Toll-like receptor-4-mediated autophagy contributes to microglial activation and inflammatory injury in mouse models of intracerebral haemorrhage


*Department of Neurology, Yongchuan Hospital, Chongqing Medical University, Chongqing, ‡Department of Neurosurgery, The 476th Hospital of PLA, Fuzhou, Fujian, and †Department of Urology, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

Aims: Much evidence demonstrates that Toll-like receptor-4 (TLR4)-mediated microglial activation is an important contributor to the inflammatory injury in intracerebral haemorrhage (ICH). However, the exact mechanism of TLR4-mediated microglial activation induced by ICH is not clear. In addition, microglial autophagy is involved other forms of nervous system injury. To explore the relationship between TLR4 and autophagy, we investigated the role of TLR4-mediated microglial autophagy and inflammation in ICH.

Methods: We detected TLR4 expression, autophagy and inflammation of microglia treated with lysed erythrocytes in vitro, and observed the cerebral water content and neurological deficit of ICH mice [TLR4−/− and wild type (WT)] in vivo.

Results: We found that lysed erythrocyte treated microglia (TLR4−/−) had reduced autophagy and inflammation compared with microglia (WT) in vitro. ICH mice (TLR4−/−) had reduced water content and neurological injury compared with ICH mice (WT). The autophagy inhibitor (3-methyladenine) decreased microglial activation and inflammatory injury due to lysed erythrocyte treatment, and improved the neurological function of ICH mice.

Conclusions: Taken together, these data suggested that TLR4 induced autophagy contributed to the microglial activation and inflammatory injury and might provide novel therapeutic interventions for ICH.

Keywords: autophagy, ICH, microglia, TLR4

Introduction

Intracerebral haemorrhage (ICH), estimated to affect over 1 million people worldwide each year, is the least treatable form of stroke and contributes substantially to the burden of cerebrovascular disease [1–3]. After ICH, a lot of changes occur in the brain, including haematoma formation, brain oedema, inflammation and microglial activation [4–6]. Thus, the inhibition of pro-inflammatory mediators offers a potentially effective therapeutic approach for ICH.

Innate immunity and inflammatory responses may contribute to neurological deficits possibly through release of endogenous ligands, which exert functions largely through Toll-like receptors (TLRs) [7–9]. The evidence demonstrates that haematoma components act on TLR4 expressed on microglia and activate gene transcription through the TLR4 downstream signalling pathway, resulting in the production of inflammatory...
factors and ultimately leading to inflammatory injury and neurological deficits [10–12].

Autophagy is an evolutionarily conserved degradation pathway which primarily functions as a cell survival adaptive mechanism during stress conditions [13–15]. Autophagy has recently been shown to be an important component of the innate immune response and TLR4 served as a previously unrecognized environmental sensor for autophagy [16–18]. However, in ICH-induced inflammatory injury, the role of TLR4 on microglial autophagy and activation remains unclear. Therefore, in the current experiment, we investigated the role of TLR4 on microglial autophagy and activation in ICH-induced inflammatory injury.

**Materials and methods**

**Animals**

C57BL/6 mice (male, 8–10 weeks, 20–24 g) were obtained from the Animal Center of the Fujian Medical University (Fuzhou, China). Transgenic line TLR4−/− mice (8–10 weeks, 20–24 g) were purchased from American Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed in individual cages with free access to sterile acidified water and irradiated food in a specific pathogen-free facility at the Fujian Medical University. Experiments were conducted in accordance with animal care guidelines approved by the Animal Ethics Committee of the Fujian Medical University.

