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Interleukin 17A exacerbates ER-stress-mediated inflammation of macrophages following ICH

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ABSTRACT

IL-17A contributes to the initiation of inflammation following intracerebral hemorrhage (ICH). Endoplasmic reticulum (ER) stress acts on protein folding and contributes to inflammatory diseases. The role of IL-17A in the regulation of ER stress following ICH has not been well characterized. In this study, macrophages were stimulated with IL-17A, and then, ER stress and downstream pro-inflammatory factors were measured *in vitro*. In addition, brain edema and brain injury in ICH mice were assessed *in vivo*. We demonstrated that IL-17A induced ER stress in macrophages and thus promoted inflammation *in vitro*. Conversely, IL-17A inhibition attenuated ER stress and neuroinflammation. Furthermore, ERK 1/2 and p38 MAPK pathways mediated IL-17A-induced ER stress in macrophages. We also showed that IL-17A inhibition significantly attenuated ER stress and brain injury in ICH mice.

In conclusion, our results demonstrate that IL 17A increases ER stress in macrophages and represents a novel mechanism in ICH.

1. Introduction

Intracerebral hemorrhage (ICH) accounts for 10%–15% of all strokes and is regarded as a common disease with high mortality and morbidity (Ovesen et al., 2015; Meyer et al., 2015; Mustanoja et al., 2015). Secondary brain injury following ICH often contributes significantly to changes in neurological functioning. Secondary injury after ICH leads to bleeding, which causes hematoma expansion and increases neuroinflammation and neuronal death, as well as contributing to the development of perihemorrhagic edema (PHE), elevated intracranial pressure (ICP), hydrocephalus, and brain atrophy (Ft et al., 2016; Duan et al., 2016; Zhou et al., 2017).

Brain inflammation following ICH is characterized by activated inflammatory cell accumulation, including macrophages and leukocytes (Ft et al., 2016; Zhou et al., 2017; Yang et al., 2016a). Macrophages are activated early following ICH and release inflammatory factors, including chemokines, proteases, cytokines and other immunoactive molecules (Yang et al., 2015; Zhang et al., 2015a; Chen et al., 2015a). Therefore, macrophages play crucial roles in the development of secondary brain damage.

IL-17A is one of the IL-17 cytokine factors and is generated by $CD4^+$ T-helper 17 (Th17) cells (Grund et al., 2012). IL-17A is involved in several inflammatory diseases, including diabetes, multiple sclerosis and systemic lupus (Hasan et al., 2013; Kurimoto et al., 2013; Basha et al., 2013). IL-17A induces the production of chemokines and cytokines that in turn recruit neutrophils and initiate an inflammatory reaction *via* adaptor proteins (Hus et al., 2013). Several studies reported that IL-17 production increased brain edema and neurologic deficits in ICH mice and functioned as a proinflammatory factor (Zhong et al., 2016; Liu et al., 2016; Rolland et al., 2013).

The endoplasmic reticulum (ER) is an intracellular organelle that contributes to membrane biosynthesis and the maintenance of intracellular organizational homeostasis (Baird et al., 2017). Much evidence demonstrates that ER stress plays an important role in many diseases, including cancer and other inflammatory and metabolic diseases (Niederreiter et al., 2013; Muralidharan and Mandrekar, 2013; Zheng et al., 2013). However, the potential of IL-17A to regulate ER stress and the specific mechanism of this regulation in macrophages following ICH is still unknown. Therefore, in the current study, we investigate whether IL-17A could induce ER stress in macrophages and promote inflammation following ICH.

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2. Materials and methods

2.1. Animals

Male C57BL/6 mice (8–10 weeks, 20–24 g) were bought from the Animal Institute of the Chongqing Medical University (Chongqing, China). Animals were feed in separated cages with free access to purified water and clean food in a specific pathogen-free cage at the Chongqing Medical University.

2.2. Preparation of BMDMs

The method was mentioned according to previous study (Song et al., 2018). BMDMs (bone marrow derived macrophages) were isolated from the marrow of the femurs and tibias of C57BL/6 mice. The legs of the animals were sprayed with 70% EtOH, and the skin and muscle tissue were removed from the bones. The bones were sprayed with 70% EtOH, transferred to a sterile-flow hood and cut at both ends. The marrow was flushed out into a sterile falcon tube in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal bovine serum (FBS; 50 ml; 10%) and penicillin-streptomycin (5 ml; 1%; Gibco). The cell suspension was triturated using a sterile Pasteur pipette, filtered through a nylon mesh filter into a sterile tube and centrifuged (400 × *g*, 5 min). The supernatant was removed, and the pellet was resuspended in red blood cell lysis buffer (Sigma-Aldrich, Gillingham). The suspension was centrifuged (400 × *g*, 5 min), the supernatant was discarded and the cells were washed using DMEM and centrifuged once more (400 × *g*, 5 min). The pellet was resuspended in 20 ml of DMEM supplemented with L929-conditioned media (20%). Cells were seeded in sterile cell culture flasks (T175 cm^2 flasks). On day 2, non-adherent cells were removed from the flask, the media was replaced and the remaining adherent cells were maintained in culture for a further 6 days.

