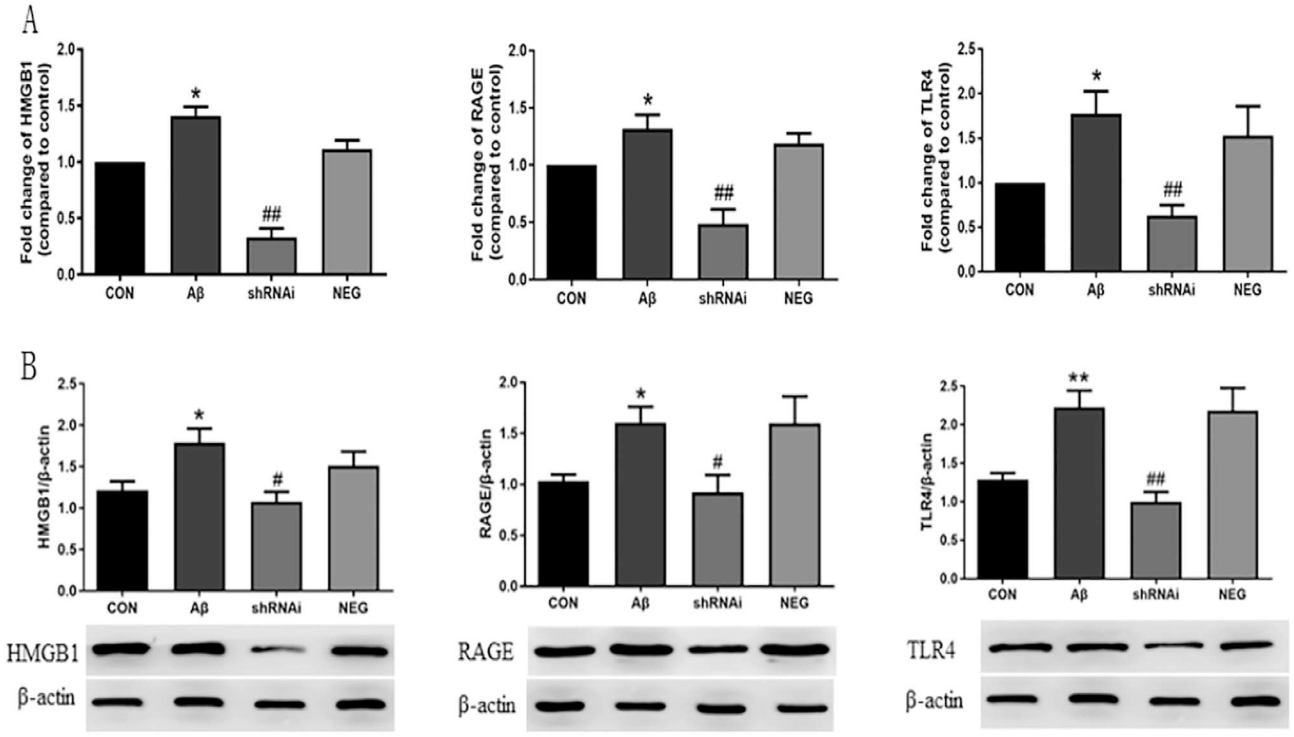

Fig. 4. High mobility group box 1 protein (HMGB1) short hairpin RNA (shRNA) reduces RAGE and TLR4 expression after $A\beta_{25–35}$ treatment in hippocampal neuronal cells. The expression of advanced glycosylation end product-specific receptor (RAGE) and toll-like receptor 4 (TLR4) in hippocampal neuronal cells pre-treated by HMGB1 shRNA was determined using real-time quantitative PCR (RT-qPCR) (A) and western blot analysis (B). CON = control, NEG = negative control, Aβ = $A\beta_{25–35}$ treatment, shRNAi = HMGB1-shRNA interference. The data are expressed as means ± standard deviations (n = 6). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. CON. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ vs. Aβ.

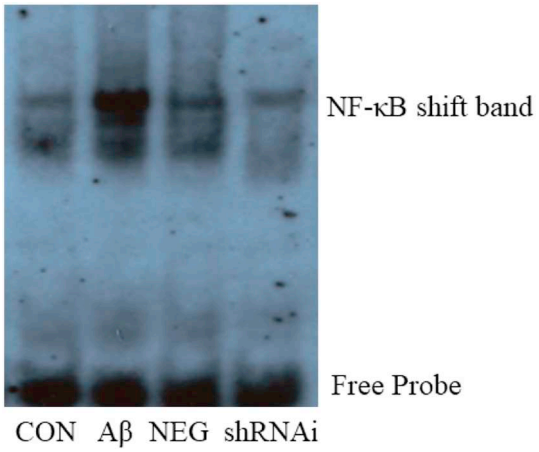

Fig. 5. High mobility group box 1 protein (HMGB1) short hairpin RNA (shRNA) decreased nuclear factor (NF)-κB activity after $A\beta_{25–35}$ treatment in hippocampal neuronal cells. NF-κB activation was assessed in hippocampal neuronal cells after $A\beta_{25–35}$ treatment, using an electrophoretic mobility shift assay with a specific κB probe. A large increase in DNA-protein complexes was observed in the Aβ group and a decrease was observed in the shRNAi group. CON = control, NEG = negative control, Aβ = $A\beta_{25–35}$ treatment, shRNAi = HMGB1-shRNA interference. Results were obtained by conducting 2 independent experiments.