**Primary cell cultures**

Cortical neuronal cultures were prepared from whole cerebral cortices of C57BL/6 mouse embryos (E16) [both wild-type (WT) and TLR4−/− mice]. After removal of the meninges, tissue was digested by 0.005% trypsin/0.002% EDTA (10 min, 37°C), mechanically dissociated, and centrifuged at 1000 g for 5 min. The cell pellet was resuspended in neurobasal medium (Gibco, Oakland, CA, USA) containing B27 serum free supplement (Gibco) and 500 nM L-glutamine. A total of 2 × 10^5 cells per well were seeded on sterile poly-L-lysine (Sigma-Aldrich, Saint Louis, MO, USA) coated glass coverslips in a 24-well plate and incubated at 37°C and 5% CO_2_. After 1 h, culture medium was changed completely. Purity of neuronal cultures was >95% as confirmed by random staining with neuronal and glia markers. Five days after plating, neurons had developed a dense network of extensions. For primary microglia cells, cerebral hemispheres of 1-day-old postnatal mice were digested with 0.1% trypsin. The cells were seeded into a six-well plate coated with poly-L-lysine and fed with Dulbecco’s Modified Eagle Media (DMEM; Sigma, St. Louis, MO, USA) containing 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA). Culture media were refreshed twice per week for 2 weeks. Microglia was detached by gentle shaking and filtered through a nylon mesh to remove astrocytes. After centrifugation at 1000 g for 10 min, the cells were resuspended in fresh DMEM supplemented with 10% FBS and plated at a final density of 5 × 10^5 cells/ml on a poly-L-lysine-coated six-well culture plate. The following day, cells were subjected to the experiments. The cell purity was determined by immunohistochemical staining using microglia-specific antibody CD11b. The microglia cultures used were >95% pure.

**Cell treatment**

Microglia (1 × 10^5) was stimulated with 10 μl erythrocyte lysis (the endotoxin levels have been assessed by Limulus amebocyte assay, and less than 0.5 endotoxin unit/μg of lysis ). After 3 days, the supernatants were removed and further analysed for cytokine production with ELISA. Neurone was cultured in a 96-well plate with 1 × 10^4 cells per well. For the toxicity experiments, neurone was serum-starved for 4 h and then treated with a mixture of microglia-conditioned medium. For MTT and apoptosis assays, cells were treated for 48 h.

**Transient transfection and identification of autophagy**

Green fluorescence protein (GFP)-tagged LC3 expression vector has recently been utilized to demonstrate the occurrence of autophagy. Microglia were seeded (1 × 10^4 cells/well) in 96-well plates overnight, then GFP-LC3 expressing plasmids were transiently transfected into the cells using Fugene HD transfection reagent (Roche) according to the manufacturer’s instructions. Cultures for 24 h, ensuring the expression of GFP-LC3, the cells were subjected to 10 μl erythrocyte lysis for 3 days and in some experiment, 3-methyladenine (3-MA) was also added. At the end of the treatment, autophagy was detected by counting the percentage of cells with GFP-LC3-positive dots under fluorescence microscope (Olympus IX71). A minimum of 200 cells per sample were counted in triplicate for each experiment.
Electron microscopy

The cells were fixed in 2.5% glutaraldehyde acid in 0.1 M phosphate buffer saline (PBS) buffer (pH 7.4) for 2 h or more, incubated in 1% osmium tetroxide in 0.1 M PBS buffer (pH 7.4) for 2 h, dehydrated in solutions of ethanol and acetone, then embedded in Araldite and finally solidified. Fifty- to 60-nm sections were cut on a LKB-I ultramicrotome and picked up on copper grids, post-stained with uranyl acetate and lead citrate, observed in a Philips CM-120 TEM.

Western blot analysis

At the end of the designated treatments, microglia was lysed in RIPA lysis buffer (Beyotime, P0013B) with 1 mM PMSF. Equal amounts of protein was separated by SDS-PAGE and transferred on to NC membrane. After blocking with 5% nonfat milk, the membrane was probed with anti-LC3 (Novus Biologicals, Inc., Denver, CO, USA), developed with the BeyoECL Plus substrate system (Beyotime, China, P0018). Blots were stripped and re-probed with β-actin antibody (Santa Cruz, Los Angeles, CA, USA) to confirm equal protein loading.

Enzyme-linked immunosorbent assay (ELISA)

Microglia (1 × 10⁵) was stimulated with 10 μl erythrocyte lysis. After 3 days, the supernatants were removed and further analysed for cytokine production with ELISA. ELISA was performed as per the manufacturer’s instructions (Dakewe Biotech, Shenzhen, China) to assess the concentrations of tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in the culture supernatant.

