Research report

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The pathological role of NLRs and AIM2 inflammasome-mediated pyroptosis in damaged blood-brain barrier after traumatic brain injury

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Abstract

Pyroptosis is a highly specific type of inflammatory programmed cell death that different from necrosis or apoptosis. It is initiated by cellular detection of acute damage via recognizing pathogen-associated molecular patterns (PAMPs) by NOD-like receptors (NLRs) or AIM2-like receptor (AIM2). NLRs and AIM2 could trigger the formation of a multi-protein complex, known as inflammasome. It also contains apoptotic speck-containing protein (ASC) and pro-Caspase-1, and could process the signals to induce a cascade of inflammatory response. Recently, growing evidence showed that inflammasome-mediated pyroptosis is involved in the pathogenesis of traumatic brain injury (TBI). However, less attention has been paid to their particular roles in regulating blood-brain barrier (BBB) damage, the central pathological change in secondary brain damage of TBI. Thus, we designed this research to explore the impact and mechanism of NLRs and AIM2 inflammasome-mediated pyroptosis in BBB after TBI. We employed the controlled cortical impact (CCI) mice model and manipulated the severity of pyroptosis in BBB using Caspase-1 inhibitor, Ac-YVAD-cmk. We found that TBI led to NLRs and AIM2 inflammasome-mediated pyroptosis in brain microvascular endothelial cells (BMVECs) from injured cerebral cortex. Ac-YVAD-cmk treatment inhibited pyroptosis in injured BMVECs by suppressing the expression of essential inflammasome subunit – Caspase-1 and pivotal downstream pro-inflammatory cytokines (IL-1β and IL-18), as well as hindering GSDMD cleavage and ASC oligomerization. In addition, inhibiting pyroptosis could alleviate TBI-induced BBB leakage, brain edema, loss of tight junction proteins, and the inflammatory response in injured BMVECs. These effects contributed to improving the neurological outcome of CCI mice. In conclusion, NLRs and AIM2 inflammasome-mediated pyroptosis could aggravate BBB damage after TBI. Targeting and controlling pyroptosis in injured BBB would be a promising therapeutic strategy for TBI in the future.

Keywords

Traumatic Brain Injury; Blood-brain Barrier; Pyroptosis; Inflammasome; NOD-like receptors; AIM2
1. Introduction

Traumatic brain injury (TBI) is the most common cause of injury induced death and long-term disability, especially in children and young adults (Rubiano et al., 2015). More than 50 million people worldwide are affected by a new TBI case annually, with a global economic cost of approximately $US 400 billion (Maas et al., 2017). With the acceleration of urbanization, the increase of traffic accident, and the frequent occurrence of local wars, the incidence of TBI increases prominently each year. It is estimated that TBI will become the third leading cause of disease burden by 2020 (Feigin et al., 2013).

The pathological development of TBI includes two general stages: primary brain damage and secondary brain damage. The primary damage is impact related, which triggers acute pathological changes, such as brain contusions, intracerebral hemorrhage and shearing injuries. A series of secondary processes, including oxidative stress, metabolic imbalance and impaired cerebral blood flow were then induced within hours to days (Maas et al., 2017). They progress, interact and initiate the secondary brain damage, an expansion of the primary damage in surrounding tissue with pericontusional brain swelling. BBB breakdown plays a central role in the development of secondary brain damage after TBI. It frequently follows head trauma, and lasts from days to weeks, or even years after the acute event (Nasser et al., 2016). Once BBB is disrupted, brain edema, local inflammation, cell death and neuronal hyperexcitability often occur, which will set off a cascade of pathological processes, including neurodegeneration, gliacytes dysfunction and neovascularization. Thus, BBB damage could not only result in acute complications, like intracranial hypertension, brain hernia, coma, seizures and death, but also contribute to long-term complications that lead to poor neurological prognosis, such as Alzheimer disease, epilepsy and cognitive/behavioral disabilities (Shlosberg et al., 2010; Levin and Smith, 2013). For clinical treatment, the primary damage is considered to be untreatable for short of time window, whereas the ‘rolling’ pathology of the following secondary
damage allows an opportunity for intervention. From this, alleviating BBB damage after TBI is an important strategy to attenuate secondary brain damage and improve the prognosis of TBI (Thal and Neuhaus, 2014).