2.3. Primary cell cultures

The method was mentioned according to previous study (Zhang et al., 2015b). Cortical neuronal cultures were got from whole cerebral cortices of C57BL/6 mouse embryos (E16). Brain tissue was diced into small fragments and incubated in 0.25% trypsin and 200 μg/ml DNase I in PBS. The suspension was then filtered and centrifuged. The pellet was resuspended in PBS and recentrifuged, and after a final wash in feeding medium, the cells were plated into T75 flasks coated with polyornithine (10 μg/ml). The plating density was 80 million cells in 25 ml of medium. To obtain neuron-enriched cultures, cells in the flasks were treated with at least three cycles of 25 μM cytosine arabinoside (2 d on, 3 d off) to kill dividing astrocytes. The feeding medium during this time was minimum essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, and 10% dextrose. All culture medium supplies were from Invitrogen (Burlington, Ontario, Canada). The resulting cells after three cycles of cytosine arabinoside treatment were neurons in excess of 90% purity, with astrocytes, microglia, and precursor cells forming the rest. The neuron-enriched cultures, henceforth called HFNs, were re-trypsinized and plated at 100,000 cells/well in 16-well Lab-tek slides (Nunc, Naperville, IL) in the above medium. Purity of neuronal cultures was > 95% as identified by staining with neuronal and glia markers. 5 days after plating, neurons had grown a dense network of extensions.

2.4. Construction of recombinant adenovirus encoding CHOP RNAi or ATF4 RNAi

A recombinant adenovirus vector encoding CHOP RNAi or ATF4 RNAi was constructed using an Adeno-XTM Expression System (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, CHOP RNAi or ATF4 RNAi cDNA was cloned into a pDC315 shuttle vector and sequenced. The target sequence was selected: shRNA CHOP: 5′-GCUAGCUGAAGAGAAUGAATT-3′. shRNA ATF4: 5′-UUCAUU C UCUUCAGCUAGCTT-3′; shRNA control: 5′-AGCT TTTCCAAAAAG CTGTGATC-3′. The desired replication-deficient adenovirus containing full-length CHOP RNAi or ATF4 RNAi cDNA was generated by homologous recombination by co-transduction of the plasmids pDC315 and pBHG1oXE1, 3Cre in HEK 293 cells using DOTAP liposome reagent (Roche, Mannheim, Germany). After several rounds of plaque purification, the adenovirus containing the CHOP RNAi or ATF4 RNAi gene was amplified and purified from cell lysates by banding twice in CsCl density gradients. Viral products were desalted and stored

2.5. Adenovirus-mediated CHOP RNAi or ATF4 RNAi transfer

The transduction of macrophages with Ad-CHOP RNAi or Ad-ATF4 RNAi was conducted in 6-well plates with 1×10^6 macrophages cells/well in 3 mL of RPMI-1640 medium containing 10% FBS. Virus was added to the wells at an MOI of 200, and the macrophages were harvested after 24 h of incubation.

2.6. Cell treatment

Macrophages (1×10^5) were stimulated with PBS, IL-17 A (25 ng/ml, BioLegend), or IL-17 A (25 ng/ml) + anti-IL-17 A (25 ng/ml, BioLegend) for 12 h according to previous study (Li et al., 2013). After that, culture medium was replaced with fresh DMEM and macrophages were cultured for 12 h. Then, the supernatants were removed and further analyzed for inflammatory factor production with ELISA. Neurons were cultured in a 96-well plate with 1×10^4 cells per well. For the toxicity experiments, neurons were serum starved for 4 h and then treated with a mixture of macrophage-conditioned medium for 48 h.

2.7. Transmission electron microscopy

Macrophages were collected and fixed in a solution containing 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate for 2 h, postfixed with 1% OsO_4 for 1 h, washed and stained in 3% aqueous uranyl acetate for 1 h. The samples were then washed again, dehydrated with a graded alcohol series, and embedded in Epon-Araldite resin. Ultrathin sections were cut on a Reichert ultramicrotome, counterstained with 0.3% lead citrate and examined on a Philips EM420 electron microscope.

2.8. qRT-PCR

The differential gene expression was further evaluated by SYBR green-based qRT-PCR in mouse inner cDNA samples. qRT-PCR primers and probes of each gene were designed using ABI Primer Express Software v2.0 (Applied Biosystems, Foster City, CA, USA). qRT-PCR analysis of the cDNA samples was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems) following the standard procedure. The sequences of primers used were shown as following: ATF4, 5′- CCCCTTCACCTTCTTACAACC -3′(forward), 5′- GGG C TCAT ACAGATGCCACTA -3′ (reverse); CHOP, 5′- AATCAGAG CT GGAACCT GAGGA -3′ (forward), 5′- TGCT TT CA GGTGTGGTGA TGTATG -3′ (reverse); β-actin, 5′- CTACAATGAGCTGCGTGTGG -3′ (forward); 5′-AAGGAAGGCTGGAAGAGTGC-3′ (reverse). Data were analyzed using the ABI Prism 7500 SDS software (Applied Biosystems). Quantitative expression data of each specific target were obtained for each cDNA sample. Expression of GADPH was used as endogenous normalization control. The comparative threshold of cycle (CT) method was used to determine any difference. All values were $\log^2$ transformed before statistical analysis.