Scratch assay

A total of 5 × 10⁵ microglia was grown in six-well plates as 60% confluent monolayers and was wounded with a sterile 100 μl pipette tip. Thereafter, the cells were stimulated with 10 μl erythrocyte lysis or controls. Migration into the open scar was documented with microphotographs at different time points after wounding. The number of migrating cells was quantified by counting all cells within a 0.4-mm² region in the centre of each scratch. A minimum of five individual cultures was used to calculate the mean migratory capacity of each cell culture condition.

Transwell migration assay

The Costar Transwell System (8-μm-pore-size polycarbonate membrane) was used to evaluate vertical cell migration. Microglia cells (1 Mio) in 1.5 ml serum-free medium were added to the upper well, and 2.6 ml serum-free medium was added to the lower chamber. Ten microlitres of erythrocyte lysis or controls was added to the lower chamber medium. At the end of a 24-h incubation period, cells that had migrated to the lower surface were quantified by counting the migrated cells on the lower surface of the membrane using microscopy.

MTT assay

Cell viability of neurone was assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich) assay. After 48 h, MTT reagent was added to the wells, incubated for 4 h at 37°C, 5% CO₂. After centrifugation, the supernatant was removed from each well. The coloured formazan crystal produced from MTT was dissolved with 0.15 ml DMSO, then the optical density (OD) value A490 was measured by the multiscanner autoreader (Dynatech MR 5000; Dynatech Laboratories, Chantilly, VA, USA). The absorbance was measured at 570 nm. The mean of readings of triplicate wells was taken as one value. The OD value for the control cultures was considered as 100% viability and viability in other samples is expressed as a percentage of viability in the control cultures.

Flow cytometric analysis of apoptosis

An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Oncogene Research Products, Boston, MA, USA) was used to detect apoptosis. The cells were seeded in 100-ml flasks and incubated until there was about 90% confluence in DMEM supplemented with 10% foetal bovine serum. Then the cells were harvested, washed with ice-cold PBS twice, and resuspended in binding buffer (10 mM of Hapes, pH 7.4, 1.50 mM of NaCl, 2.5 mM of CaCl₂, 1 mM of MgCl₂, 4% bovine serum albumin). Annexin V-fluorescein isothiocyanate (0.5 mg/ml) and propidium iodide (0.6 mg/ml) were then added to a 250-ml aliquot (1 × 10⁵ cells) of this cell suspension according to the protocol of the manufacturer. After a 15-min incubation in the dark at room temperature,
stained cells were immediately analysed on a flow cytometer (Beckman Coulter, Fullerton, CA, USA). All of the samples were assayed in triplicate, and the cell apoptosis rate calculated using the following formula: apoptosis rate = (apoptotic cell number/total cell number) × 100%.

Caspase assay
To detect caspase-3-like protease activities, the ApoAlert caspase-3 colorimetric assay kit (Clontech, Palo Alto, CA, USA) was used. Cytosolic lysates were prepared 48 h following transfection and incubated with 50 mm p-nitroanilide (pNA) conjugated to the caspase cleavage site Asp–Glu–Val–Asp (DEVD) for 1 h at 37°C. Hydrolysed pNA was detected using a Multiscan MS colorimeter (Thermo Labsystems, Vantaa, Finland) at 405 nm. For control experiments, the lysates were incubated with 10 mm of the caspase-3 inhibitor DEVD-fmk (Clontech) for 30 min, before addition of the substrate.

ICH model
Briefly, mice were anaesthetized with an intraperitoneal injection of 400 mg/kg chloral hydrate and fixed on a mouse stereotaxic frame (Stoelting, Tolang, WI, USA). A 20-μl volume of autologous non-anticoagulated blood was collected from the tail vein of the mouse and then injected into the caudate nucleus at 2 μl/min under stereotactic guidance at the following coordinates relative to bregma: 0.8 mm anterior; 2 mm left lateral and 3.5 mm deep during a period of 10 min. The needle was held in place for 10 min after injection, and the microsyringe was pulled out after the blood had coagulated. The craniotomy was then sealed with bone wax, and the scalp was closed with sutures. Body temperature was maintained at 37°C throughout the procedure, and the mice were given free access to food and water after they woke up. The mice that died because of anaesthesia were excluded.

Administration of 3-MA
To study the effects of the autophagy inhibitor 3-MA, mice were received an intracerebral ventricular injection of 3-MA (2 μl of a 30 mg/ml solution prepared in 0.9% NaCl) or vehicle (0.9% NaCl, 2 μl) 10 min after ICH.