Although BBB breakdown is considered to be potentially treatable, and it has aroused great interest for research on alleviating associated brain injury for years, no accepted therapeutic protocols are available for the prevention or treatment on BBB damage in the clinical setting of TBI till now. Corticosteroids are well-known agents with antiedematous properties acting on BBB. Since their introduction in 1960s, they have been widely used in controlling brain edema after TBI. High-dose corticosteroids pulse therapy was thought to be effective in a period, whereas a clinical trail conducted in more than 40 countries involving 10,008 cases (MRC CRSAH) suggested that the treatment have no beneficial effect on TBI patients (Edwards et al., 2005). Our recent research explained that corticosteroids could aggravate neuronal death in hypothalamus after TBI, thus suppress the stress response of HPA axis, and induce critical illness-related corticosteroid insufficiency (CIRCI) that results in poor prognosis (Chen et al., 2013; Chen et al., 2014). Therapeutic hypothermia therapy (also called target temperature management) is a cornerstone in TBI treatment. Since 1990s, scientists have found that mild reductions in brain temperature can reduce BBB disruption, and improve the histopathological and neurological outcomes for experimental TBI animals (Jiang et al., 1992). These results were confirmed by a number of single institutional clinical trails. However, larger randomized multicenter trails failed to prove the benefits of hypothermia therapy, suggesting that it is limited by patients’ selection and administration time (Dietrich and Bramlett, 2016). Molecular therapy, such as erythropoietin and progesterone treatment, was widely reported to be effective in alleviating BBB damage in animal models (Shlosberg et al., 2010). But phase III clinical trials failed to find their therapeutic effects on TBI patients (Robertson et al., 2014; Skolnick et al., 2014). In addition, hot-spot
stem cell therapy, including transplantation of mesenchymal stromal cells (Menge et al., 2012), multipotent adult progenitor cells (Walker et al., 2010), and endothelial progenitor cells (Huang et al., 2013) have been proved to restore BBB integrity and promote the functional recovery after TBI (NCT02028104). However, problems on ethics and biosafety have long been concerned, which largely limited its further development and clinical applications. Consequently, it is significant for exploring novel mechanism that regulating the development of BBB damage after TBI, in order to find new therapeutic targets and design specific treatment methods.

Pyroptosis is a highly specific type of inflammatory programmed cell death that different from necrosis or apoptosis. It is initiated by cellular detection of acute damage via recognizing extracellular and intracellular pathogen-associated molecular patterns (PAMPs) by NOD-like receptors (NLRs) or AIM2-like receptor (AIM2). NLRs and AIM2 could trigger the formation of a multi-protein complex, known as inflammasome that also contains apoptosis-associated speck-like protein (ASC) and pro-Caspase-1, and process the signals to induce a cascade of inflammatory response (Man et al., 2017). Pyroptosis leads to plasma-membrane rupture and the release of damage-associated molecular pattern (DAMPs) molecules into the extracellular space. The released DAMPs, including ATP, damaged DNA, ASC dimers/oligomers and cytokines, could recruit immune cells and further perpetuate inflammatory cascade (Zhang et al., 2018). These processes are in marked contrast to the packaging of cellular contents and non-inflammatory phagocytic uptake of membrane-bound apoptotic bodies that characterizes apoptosis (Baroja-Mazo et al., 2014; Franklin et al., 2014). In addition, the process of cell death led by pyroptosis also features the maturation of Caspase-1, and the release of pro-inflammatory IL-1β and IL-18, which is totally different from apoptosis that dependent on the activation of Caspase-3, Caspase-8 and Caspase-9.
Recently, growing evidence have indicated that inflammasome-mediated pyroptosis is involved in the pathological development of TBI. The activation of cytoplasmic inflammasome complex has been regarded as an essential step of neuroinflammation in secondary brain damage (Mortezaee et al., 2018). Among multiple inflammasome complexes, NLRs and AIM2, especially NLRP1 and NLRP3 appear to be the most relevant receptors that functioning in TBI pathogenesis. They could be detected in neurons, astrocytes and microglia in injured brain, which contribute to inducing inflammatory response and neuronal death, as well as aggravating neurological outcome (Lee et al., 2018; Liu et al., 2018). However, less attention has been paid to their particular roles in brain microvascular endothelial cells (BMVECs), which destruction or dysfunction were strongly associated with BBB damage, the central pathological change in secondary brain damage after TBI. Thus, we designed this research and focused on studying the impact and mechanism of NLRs and AIM2 inflammasome-mediated pyroptosis on BBB. The results could expand the previous understanding on the pathophysiology of secondary brain damage, thus open a new avenue of therapeutic strategies for TBI by targeting and controlling pyroptosis in injured BBB.

