Interleukin 13 Affects the Recovery Processes in a Mouse Model of Hemorrhagic Stroke with Bilateral Tibial Fracture

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Abstract

As one form of stroke, intracerebral hemorrhage (ICH) is a fatal cerebrovascular disease, which has high morbidity and mortality, and lacks of effective medical treatment. An increased infiltration of inflammatory cytokines coupled with pyroptotic cell death is involved in the pathophysiological process of ICH. However, little is known about whether concomitant fracture patients have the same progression of inflammation and pyroptosis. Hence, we respectively established mouse ICH model and ICH with bilateral tibial fracture model (MI) to explore the potential cross-talk between the above two injuries. We found that MI obviously reversed the expressions of pyroptosis-associated proteins, which were remarkably up-regulated at the acute phase after ICH. Similar results were observed in neuronal expressions via double immunostaining. Furthermore, brain edema was also significantly alleviated in mice who suffered MI, when compared with ICH alone. To better clarify the potent mechanisms mediated this cross-talk, recombinant mouse interleukin-13 (IL-13) was used to investigate its effect on pyroptosis in the mouse MI model, in which lower level of IL-13 was observed. Remarkably, IL-13 administration re-awakened cell death, which was mirrored by the re-upregulation of pyroptosis-associated proteins and PI positive cell counts. The results of hemorrhage volume and behavioral tests further confirmed its critical role in regulating the neurological functions. Besides, IL-13-treated MI group showed poor outcomes of fracture healing. To sum up, our research indicates that controlling the IL-13 content in acute phase would be a promising target in influencing the outcomes of brain injury and fracture, and meanwhile, provides new evidence in repairing compound injuries in clinics.

Introduction

Intracerebral hemorrhage (ICH) is defined as spontaneous, nontraumatic bleeding into the parenchyma of the brain [1]. It is a serious disease which lead to severe disability and even death that substantially impacting public health. Previous researches have shown a mortality rate of up to 40% within a month of ICH [2, 3], which accounted for 10–15% of strokes worldwide each year [4]. The increased intracranial pressure caused by hematoma which was formed quickly along with blood into the brain parenchyma plays an important role in the primary injury of ICH, and consequently disruption of physiological structure via compressing surrounding brain tissue. Thereafter, the secondary injuries induced by the toxic products of blood, excitotoxicity, oxidative stress, and inflammation occur. These insults trigger irreversibly damage to the neurovascular unit, and finally to death [5].

Apart from affecting the central nervous system, many studies have reported that stroke is a risk factor of bone fracture[6, 7]. It has been proved that the risk of fracture is increased by 2–4 folds in stroke patients compared to healthy people [8, 9]. At the same time, up to 75% of all patients with stroke fall within 6 months and approximately 1%-15% experience a fall-related fracture [10, 9]. Based on the available data, nearly 167,000-250,000 patients worldwide will experience a fall-fracture within the first day of the stroke [11]. Therefore, it is necessary to explore the relationship between fracture and stroke. Recent researches indicated that bone fracture can aggravate neural impairment caused by ischemic stroke [11, 12].

However, there remains obscured about the pathophysiological mechanisms of hemorrhage stroke if accompanied with bone fracture.

As a manner of programmed cell death (PCD), pyroptosis has been involved in the pathophysiological process of brain injury [13–15], as well as bone destruction [16, 17]. The inflammasomes act as initiators are critical for the activation of pyroptosis. In the classical pathway of pyroptosis, the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3) inflammasomes, consisted of NLRP3, apoptosis-associated speck-like protein (ASC) and caspase 1, are a platform that activates caspase 1. Activated caspase 1 then cleaves the gasdermin D (GSDMD) into N-terminal gasdermin domain and C-terminal gasdermin domain. Upon activation, N-terminal domain forms a pore in the plasma membrane, causes cell swelling and leads to cell rupture accompanied by the release of cellular contents [18, 19]. Although the important action of pyroptotic cell death in different pathophysiological processes, there is still no research about cerebral hemorrhage with fracture.