Histochemical evaluation of microglia activation
Three days after ICH, the animals were deeply anaesthetized with pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After the mice were perfused and fixed, the perihæmatomal region of cerebral tissues were collected, fixed in 4% paraformaldehyde for 24 h, dehydrated in 30% sucrose solution for 48 h, embedded, frozen, and cut into 25-μm sections using a Leica CM1900 cryostat. The perihæmatomal region was treated with 3% H2O2 in 0.01 M phosphate-buffered saline (PBS) and pre-incubated in 5% normal goat serum. The samples were then incubated in a primary antibody solution containing rat anti-CD11b antibody (Serotec, Fullerton, CA, USA, 1:200) overnight at 4°C. After washing, the samples were incubated in a secondary IgG antibody (1:200) for 1 h at room temperature (RT). Finally, the sections were incubated in horseradish peroxidase (HRP)-Streptavidin (1:200) for 1 h at RT, and the colour reaction was conventionally developed with diaminobenzidine (DAB) and H2O2.

Evaluation of neurological scores
The neurological scores were determined by Neurological Severity Scores, a composite of motor, sensory, reflex and balance tests. Neurological function was graded on a scale of 1 to 18; a score of 1 point is awarded for the inability to perform the test or for the lack of a tested reflex. The higher the score, the more severe the injury (normal score: 2–3; maximal deficit score: 18).

Brain water content measurement
Brain water content was measured in mouse cerebral tissues after ICH. Briefly, mice were randomly sampled from each group and anaesthetized by intraperitoneal injection with chloral hydrate (n = 5). Next, the cerebral tissues were removed, and the surface water on the cerebral tissues was blotted with filter paper. The brains were divided into five parts (ipsilateral and contralateral cortex, ipsilateral and contralateral basal ganglia, and cerebellum). Brain samples were immediately weighed on an electric analytic balance to obtain the wet weight and then dried at 100°C for 24 h to obtain the dry weight. Brain water content was calculated using the following formula: brain water content (%) = (wet weight – dry weight)/wet weight × 100%.
Statistical analysis

The statistical significance of differential findings between experimental groups and controls were determined by t-test and considered significant if $P < 0.05$.

Results

Erythrocyte lysis induces TLR4-mediated microglia activation

To test the hypothesis ICH trigger microglia activation via TLR4, we investigated the effect of erythrocyte lysis on TLR4 expression and cytokine release in vitro. First, using primary cultured microglia cells, we found that erythrocyte lysis treatment significantly increased TLR4 expression ($P < 0.01$) in cultured microglia (Figure 1A). Next, we investigated the effects of erythrocyte lysis stimulation on microglia culture derived from TLR4−/− mice. We observed that administration of erythrocyte lysis significantly induced the expression of TNF-α, IL-1β and IL-6 (Figure 1B) in microglia from WT mice but not from TLR4−/− mice. These data suggest that ICH might promote microglia activation via TLR4 and genetic depletion of TLR4 in microglia lead to suppression ICH-induced microglia activation.

Erythrocyte lysis induces TLR4-mediated microglial autophagy

TLR4 downstream signalling pathway could promote the production of inflammatory factors. Microglia can make themselves adapt to adverse conditions, such as hypoxia and nutrient deprivation, by activating autophagy. To identify whether erythrocyte lysis induces TLR4-mediated microglia autophagy, we next analysed the occurrence of microglia autophagy with an expression vector encoding GFP-LC3 which is concentrated in autophagic vacuoles, resulting in punctate fluorescence within cells undergoing autophagy. Microglia derived from WT mice or TLR4−/− mice was transiently transfected with GFP-LC3 plasmids. Twenty-four hours post transfection, cells were treated with erythrocyte lysis for 5 days. At the end of treatment, cells were observed under a fluorescence microscope and the cells with diffused or punctate GFP were counted. To confirm the involvement of autophagy by additional independent assays, we analysed LC3-I to LC3-II protein processing, which is a hallmark of autophagy, by Western