2. Results

2.1 TBI led to NLRs and AIM2 inflammasome-mediated pyroptosis in BMVECs from injured cerebral cortex

Brain microvascular endothelial cells (BMVECs), which are also regarded as brain capillary endothelial cells (CECs) are the major cellular component of microvascular endothelial barrier, which is a crucial element of the highly specialized BBB (Ruck et al., 2014). In order to study the pathological role of NLRs and AIM2 inflammasome-mediated pyroptosis in BBB damage after TBI, we first detected the expression of NLRP1, NLRP3, NLRC4 and AIM2 in BMVECs from injured cerebral cortex of CCI mice
using double immunofluorescence staining (Figure 1A). The tests were performed at 72 h post-CCI, when peak inflammasome-mediated pyroptosis in the whole brain develops (Ismael et al., 2018), so that the expressions of inflammasome subunits are maximized. We found that NLRP1, NLRP3, NLRC4 and AIM2 were all highly expressed in injured BMVECs (Figure 1B). In addition, the results of Western Blotting showed that abundant expression of other inflammasome subunits in injured BMVECs, including cleaved Caspase-1 and downstream pro-inflammatory cytokines - IL-1β and IL-18 were also induced by CCI (Figure 1C). Taken together, TBI could lead to NLRs and AIM2 inflammasome-mediated pyroptosis in injured BMVECs.

2.2 NLRs and AIM2 inflammasome-mediated pyroptosis in injured BMVECs after TBI could be inhibited by Caspase-1 inhibitor, Ac-YVAD-cmk

Ac-YVAD-cmk is a Caspase-1 specific inhibitor functioned by binding specifically to Caspase-1 subunits, thus blocking the assembly of NLRs and AIM2 inflammasome (Antonopoulos et al., 2013; Zhang et al., 2014; Wang et al., 2015). To study the impact of inflammasome-mediated pyroptosis on BBB damage after TBI, we manipulated the severity of pyroptosis in injured BMVECs using Ac-YVAD-cmk treatment. As shown in figure 2A and 2B, Ac-YVAD-cmk suppressed the increased expression on essential inflammasome subunit – Caspase-1, and pivotal downstream pro-inflammatory cytokines – IL-1β and IL-18 that directly results in the onset of cell death. Gasdermin D (GSDMD) is a generic substrate for Caspase-1. Cleavage of GSDMD by the inflammatory caspases could promote the formation of membrane pores, and determine pyroptosis by releasing pro-inflammatory cytokines and cleaved gasdermin-N domain that bears intrinsic pyroptotic activity (Shi et al., 2015; Liu et al., 2016). We found that TBI induced GSDMD cleavage in injured BMVECs, characterized by decreased expression of uncleaved GSDMD and increased expression of cleaved GSDMD. Ac-YVAD-cmk treatment could
reverse the effect, which promoted the expression of uncleaved GSDMD, and inhibited the expression of cleaved GSDMD (Figure 2A, 2C).