In this study, we used type IV collagenase to establish the mouse acute intracerebral hemorrhage model. Then bilateral tibial fracture with an intramedullary fixation was performed immediately after ICH. We aim to elucidate the effect of a concomitant bilateral tibial fracture on ICH-related brain damage and the potential mechanisms, which also influence the fracture healing process. Furthermore, our results are expected to provide a precise therapeutic target on ICH and fracture.

Materials And Methods

Animals

Adult male C57BL/6J mice (6–8 weeks, body weight 25–30 g) were obtained from the SLAC company (Shanghai, China). Mice are kept in cages (four mice per cage), fed with standard laboratory fodder and water and maintained under a 12–12 h light-dark cycle with controlled room temperature (25°C) and humidity (50%). All animal experiments were approved by the Institutional Animal Use and Care Committee at Soochow University and were implemented under the guidelines of Animals Use and Care of the National Institutes of Health (NIH) and the Animal Research: Reporting in Vivo Experiments (ARRIVE).

Intracerebral Hemorrhage and Bilateral Tibial Fracture Model

The mouse ICH model was established as our previously reported [13,20]. In brief, mice were fixed in stereotaxic frame (David Kopf Instruments, Tujunga, California) after anesthetized with 4% chloral hydrate by intraperitoneal injection. Then carried out craniotomy after exposed the skull. Subsequently, 0.5 μ L (0.1 μ L/min) saline containing 0.05 units of bacterial collagenase (type IV, Sigma, USA) was injected into the left striatum at coordinates 1.0 mm anterior and 2.0 mm lateral to bregma and 3.5 mm

below the cortical surface, using a microinjection system (LongerPump, China). The needle was kept for another 5 min after injection to prevent leakage. Needle was inserted into the striatum after craniotomy but the collagenase was not injected in sham group (Sham).

Tibial fracture (TF) surgery was performed according to the literature described previously [11]. Briefly, the bilateral tibia with an intramedullary fixation was fractured after mice anesthetized. The model of ICH accompanied by fracture (MI) was performed fracture surgery immediately after ICH. After the surgery, all mice were placed next to a heater to maintain their normal body temperature and put back to cages when they recovered from anesthesia. All of the above operations were performed under aseptic conditions.

Experimental Design and Reagent

In experiment 1, to explore whether a concomitant bilateral tibial fracture affect the progressions of ICH, the detailed experimental groups were divided as follows: Sham, ICH, TF and MI.

In experiment 2, to determine the effect of IL-13 in the pathophysiological process of MI model, the detailed experimental groups were divided as follows: MI with PBS and MI with IL-13. The detailed experiment design was showed in Fig. 1.

Recombinant mouse IL-13 were obtained from Novoprotein company (Shanghai, China). For MI + IL-13 group, IL-13 (0.2 μ g / 2 μ L per mouse) was injected into the contralateral lateral ventricle (1.0 mm rear and 1.0 mm lateral to bregma and 2.5 mm below the cortical surface) within 5 min after ICH treatment. The needle was kept for another 5 min after injection to prevent leakage.

Western Blotting

Mice were deeply euthanized after surgery and ipsilateral striatum was sampled (n = 5 per group). Proteins were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, China) and allowed to lyse for 30 min on ice. Then the brain tissues were lysed by ultrasonic homogenizer (Scientz Biotechnology, China). After that, the homogenate was centrifuged at 12000 revolutions per min for 25 min at 4°C. The protein concentration in the supernatant was measured using NanoDrop 2000 spectrophotometers (Thermo Fisher Scientific, USA). 60 μ g proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto hybond-polyvinylidene difluoride (PVDF) membranes. The PVDFs were blocked with 5% bovine serum albumin (BSA), then the blots were probed with respectively primary antibodies overnight at 4 °C. After washing with tris buffered saline-tween (TBST), the PVDFs were incubated with corresponding horseradish peroxidase (HRP) –conjugated secondary antibody for 2 h at room temperature. ECL chemiluminescence system (Clinx Science Instruments, China) were used for immunodetection. Image J (NIH, USA) software was used for the analysis of band density.