Figure 1. Erythrocyte lysis induces TLR4-mediated microglia activation. (A) Microglia (1 × 10^5) (WT or TLR4−/−) was stimulated with 10 μl PBS, 10 μl erythrocyte lysis, polymixin B (PMB, 10 μg/ml), LPS (2 μg/ml), 10 μl erythrocyte lysis + polymixin B (PMB, 10 μg/ml), or LPS (2 μg/ml) + polymixin B (PMB, 10 μg/ml). After 3 days, microglia was lysed and analysed the TLR4 expression with Western blot analysis. (B) Microglia (1 × 10^5) (WT or TLR4−/−) were stimulated with 10 μl erythrocyte lysis or controls. After 3 days, the supernatants were removed and further analysed for TNF-α, IL-1β and IL-6 cytokine production with ELISA. Experiments performed in triplicate showed consistent results. Data are presented as the mean ± SD of three independent experiments. *$P < 0.05$. © 2014 British Neuropathological Society

NAN 2015; 41: e95–e106
blot. Levels of endogenous LC3-II from 1 day to 5 days were markedly increased in microglia (WT) treated with erythrocyte lysis as compared with microglia (TLR4−/−) treated with erythrocyte lysis or other controls (Figure 2A). As shown in Figure 2B, microglia (WT) treated with erythrocyte lysis exhibited significant high percentage of punctate GFP, while microglia (TLR4−/−) treated with erythrocyte lysis or other controls showed primarily diffused. As another independent assay of autophagy, microglia was processed for transmission electron microscopy after treatment. Microglia (WT) treated with erythrocyte lysis revealed a marked accumulation of autophagosomes as compared with that of microglia (TLR4−/−) treated with erythrocyte lysis or other controls (Figure 2C, black arrows).

**Autophagy is involved in TLR4-mediated microglia activation**

To identify whether autophagy is involved in TLR4-mediated microglia activation by erythrocyte lysis, we analysed the TNF-α, IL-1β and IL-6 cytokines. The results demonstrated that administration of erythrocyte lysis significantly induced the expression of TNF-α, IL-1β and IL-6 of microglia (WT) but not those of microglia (TLR4−/−) (Figure 3). However, the 3-MA could attenuate cytokines expression of microglia (WT) but not those of microglia (TLR4−/−). Thus, these data suggest that autophagy was activated significantly in response to erythrocyte lysis, which may likely correlate with TLR4.

**Autophagy involved in TLR4-mediated microglia migration**

To explore the effect of autophagy on TLR4-mediated microglia migration, we first cultured microglia on plastic dishes until 60% confluence and then created a scratch with a pipette tip. Microglia (WT or TLR4−/−) was stimulated with erythrocyte lysis or controls for 12 h, and then, migration into the cell-free scratch area was documented. The statistical analysis of five independent experiments revealed a significantly reduced number of migrating microglia (TLR4−/−) compared with that of microglia (WT) when stimulated with erythrocyte lysis. As an independent measure of microglia motility and to study the long-term effects of erythrocyte lysis, we performed transwell migration assays over a period of 24 h. Similar as in the scratch assays, erythrocyte lysis caused a significant attenuation of migrating microglia (TLR4−/−) compared with that of microglia (WT). However, 3-MA could attenuate migration of microglia (WT) but not that of microglia (TLR4−/−) (Figure 4). These data suggest that autophagy was involved in TLR4-mediated microglia migration.

**Autophagy is involved in TLR4-mediated microglia inflammatory injury to neurone cells**

The activated microglia can cause toxicity to neurones. We next explored whether autophagy induced activated microglia inflammatory injury to neurone cells through TLR4. We used an MTT assay to examine erythrocyte lysis-induced toxicity of microglia to neurone. We found that when neurone was treated with conditioned medium from erythrocyte lysis-treated microglia (WT), there was a significant decrease in cell viability. However, when the cells were treated with conditioned medium from microglia (TLR4−/−) treated with erythrocyte lysis, the cell viability was increased (Figure 5A). In addition, we also analysed the apoptosis of neurone and the specific apoptotic pathways. Two days after neurones were treated with conditioned medium from erythrocyte lysis treated microglia, cell apoptosis ratio and caspase activity were detected by flow cytometry and colorimetric assay. The results demonstrated that when neurone was treated with conditioned medium from erythrocyte lysis treated microglia (WT), there was a significant increase in cell apoptosis rate and caspase-3 activity compared with that of microglia (TLR4−/−) (Figure 5B, C). However, 3-MA could attenuate inflammatory injury to neurone cells of microglia (WT) but not that of microglia (TLR4−/−). Thus, the results might demonstrate that autophagy induced activated microglia inflammatory injury to neurone cells through TLR4.