ASC, the apoptosis-associated speck-like protein, could bridge the inflammasome sensors and Caspase-1. Upon inflammasome activation, the recruitment of pro-Caspase-1 triggers supramolecular oligomerization of ASC monomers into large interweaving fibrils (dimers or oligomers), also termed ASC-speck or pyroptosome (Fernandes-Alnemri et al., 2007). As a hallmark of pyroptosis, ASC-speck/pyroptosome contributes to caspase-1 cleavage and release of mature IL-1β (Dick et al., 2016). Therefore, we detected the expression changes on ASC monomers, dimers and oligomers in BMVECs after TBI and Ac-YVAD-cmk treatment (Lugrin and Martinon, 2017). The results showed that the expression of ASC monomers was increased in injured BMVECs, and was not suppressed by Ac-YVAD-cmk treatment. In contrast, the increased ASC dimers and oligomers after TBI could be inhibited by Ac-YVAD-cmk treatment, suggesting that the oligomerization of ASC was blocked (Figure 2D, 2E). Furthermore, the increased expression on NLRs and AIM2 in injured BMVECs after TBI was not inhibited by Ac-YVAD-cmk treatment (Figure 2F, 2G). These findings confirmed that Ac-YVAD-cmk could inhibit pyroptosis in injured BMVECs by blocking Caspase-1 recruitment and ASC oligomerization, despite of the increased expression on NLRs, AIM2 and ASC monomers after TBI.

2.3 Inhibiting pyroptosis by Ac-YVAD-cmk treatment alleviated BBB damage after TBI

To demonstrate the impact of pyroptosis on regulating BBB damage, we evaluated BBB permeability after Ac-YVAD-cmk treatment at 72 h post-CCI, using Evans Blue (EB) dye extravasation assay and brain water content measurement. EB solution has a high affinity for the albumin in blood circulation, which gives rise to a high-molecular complex (68.5 kDa) with limited penetration of the intact BBB. Therefore, the degree of EB dye extravasation represents the permeability of high molecular weight
substances from injured BBB. In our tests, remarkable EB leakage from injured hemisphere was observed to be induced by TBI, and was alleviated after Ac-YVAD-cmk treatment (Figure 3A, 3B). Brain water content is an indicator for the permeability of low molecular weight substances from injured BBB. Consistent with the results of EB dye extravasation assay, we found that the percentage of brain water content in injured hemisphere was increased after TBI for the development of brain edema. It could also be attenuated by Ac-YVAD-cmk treatment (Figure 3C).

Tight junction proteins, such as Occludin, Claudin-5 and ZO-1, are the components that connect BMVECs, which sealed the intracellular gaps among BMVECs and maintain the integrity and stabilization of BBB [27]. Their expressions has been shown to be decreased in injured brain after TBI in our previously research (Ge et al., 2015). We quantified the expression levels of Occludin, Claudin-5 and ZO-1 in injured BMVECs at 72 h post-CCI, and found that their expressions were promoted by Ac-YVAD-cmk treatment (Figures 3D, 3E). Consequently, these results suggested that inhibiting pyroptosis by Ac-YVAD-cmk treatment could alleviate BBB damage after TBI.