Immunofluorescence Staining

Mice were perfused under deep anesthesia with ice-cold PBS whereafter perfusion with 4 % formalin at 24 h after surgeries (n = 5 per group). The brain was removed and soaked in formalin overnight at 4°C and dehydrated with 10-30% sucrose in the next 3 days. Frozen brains which embedded in optimal cutting temperature (OCT, Sakura, Japan) were cut into 10 µm per slice from the front of bleeding area to the end continuously using freezing microtome (Thermo Fisher Scientific, USA). Slices were blocked by 5% BSA for 2 h. Then the slices were incubated with corresponding primary antibodies at 4°C overnight. After phosphate buffer saline-tween (PBST) washed, the slices were incubated with double fluorochrome conjugated antibodies at room temperature for 2 h. Thereafter, the cell nuclei were stained with DAPI (Beyotime Biotechnology, China) for 30 sec. Images were taken with a fluorescence microscope (Nikon, Japan), and the images were processed with Image J software.

Immunohistochemistry Staining

The preliminary treatment of the slices as described above (n = 5 per group). After incubated with respectively primary antibodies at 4°C overnight, the slices were hatched with HRP conjugated secondary antibody for 1 h at room temperature. Using dimethylbenzidine (DAB) to coloration, then the slices were washed and nuclei were stained with hematoxylin. After water rinsing and dehydrating with concentration gradient ethanol (75-100%), we used xylene to transparency, then cover glasses and neutral resin were used to seal the slices. Images were taken with an optical microscope (Nikon, Japan)

Propidium Iodide Staining

As previously described [20], loss of plasma membrane integrity was evaluated by intraperitoneal injection of propidium iodide (PI, 5 mg/mL, Beyotime Biotechnology, China) for 200 µL per mouse 1 h before sacrificed (n = 5 per group). The brains were removed quickly and frozen in liquid nitrogen vapor, then cut into 10 µm frozen slices. Sections were incubated with DAPI for 30 sec to stained cell nuclei. Images were photographed and analyzed by fluorescence microscope. PI positive cells in mice were counted in 200 × fields in the peripheral area of hematoma randomly.

Bleeding Volume Measurement

The frozen sections were prepared as previously described (n = 5 per group). Sections were incubated in prussian blue dye (Sangon Biotech, Shanghai) for 1 h at room temperature after dehydrated with 95% ethanol for 10 min and sections were subjected to washing by PBST. Subsequently, 0.3% hydrogen peroxide-methanol solution were used for 15 min then washing sections. We used DAB to enhance the coloration until the hemorrhagic focus turned brown, then wash the dye away with running water. The

process of dehydration, transparency and seal were described above. Finally, the bleeding volume was analyzed by Image J software.

Brain Water Content Measurement

Mice were sacrificed 24 h after surgery ($n = 5$ per group). The brains were removed and the cerebellum was isolated first, followed by the separation of the ipsilateral (lesion side) and contralateral hemispheres along the sagittal suture. These three parts of tissue samples were immediately weighed on an electronic analytical balance (Mettler Toledo, Switzerland) to the nearest 0.1 mg and the wet weight were recorded. Then these samples were dried in a drying oven at 160°C for 24 h and weighed again to obtain the dry weight. The percentage of brain water content was: $(\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$.

Behavioral Tests

Corner test was performed to detect sensorimotor and postural asymmetries after injury ($n = 7$ per group). Mice were allowed to crawl into a 30° corner. When mice entered the corner, they stood up against the wall and turned to face the open end. For normal mice, the number of right and left turns would be equal. The lesion side of ICH mice lost the ability to sense stimulation, so they used the contralateral limb more, causing them to move toward the injured side (on the left in this test) more frequently. Test was repeated 10 times for each mouse. The percentage of turning direction was: $(\text{left turns} / \text{total turns}) \times 100\%$.

For the wire grip test, it was aimed to evaluate the motor function deficits after injury ($n = 7$ per group). A metal wire (45 cm long) was suspended 45 cm above a soft platform. The mice's tails were gently grasped, then slowly released after they catch the metal wire with their forelimbs. The experiment was repeated three times for each mouse, with a 10-minute rest interval. An average value was calculated for each mouse on every day of testing. The scoring criterion was based on previously described [21]. Mice were trained for five consecutive days prior to surgery for both corner test and wire grip test.