**Autophagy inhibitors reduce microglia activation in ICH**

In mice subjected to ICH for 3 days, the perihematomatol region of cerebral tissues was analysed, and microglia activation was observed. The results demonstrated that ICH could promote the microglia activation, including swollen and hypertropic cell bodies as well as thick and short processes.

To determine the contribution of autophagic mechanisms to microglia activation, the effects of intracerebroventricular (i.c.v.) injection of the autophagy
Figure 2. Erythrocyte lysis induces TLR4-mediated microglial autophagy. (A) Microglia (1 × 10⁵) (WT or TLR4−−/) was stimulated with 10 μl PBS, 10 μl erythrocyte lysis, 10 μl erythrocyte lysis + 3-MA (5 μM), LPS (2 μg/ml) or LPS (2 μg/ml) + 3-MA (5 μM). After 3 days, the whole cell lysates were subjected to Western blot for analysis of LC3-I to LC3-II. (B) Microglia (1 × 10⁵) (WT or TLR4−−/) transiently expressing GFP-LC3 was stimulated with erythrocyte lysis or controls mentioned above for 3 days, and analysed by fluorescence microscopy. The number of GFP-LC3 puncta in each cell was counted. (C) Ultrastructural alterations of microglia. Electron micrographs showed the ultrastructure of microglia (WT or TLR4−−/) stimulated with erythrocyte lysis or controls mentioned above for 3 days. Arrows indicate the autophagic vacuoles in the cytoplasm. Magnification, × 10 000. Experiments performed in triplicate showed consistent results. Data are presented as the mean ± SD of three independent experiments. *P < 0.05.
inhibitors 3-MA administered immediately after the onset of ICH were examined. We found that administration of 3-MA significantly reduced microglia activation of mice (WT) but not those of mice (TLR4−/−) (Figure 6) (P < 0.05). These analyses showed that autophagy inhibitors could inhibit microglia activation through TLR4 after ICH.

**Autophagy inhibitors reduce brain damage in ICH**

In mice subjected to ICH for 3 days, the water content in mice brains and neurological injury were observed. The results demonstrated that water content in mice brains and neurological injury subjected to ICH significantly increased compared with sham-operated animals (Figure 7) (P < 0.05). To determine the contribution of autophagic mechanisms to neuronal death, the effects of i.c.v. injection of the autophagy inhibitors 3-MA administered immediately after the onset of ICH were examined. We found that administration of 3-MA significantly reduced water content and neurological injury of mice (WT) but not those of mice (TLR4−/−) (P < 0.05). These analyses showed that autophagy inhibitors could ameliorate the neurological symptoms through TLR4 after ICH.
Intracerebral haemorrhage is a common type of fatal stroke, accounting for about 15% to 20% of all strokes [19–21]. Haemorrhagic strokes are associated with high mortality and morbidity, and increasing evidence shows that innate immune responses and inflammatory injury play a critical role in ICH-induced neurological deficits [22–24]. Although there is much research on the events following ICH, the initial cellular events triggering innate immune and inflammatory responses are complex. Therefore, a strategy for inhibiting the inflammatory response is needed.

Toll-like receptors belong to a large family of pattern recognition receptors that play a key role in innate immunity and inflammatory responses [25,26]. Microglial activation in response to ICH contributes to ICH-induced brain injury by releasing cytokines and inhibition of microglial activation has been shown to improve neurological function in animal models of ICH [27,28]. Exogenous haemin treatment of cultured microglia increases expression of TLR4, as well as pro-inflammatory cytokines. This effect is completely abolished by knockout of TLR4 or treatment with anti-TLR4 antibodies, suggesting that TLR4 mediates haemin-stimulated microglial activation [12].