2.4 Inhibiting pyroptosis suppressed the inflammatory response in injured BMVECs after TBI

The impact of pyroptosis on regulating the inflammatory response in injured BMVECs was evaluated by measuring the expression levels of inflammatory mediators (TNF-α, IL-6 and IL-10) at 72 h post-CCI. We found that TBI led to the inflammation in injured BMVECs, characterized by increased levels of pro-inflammatory TNF-α and IL-6, and decreased level of anti-inflammatory IL-10. Ac-YVAD-cmk treatment could inhibit the expression of TNF-α and IL-6, and promote that of IL-10, thus exert an anti-inflammatory effect (Figure 4A). In addition, we quantified the expression changes on NF-κB signaling factors in injured BMVECs at 72 h post-CCI. NF-κB signaling plays a central role in controlling inflammatory response, which regulates the expression of abundant downstream inflammatory
mediators. In unstimulated cells, it is sequestered in the cytoplasm by IκB inhibitory proteins via blocking the nuclear localization signals. On cellular stimulation by immune and proinflammatory responses, NF-κB-activating agents can induce the phosphorylation of IκBα, and target it for rapid degradation that releasing NF-κB to the nucleus, where it regulates downstream gene expression (Courtois and Gilmore, 2006). In our tests, we observed that Ac-YVAD-cmk treatment suppressed the activation of NF-κB signaling after TBI by inhibiting the expression of NF-κB and phosphorylated IκBα (p-IκBα), and promoting that of total IκBα (Figure 4B-4D). Therefore, inhibiting pyroptosis by Ac-YVAD-cmk treatment could suppress the inflammatory response in injured BMVECs after TBI.

2.5 Inhibiting pyroptosis could improve the neurological outcome after TBI

The neurological function of CCI mice was evaluated by the modified Neurological Severity Score (mNSS) test, Morris Water Maze (MWM) test and novel object recognition test. In the mNSS test, lower neurological score demonstrates better neurological function. As shown in Figure 5A, no difference on the neurological score was observed between the CCI + YVAD group and the CCI + DMSO group at 1 d post-CCI. We found that the recovery of neurological function began at 3 d and lasted to 14 d post-CCI, when CCI mice still suffered from residual neurological deficiencies. Besides, the neurological score at 3 d, 7d and 14 d post-CCI was decreased in the CCI + YVAD group, compared with the CCI + DMSO group. It indicated that Ac-YVAD-cmk treatment could improve the recovery of neurological function after TBI.

In the MWM test, the spatial acquisition trial was performed from 14 d to 17 d post-CCI to test spatial learning ability. Escape latency, which represents the capability to navigate from a start location to a submerged platform, was gradually decreased in the testing procedure, indicating that a spatial memory was established (repeated-measures ANOVA, F (3, 72) = 559.19, P < 0.001). The
probe trial was conducted at 18 d post-CCI to test the retrograde reference memory, in which more time spent in the goal quadrant indicates better memory. We found that CCI led to an obvious increase on escape latency, and a decrease on time spent in the goal quadrant. Compared with the CCI + DMSO group, the CCI + YVAD group displayed a decrease on the escape latency from 15-17 d post-CCI, and an increase on the time spent in the goal quadrant (Figure 5B, 5C). In addition, no difference was observed on the swim speed among all groups, which proved that the different performance of CCI mice was not due to their motor impairments. Thus, these findings suggested that Ac-YVAD-cmk treatment could improve the recovery of spatial learning ability after TBI.

In the object recognition test, the preference on exploring new object provides an indicator for cognitive memory. As presented in Figure 5D, CCI mice showed a reduced exploring time on the novel object. In addition, compared with the CCI + DMSO group, the index of exploring time on the novel object over the total exploring time was increased in the CCI + YVAD group, suggesting that Ac-YVAD-cmk treatment could improve the cognitive memory after TBI.

Taken together, the results of above behavioral tests on CCI mice indicated that Ac-YVAD-cmk treatment could improve the neurological outcome after TBI.