Systemic blocking of HMGB1 by anti-HMGB1 mAb during the perioperative period prevents postoperative neurocognitive dysfunction in aged rats [38]. HMGB1 siRNA administration has protective effects in a rat lipopolysaccharide-induced acute lung injury model [39]. The wide application of gene editing technology is very helpful for the determination of target genes [40]. In the present study, HMGB1 shRNA delivered by lentivirus, which has high transfection efficiencies, attenuated HMGB1 expression, followed by attenuation of RAGE and TLR4 expressions. Furthermore, activation of NF-κB decreased, resulting in down-regulation of pro-inflammatory cytokines, similarly to the results of previous studies. Together, the evidence suggests that the HMGB1-mediated RAGE/TLR4-NF-κB pathway participates in neuroinflammation induced by $A\beta_{25–35}$ treatment.

Our study has certain limitations. Firstly, we did not apply HMGB1 shRNA to AD transgenic mice and hence specific targets of HMGB1 in these signals are not known and warrant further study. Secondly, the translocation of HMGB1 is likely to be a more complicated process [11] and hence it remains unclear acetylation positions of HMGB1 in $A\beta_{25–35}$-induced hippocampal neuroinflammation. Thus, future studies are needed to clarify these issues.

Our findings indicate that HMGB1 may mediate the pathogenesis of AD through activation of RAGE/TLR4 signaling. Furthermore, shRNA targeting HMGB1 is a promising therapeutic strategy for preventing AD onset and progression. The basic science work in hippocampal neuron cultures may help future research directions toward clinical applications. However, more detailed mechanisms and clinical applications of HMGB1 inhibitors during inflammation require further investigation.

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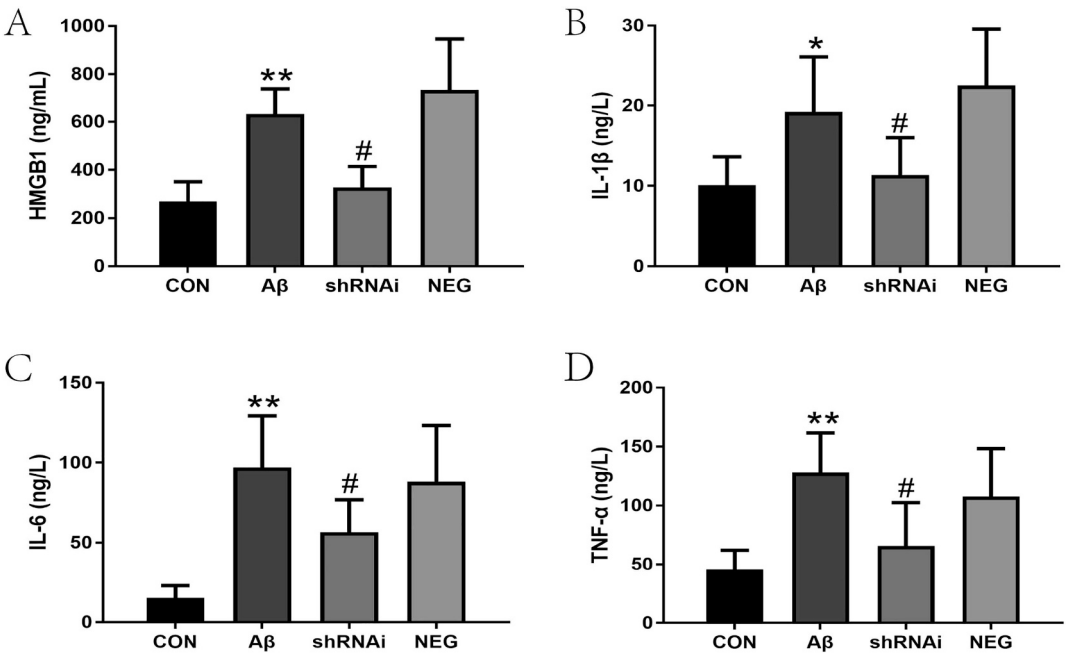

Fig. 6. High mobility group box 1 protein (HMGB1) short hairpin RNA (shRNA) reduces inflammatory cytokine secretion after $A\beta_{25–35}$ treatment. Expressions of pro-inflammatory cytokines such as HMGB1 (A), interleukin (IL)-1β (B), IL-6 (C), and tumor necrosis factor (TNF)-α (D) were assessed using ELISA on hippocampal neuronal cell culture supernatant following pre-treatment with HMGB1 shRNA and $A\beta_{25–35}$ treatment. CON = control, NEG = negative control, Aβ = $A\beta_{25–35}$ treatment, shRNAi = HMGB1-shRNA interference. The data are presented as means ± standard deviations (n = 8). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control $^{\#}P < 0.05$ vs. Aβ.

Conflict of interest

None.

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