Autophagy is a fundamental cell biological process with impact on ageing, development, cancer, neurodegeneration, metabolic disorders, infection and immunity [29–31]. Autophagy plays an evolutionarily conserved role in the host defence against pathogens. Toll-like receptors that detect conserved molecular features shared by pathogens regulate several innate immune responses including autophagy [32,33]. Recent research demonstrates that autophagy reported in response to TLR4-stimulation in macrophages is selective autophagy of aggresome-like induced structures [33,34]. In addition, the related evidence highlights a pivotal role for TLR4-mediated basal immunity, particularly autophagic activity, in the resolution of inflammation and fibrosis after chemical-induced lung injury [35]. However, in ICH-induced inflammatory injury, the role of TLR4 on microglial autophagy and activation remains unclear.

In our study, we used two different models to assess the effect of haematoma formation on microglial autophagy and activation – the erythrocyte lysis in vitro and ICH in vivo. We first investigated the effect of erythrocyte lysis on the expression of TLR4 and cytokine release in vitro. We
Autophagy is involved in TLR4-mediated microglia activation in ICH. Three days after ICH, mice were deeply anaesthetized and transcardially. The brains were removed and post-fixed. The perihaematoma region of cerebral tissue was collected, and microglia was analysed with anti-CD11b antibody. Experiments performed in triplicate showed consistent results.

Figure 6. Autophagy is involved in TLR4-mediated microglia activation in ICH. Three days after ICH, mice were deeply anaesthetized and transcardially. The brains were removed and post-fixed. The perihaematoma region of cerebral tissue was collected, and microglia was analysed with anti-CD11b antibody. Experiments performed in triplicate showed consistent results.
found that erythrocyte lysis treatment significantly increased TLR4 and cytokine expression in microglia from WT mice but not from TLR4−/− mice. To further identify whether autophagy was involved in TLR4-mediated microglial activation, we analysed microglial autophagy. We found that erythrocyte lysis promoted microglia autophagy via TLR4 and autophagy was involved in TLR4-mediated microglial inflammatory injury and migration.

To study the effect of microglial autophagy on neurological function, we utilized the in vivo ICH mouse model. In addition, we found that ICH could promote microglial activation and administration of 3-MA significantly reduced water content and neurological injury of WT mice but not those of knockout mice (TLR4−/−). These analyses showed that autophagy inhibitors could ameliorate neurological symptoms through TLR4 after ICH.

In summary, the present study showed that ICH induced microglial autophagy and activation, and that TLR4 plays an important role in the processes. In addition, the understanding of the mechanisms of ICH-induced autophagic cell activation may provide novel therapeutic interventions for ICH induced inflammatory injury.

Conflict of interest
None of the authors has any conflict of interest.

Author contributions
Zhao Yang, Nan Zhang and Yongping Liu completed the study. Hanchao Shen, Chuangan Lin and Li Lin designed the study. Bangqing Yuan wrote the study.

Acknowledgements
We thank Dr Tamotsu Yoshimori for providing the GFP-MAP1LC3B plasmid. This study was supported by a grant from National Natural Science Foundation of China (NSFC, No. 81200908).

Reference
6 Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. Prog Neurobiol 2010; 92: 463–77


15 Wellnitz K, Taegtmeyer H. Mechanical unloading of the failing heart exposes the dynamic nature of autophagy. *Autophagy* 2010; 6: 155–6


17 Seay MD, Dinesh-Kumar SP. Autophagy takes its TOLL on innate immunity. *Cell Host Microbe* 2007; 2: 69–70


25 Jo EK. Mycobacterial interaction with innate receptors: TLRs, C-type lectins, and NLRs. *Curr Opin Infect Dis* 2008; 21: 279–86


32 Campbell GR, Spector SA. Toll-like receptor 8 ligands activate a vitamin D mediated autophagic response that inhibits human immunodeficiency virus type 1. *PLoS Pathog* 2012; 8: e1003017

33 Fujita K, Srinivasula SM. TLR4-mediated autophagy in macrophages is a p62-dependent type of selective autophagy of aggresome-like induced structures (ALIS). *Autophagy* 2011; 7: 552–4


Received 5 December 2013
Accepted after revision 8 August 2014
Published online Article Accepted on 3 September 2014
学霸图书馆
www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页　文献云下载　图书馆入口　外文数据库大全　疑难文献辅助工具