3. Discussion

Pyroptosis is a recently identified inflammasome-mediated and Caspase-1-dependent programmed cell death, which is stimulated by a range of microbial infections or non-infectious stimuli, such as TBI. The present study is the first report that focused on studying the impact and mechanism of NLRs and AIM2 inflammasome-mediated pyroptosis in damaged BBB after TBI. The major discovery are that (1) TBI led to NLRs and AIM2 inflammasome-mediated pyroptosis in BMVECs from injured
cerebral cortex; (2) The Caspase-1 inhibitor, Ac-YVAD-cmk could inhibit pyroptosis in injured BMVECs by suppressing the expression of essential inflammasome subunit – Caspase-1 and pivotal downstream pro-inflammatory cytokines (IL-1β and IL-18), as well as hindering GSDMD cleavage and ASC oligomerization; (3) Inhibiting pyroptosis could alleviate TBI-induced BBB leakage, brain edema, loss of tight junction proteins, and the inflammatory response in injured BMVECs. These effects contributed to improving the neurological outcome of CCI mice. In conclusion, NLRs and AIM2 inflammasome-mediated pyroptosis could aggravate BBB damage after TBI. Targeting and controlling pyroptosis in injured BBB would be a promising therapeutic strategy for TBI in the future.

NLRs are the receptors that respond to a variety of pathogen-associated molecular patterns (PAMPs). Among the NLRs, the signal specificity and functional roles of NLRP1, NLRC4, especially NLRP3 aroused the most interest in research on inflammasome and pyroptosis (Awad et al., 2018). NLRP1, also known as NALP1, is the first inflammasome that has been described in detail (Chavarria-Smith and Vance, 2015). An in-vivo study in 2009 first reported its roles in TBI. It proved that NLRP1 inflammasome in neurons of the cerebral cortex is critical for the activation of innate inflammatory response, and administrating an anti-ASC antibody could decrease the contusion volume of injured brain (de Rivero Vaccari et al., 2009). Clinical relevance toward the pathogenic role of NLRP1 in TBI was then provided, in which higher levels of NLRP1 and Caspase-1 were observed in the cerebrospinal fluid (CSF) of TBI patients with a poor prognosis, while lower expressions of the proteins were associated with patients that had a favorable outcome (Adamczak et al., 2012). These results were also confirmed by another study, in which NLRP1 inflammasome proteins (NLRP1, ASC and Caspase-1) were detected in exosomes derived from the CSF of TBI patients (de Rivero Vaccari et al., 2016). In addition, hypothermia therapy was proved to inhibit NLRP1 inflammasome signaling in cerebral cortical neurons of TBI rats, thus it could suppress the inflammatory response in injured brain.
and improve the histopathological outcome (Tomura et al., 2012). This research from a side showed the potential of treatment that targeting NLRP1 inflammasome for TBI.

Among all inflammasome complexes, NLRP3 inflammasome is the best characterized, which has been demonstrated a crucial role in TBI (Zhou et al., 2016). Assembly of NLRP3 inflammasome complex with expression of ASC and Caspase-1, and processing of IL-1β and IL-18 has been detected in cerebral cortex of TBI rats (Liu et al., 2013). Besides, increased level of NLRP3 was observed in the CSF of severe TBI patients, suggesting that it could be regarded as an indicator for TBI prognosis (Wallisch et al., 2017). From this, therapeutic methods targeting NLRP3 inflammasome-mediated pyroptosis for TBI was widely studied. Propofol, telmisartan, traditional Chinese medicine like mangiferin and resveratrol, as well as hyperbaric oxygen treatment were all reported to be effective in inhibiting NLRP3 inflammasome on TBI animals (Ma et al., 2016; Wei et al., 2016; Fan et al., 2017; Qian et al., 2017; Zou et al., 2018). In addition, selective NLRP3 inhibitors – MCC950 and BAY 11-7082 were designed, which showed remarkable protective effect on alleviating brain damage and inflammatory response, and improving the cognitive outcome after TBI (Irrera et al., 2017; Ismael et al., 2018). For the mechanism research on NLRP3 inflammasome, its activation in neurons and microglia after TBI was further elucidated (Lee et al., 2018; Liu et al., 2018). Ma found that deletion of NOX2, a major contributor to oxidative stress in TBI pathology, could attenuate the assembly and activity of NLRP3 inflammasome via a mechanism that associated with TXNIP, a sensor of oxidative stress (Ma et al., 2017). Lin suggested that omega-3 fatty acids could ameliorate TBI-induced inflammation and behavior deficits by inhibiting NLRP3 inflammasome-mediated pyroptosis through activating G protein-coupled receptor 40 (Lin et al., 2017). Based on these findings, we concluded that NLRP1 and NLRP3 inflammasome may work together to play a significant role in regulating pyroptosis.
and inflammation after TBI. Our present study further clarified their impact in traumatic BBB damage, suggesting that they could be served as candidate therapeutic targets.