The Morris-water maze (MWM) test was performed as previously described [22]. A circular pool with a diameter of 120 cm was filled with water at a depth of 30 cm and the temperature of the water was maintained at about 25°C. Then a circular transparent platform about 8 cm in diameter was placed 1 cm below the surface of the water, about 30 cm from the wall of pool. The pool is divided into four quadrants, with four highly visible cues located on the walls. Mice were trained for five consecutive days ($n = 7$ per group). Before each training, the mice were subjected to recognize the visible cues on the wall of the pool, then they were gently put in the water and the timing began immediately. Mice were given 90 sec to find the platform. Once they found it, they were allowed to stay for 3 sec, then recorded how long it took. If they could not find the platform within 90 sec, the time was recorded for 90 sec and mice were placed on the platform for 15 sec. Each mouse was trained twice a day with an interval of more than 30 min. At the end of the training, they were placed next to a heater to restore their body temperature. When motor function was restored after surgery, we began to test the spatial memory ability of mice. The

escaped latency (the time it took the mice to get on the platform) and crossing number (the number of crossed the original position of the platform after removing it) were used to assess spatial memory ability.

Micro-CT Analysis

As previously described [23], tibia collected from different groups were subjected to Micro-CT (micro-computed tomography) scanning (SkyScan 1176, Aartselaar, Belgium). The parameters of X-Ray were set at a current of 500 μ A with the voltage of 50 kV and the scanning per layer was 9 μ m. Cross sections of fracture site were selected for quantification of bone mineral density (BMD, mg/cc) and bone volume as a proportion of total tissue volume (BV/TV, %).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. Students' t-test was used for the comparison between two groups, and one-way ANOVA followed by Tukey's multiple comparisons test was used in which there were more than two groups. Behavioral tests (Corner test and wire-grip test) were analyzed by two-way ANOVA (time and treatment) followed by Tukey's multiple comparisons test. $p < 0.05$ was considered statistically significant. All data were processed with GraphPad Prism 7 (GraphPad Software Inc., San Diego, USA).

Results

Concomitant Tibial Fracture Inhibits Neuronal Pyroptosis and Brain Edema at the Acute Phase

Previous researches have reported that pyroptotic cell death was involved in the pathophysiological process of ICH [24, 13]. In order to explore the effect of tibial fracture on pyroptosis induced by ICH, mice in different models were sacrificed at 24 h post-injury. Western blot results showed that the level of pyroptosis associated proteins, including ASC ($p < 0.0001$), caspase 1 ($p < 0.01$), GSDMD ($p < 0.01$) and IL-1 β ($p < 0.0001$) were remarkably elevated after ICH. While, compared with ICH group, decreased expression levels of these proteins were observed in the MI group, and no changes were examined in the TF group relative to Sham group (Fig. 2a, b). Similar results were found in immunofluorescence staining. We found that the ratio of NeuN (neuron marker) which was double-labelled with ASC or caspase 1 in the MI group was significantly reduced respectively, comparing to the ICH group (Fig. 2c, d). There were also no obvious changes in the TF group (Fig. 2a-d).

Brain edema was another typical pathological change in the acute phase post ICH which increased the risk of death. Then we measured the brain water content in different groups. There were no differences at the contralateral hemisphere and cerebellum among the four groups (Fig. 2f, g). In the ipsilateral hemisphere, ICH led to a significantly higher brain water content than Sham group ($p < 0.0001$), whereas

obviously reduced brain edema was observed in the MI group ($p < 0.001$) relative to ICH group (Fig. 2e). Isolated bone fracture did not affect the brain water content (Fig. 2e-g).

Concomitant Tibial Fracture Reduces the IL-13 Expression in the Brain at the Acute Phase

Our previous studies have proved that the increased expression of IL-13 in ICH animal models [13]. However, its changes or effect in regulating pyroptosis in MI model has not been illustrated so far. The results showed that ICH induced the increased expression level of IL-13 ($p < 0.01$), while MI remarkably reversed the upregulation of IL-13 in the brain tissue ($p < 0.05$, Fig. 3a). Meanwhile, the immunofluorescence staining in the peripheral area of hemorrhage showed that the percentages of IL-13 positive in neurons were increased in ICH group compared with Sham group ($p < 0.001$), and this tendency was reversed in the MI group ($p < 0.001$, Fig. 3b). Based on our obtained findings, we hypothesized that IL-13 would be a novel target in regulating pyroptosis in the MI model, and link with the recovery process of ICH and bone fracture.

IL-13 Administration Re-activates the Process of Pyroptosis and Increases the Cell Death in the Brain

In order to verify our hypothesis, recombinant IL-13 was injected through contralateral intracerebroventricular and then the process of pyroptosis was assessed in the brain tissues post MI injury. In the presence of IL-13 treatment, the expression levels of caspase 1, IL-1 β and ASC were rose again via immunoblotting and immunostaining, respectively (Fig. 4a, b). Meanwhile, a range of pyroptosis proteins expressed by neurons were further examined by double immunostaining (Fig. 5a-e). Similar results of these proteins were obtained in the presence of IL-13 treatment: namely the expressions of these proteins in neuron elevated significantly (NLRP3, ASC, caspase 1, GSDMD and IL-1 β).

Other manner of PCD was also involved in the pathophysiological process of ICH. Therefore, using PI staining, which mirrored the plasma membrane permeability and cell insult, we then detected the fold changes of cell death. As shown in the Fig. 5f, IL-13 treatment increased the ratio of PI positive cells, compared with the PBS-treated MI group. All these changes confirmed our assumption that concomitant bone fracture could suppress neuronal pyroptosis via decreasing the expression of IL-13.

IL-13 Administration Exacerbates Hemorrhage Volume and Delays the Recovery Process of Neurological Functions and Fracture Healing

Along with the administration of IL-13, using prussian blue reaction method, the data demonstrated that injection of recombination IL-13 could aggravate the volume of bleeding ($p < 0.001$, Fig. 6a), it indicated that bone fracture after ICH had neuroprotective effect. In order to evaluate whether IL-13 in bone fracture after ICH exacerbate motor dysfunction, we performed corner test and wire grip test. In corner test, MI + IL-13 group made more left turn than MI + PBS group on days 1–3 post ICH ($p < 0.01$, $p < 0.05$ and $p < 0.05$, Fig. 6b), which means IL-13 aggravated the damage in this pathological process. In the wire-grip test, MI + IL-13 group got less scores than MI + PBS group on days 1–3 post ICH ($p < 0.01$, $p < 0.05$ and $p < 0.05$, Fig. 6c). When motor function of mice recovered at day 8 post-injury, we tested the spatial memory ability. MWM test results showed that MI + PBS group took less time to find platform than MI + IL-13 group ($p < 0.01$, Fig. 6d). Then the platform was removed to record the crossing number. We observed that MI + PBS group traversed the original position of the platform frequently, which could be diminished by IL-13 ($p < 0.01$, Fig. 6e). These above behavioral results demonstrated that IL-13 administration aggravated neurological defects in the MI model. Subsequently, using micro-CT we analyzed the index of fracture healing, which was mirrored by the BMD and BV/TV. As shown in the Fig. 6f–h, the IL-13-treated MI group displayed lower BMD and BV/TV. These results both confirmed the delayed healing process of tibial fracture.

Discussion

In this study, we established a compound injury model of hemorrhagic stroke combined with tibial fracture, and focused on the effect and mechanism of interleukin-13 on the prognosis of cerebral hemorrhage and fracture healing, so as to provide basic research data for the clinical treatment of this type of compound injury. The major findings were as follows: (1) Concomitant tibial fracture alleviates ICH-induced neuronal pyroptosis and reduces brain edema at the acute phase; (2) The up-regulation of IL-13 at the acute phase in the brain post-ICH is diminished when concomitant with bilateral tibial fracture, so were the amounts of IL-13 positive neurons; (3) IL-13 administration reawakens the process of neuronal pyroptosis and promotes cell death in the MI model; (4) IL-13 administration aggravates hemorrhage volume, and deteriorates the neurological deficits and fracture healing in the MI model.

As one type of stroke and common public health problem, ICH leads to high disability and mortality in adults[4]. Also, stroke is an important risk factor for bone fracture [25, 9, 8]. The primary injury of hematoma and followed intracranial high pressure are the main factors which threatened the life of patients with cerebral hemorrhage. The secondary brain injury caused by breakdown products of hematoma, cellular debris and accumulation of cellular metabolite could also not be ignored, due to the fact that they are potential risks for neuroinflammation. Inflammatory responses crossed throughout the ICH process, from endothelial cells were activated immediately after ICH to subacute and chronic phases (days to months) after injury [26]. During the acute phase of ICH, neurotoxicity hematoma provoked the damage associated molecular patterns (DAMPs) to initiate neuroinflammation. Then the pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) were activated to promote the formation of large multiprotein complexes called inflammasomes [27, 26]. As a recently studied form of PCD, pyroptosis was participated in the pathophysiological changes of ICH and played a critical role. NLRP3

inflammasome, the most widely researched inflammasome, mediated pyroptosis via forming holes in the cell membrane by GSDMD N-terminal domain, resulted in cell membrane rupture and release of pro-inflammatory cytokines including IL-1 β and IL-18, thus triggering an inflammatory response. Animal experiments have showed that the neurological damage can be alleviated by inhibiting the occurrence of neuronal pyroptosis [28, 29]. As described previously [25, 9, 8], ICH is accompanied with fracture sometimes, but the relationship between tibia fracture and pyroptosis, and whether concomitant tibia fracture affects the pathophysiological process of ICH is remained unclear. Therefore, we investigated their crosstalk in mice acute ICH model. Similar with others findings [30, 31], the proteins of pyroptosis were increased after ICH, while concomitant tibia fracture reversed it. Then we measured the brain water content, an important indicator of evaluating brain cell death and neuronal deficits [32, 33]. We found that tibia fracture could alleviate the degree of brain edema induced by ICH. This phenomenon aroused our attention because other researchers had proven that tibia fracture could exacerbate brain insults caused by ischemic stroke in mice [11, 12, 34]. We speculated that the inconsistent results may be due to the different models, and even, the different type of stroke has different mechanisms of injury.

Next, we explored the potential mechanisms that how tibia fracture affects the pathophysiological processes of ICH. Based on our previous study [13], in which we have proved that through neutralizing the IL-13, the neuronal pyroptosis post-ICH was repressed and followed with the neuroprotective effect. We decided to further investigate the function of IL-13 again in the MI model. Numerous studies have proved that IL-13 is an anti-inflammatory cytokine which produced by Th2 cells, and triggers a shift from M1 phenotype to M2 phenotype state and attenuates the production of inflammatory mediators [35–37]. Additionally, IL-13 inhibits brain inflammation by down-regulating the expression levels of iNOS and TNF- α to protect neurons [38]. On the other hand, IL-13 could also play a pro-inflammatory role such as producing reactive oxygen species (ROS) and pro-inflammatory cytokines [39, 40], it may contribute to neuroinflammation in the course of central nervous system diseases. Besides, Park *et al.* suggested that blockade of IL-13 reduced pro-inflammatory cytokines expression, which facilitated neuronal survival in the hippocampus *in vivo* [40]. There still remained controversial about the role of IL-13. Together with the above studies including ours¹³ and the results obtained in this study, we conclude that the content of IL-13 in the brain is associated with the ameliorated neurological functions when concomitant with fracture. To further verify this hypothesis, recombinant IL-13 was used in the MI model. Thereafter, we found that pyroptosis was re-activated, along with the ascend level expressed by neurons. In addition, the amount of cell death mirrored by the PI positive cells were also increased. Subsequently, the hemorrhage volume was further enlarged. Finally, a series of behavioral tests also confirmed that IL-13 was closely related to cell death and neurological deficits after ICH. All of these evidences manifested that concomitant tibial fracture could alleviate the neurological damage caused by ICH and the central role of IL-13 in regulating neuronal pyroptosis.

Research about fracture with brain injury (such as traumatic brain injury) demonstrated that there are some growth cytokines were associated with accelerated fracture healing process in patients [41], and a significant increase in fracture healing rate in patients who had sustained TBI with fractures of the femur

and the tibia had also been reported [42]. However, the process of fracture healing when concomitant with hemorrhage stroke has not been well documented, even the effects of IL-13 in this compound injuries. In the pathological process of bone fracture, the death of osteoblasts is accompanied by the activation of osteoclasts. Sufficient osteoclasts are necessary for the establishment of fracture healing micro-environment, whenever at the acute phase to clear the debris or the chronic phase to remodel the callus. Under physiological conditions, pluripotent stem cells (PPSC) in the bone marrow eventually differentiates into osteoclasts during the stimulation of receptor activator of nuclear factor- κ B ligand (RANKL) in the bone. Alternatively, they can also differentiate into macrophage through the effects of IL-13 [43]. And we provided hypothesis that this change might alter the ratio of PPSC differentiating into osteoclasts and finally influence the outcomes of fracture healing [44]. However, in this study there were no obvious differences of IL-13 content in the plasma between the two groups whenever at 1 day or 21 day (Supplementary Fig. 1). We attribute these results to the fact that the dosage was not enough to cause the change of systematic IL-13. In addition, *in vitro* researches are needed to demonstrate the effects of IL-13 on the differentiation of PPSC isolated from the MI-treated mice in the future.

Collectively, our experiments clarified the mechanisms which concomitant fracture alleviated ICH-related brain damage, and provided a new concept of precise therapy for compound injuries in clinic.

Our findings may provide precise treatment strategy for certain type of stroke and bone fracture.

Declarations

Acknowledgement

None.

Author Contributions

Y.Y., C.G., and G.C. contributed equally to this paper. L.T., X.C. and J.L. comprehended the study, provided critical suggestions, contributed to manuscript preparation, oversaw the research program, and wrote the main manuscript. Y.Y., C.G., and G.C. performed the Western blot and immunostaining experiments and analyzed the data. Y.Y., C.G., G.C., X.C., H.X., Z.C., C.L., M.Z. and T.W. performed the brain edema test, PI staining, bleeding volume test, ELISA test, behavioral experiment and analyzed the data. Y.W. performed tibial fracture surgery, micro-CT scan and analyzed the data. All the authors listed in the manuscript have agreed upon and reviewed the manuscript and provided feedback.

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Declaration of Competing Interest

The authors declare no competing financial interest.

Availability of Data and Material

All data included in this study are available upon request by contact with the corresponding author.

Compliance with Ethical Standards

All animal procedures were approved by the Institutional Animal Use and Care Committee at Soochow University and carried out in accordance with the guidelines of Animal Use and Care of the National Institutes of Health (NIH) and the Animal Research: Reporting In Vivo Experiments (ARRIVE).

Consent to Participate

Informed consent was obtained from all individual participants included in the study.

Consent for Publication

All the authors consent for the publication of this manuscript.

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Figures

Figure 1

Experimental designs for this study. The whole experiments were divided into two parts. In experiment 1, we mainly focused on the effect of concomitant tibial fracture on the pathological changes of ICH-related brain injury in the acute phase. Mice were arbitrarily divided into four groups: Sham, ICH, TF and MI. In experiment 2, we mainly focused on the effects of IL-13 on the recovery of neurological functions and fracture healing. Mice were arbitrarily divided into two groups: PBS-treated MI group (MI + PBS) and recombinant mouse IL-13-treated MI group (MI + IL-13). ICH: intracranial hemorrhage; TF: tibial fracture; MI: ICH with bilateral tibial fracture; dpi: day post injury.

Figure 2

Concomitant tibial fracture inhibits pyroptotic cells death and alleviates brain edema in the acute phase. a Western blot bands showed the expression level of pyroptosis associated proteins ASC, caspase 1, GSDMD, IL-1 β in different groups (n = 5 mice per group). b Statistical differences are shown in the downward bar graphs. c, d Fluorescence microscopy indicated the co-location of NeuN with ASC and caspase 1 respectively (n= 5 mice per group). Right bar graphs indicate the statistical differences. Magnification is 20 $\times$. Scale bar is 50 μ m. Image J software was used to quantitative analysis. e-g Histogram showing the content of brain water in different brain regions (n= 5 mice per group). One-way ANOVA followed by post-hoc analysis. **p < 0.01, ***p < 0.001, ****p < 0.0001 represent the differences between the relevant two groups.

Figure 3

Concomitant tibial fracture reduces the expression of IL-13 in the brain, as well as the amounts of IL-13 expressed by neurons. a Western blot bands of IL-13 in different groups with the right bar graphs (n = 5 mice per group). b Fluorescence microscopy indicated the co-expression of IL-13 with NeuN in different groups (n = 5 mice per group). Bar graph indicated the statistical significance. Magnification is 20 ×. Scale bar is 50 μm. Image J software was used to quantitative analysis. One-way ANOVA followed by post-hoc analysis. * p < 0.05, **p < 0.01, ***p < 0.001 represent the differences between the relevant two groups.

Figure 4

IL-13 administration re-activates pyroptotic cells death of the brain in the MI model. a Western blot bands of caspase 1 and IL-1 β (n = 5 mice per group). b Immunohistochemical staining results showed the ASC, caspase 1 and IL-1 β positive cells in the periphery of the hematoma (n = 5 mice per group). Bar graph indicated the statistical significance. Magnification is 20 $\times$. Scale bar is 50 μ m. Image J software was used to quantitative analysis. Data was analyzed by students' t-test. * p < 0.05, ***p < 0.001 **** p < 0.0001 represent the differences between the relevant two groups.

Figure 5

IL-13 administration increases the neuronal pyroptosis and PI positive cells of the brain in the MI model. a-e Fluorescence microscopy results show the percentages of neuron expressing pyroptosis markers NLRP3, ASC, caspase 1, GSDMD and IL-1 β in different groups (n = 5 mice per group). f Quantification of PI positive cells in the periphery of the hematoma (n = 5 mice per group). Bar graph indicated the statistical significance. Magnification is 20 $\times$. Scale bar is 50 μ m. Image J software was used to quantitative analysis. Data was analyzed by students' t-test. **** p < 0.0001 represent the differences between the relevant two groups.

Figure 6

IL-13 administration exacerbates hemorrhage volume and delays the recovery of neurological functions and fracture healing in the MI model. a Mice brain sections are stained by prussian blue staining to show the bleeding volume of different groups (n = 5 mice per group). Bar graph indicated the statistical significance. Interval of 500 μ m for each brain section. Image J software was used to quantitative analysis. b Scatter diagram showing the percentages of left turn after different treatment (n = 7 mice per group). c Scatter diagram indicating the score of the wire grip test in different groups (n = 7 mice per group). d, e Histogram showing the spatial memory ability of mice after different treatment, reflected by escaped latency and crossing number (n = 7 mice per group). f Right panel indicated general view of 3D reconstruction of tibia from each group. g, h Quantifications of bone mineral density (BMD) and bone volume as a proportion of total tissue volume (BV/TV). Data were analyzed by students' t-test or two-way ANOVA followed by post-hoc analysis. * p < 0.05, ** p < 0.01, *** p < 0.001 represent the differences between the relevant two groups.

Figure 7

The graphic abstract of this study. (1). When concomitant with bilateral tibial fracture, reduced pyroptotic neuronal death at the acute phase of hemorrhagic stroke was observed, so was the neuronal IL-13. (2). IL-13 administration increased pyroptotic neuronal death, exacerbated rupture of plasma membrane integrity and consequently the aggravated neurological deficits. (3). IL-13 administration delayed fracture healing which may be due to alter the differentiation of PPSC into osteoclasts.

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