NLRC4, also known as IPAF, has been widely studied in Alzheimer’s disease (AD) (Freeman and Ting, 2016), but information about its roles in TBI is still limited. In addition, NLRC4 inflammasome was proved to be the major contributor to ASC-dependent pyroptosis in acute cerebral ischemia (Denes et al., 2015). These studies suggested that NLRC4 inflammasome-mediated pyroptosis would also play a significant role in acute brain injury diseases. Our findings that NLRC4 inflammasome was activated in injured BMVECs after TBI also confirmed this opinion.

In addition to NLRs, AIM2 is another well-documented inflammasome receptor, which could be detected in BMVECs (Nagyoszi et al., 2015). Adamczak found that application of the CSF from TBI patients to cultured neurons could induce AIM2 inflammasome activation, ASC oligomerization, Caspase-1 maturation and IL-1β secretion, suggesting that the CSF from injured brain is immunogenic and probably lead to pyroptosis in surrounding cells (Adamczak et al., 2014). In addition, a research on hyperbaric oxygen therapy indicated that the treatment could inhibit the activation of AIM2, as well as NLRs inflammasome, and suppress the inflammatory response in TBI mice (Geng et al., 2016). In the present study, our results showed that NLRP1, NLRP3, NLRC4 and AIM2 inflammasome were all activated in injured BMVECs at 72 h post-CCI. This finding expands the previous understanding on cell-type distribution of NLRs and AIM2 in the brain.

Ac-YVAD-cmk is a Caspase-1 specific inhibitor functioned by binding specifically to Caspase-1 subunits, thus blocking the assembly of NLRs and AIM2 inflammasome (Antonopoulos et al., 2013; Zhang et al., 2014; Wang et al., 2015). We confirmed its effect for the first time in an animal model of TBI, suggesting that its administration could inhibit the activation of NLRs and AIM2 inflammasome,
and the subsequent IL-1β and IL-18 expression in injured BMVECs. In addition, Ac-YVAD-cmk also suppressed the cleavage of GSDMD and the oligomerization of ASC, which indicated that it is effective in controlling pyroptosis in the setting of secondary BBB damage. Based on these findings, we observed the potential therapeutic effect of Ac-YVAD-cmk in CCI mice, and confirmed that Ac-YVAD-cmk treatment could alleviate TBI-induced BBB leakage, brain edema, loss of tight junction proteins, and the inflammatory response in injured BMVECs. For long-lasting effect, it also contributed to improving the neurological outcome, as demonstrated by improvement in the neurobehavioral tests. All of these findings proved that controlling pyroptosis in injured BBB would be a promising therapeutic strategy for TBI, and Ac-YVAD-cmk is a promising drug candidate for future clinical trials.

Our research is still limited in the power of explaining further molecular mechanism on the functioning of NLRs and AIM2 inflammasome. To address this problem, we are performing in-vivo and in-vitro experiments on BMVECs using MCC950, which is a widely reported NLRP3 specific inhibitor that suppressing NLRP3, but not NLRP1, NLRC4 and AIM2 activation (Coll et al., 2015). In addition, we propose that the design of therapeutics that selectively targeting NLRP1, NLRC4 and AIM2, should be of great interest to all researchers in the field for its importance in future mechanism research and clinical treatment. Nevertheless, our findings expand the previous understanding on the pathophysiology of secondary brain damage, thus open a new avenue of therapeutic strategies for TBI by targeting and controlling pyroptosis in BBB.

4. Experimental Procedures

4.1 Animals and grouping methods. Adult male C57BL/6 mice (aged 12 wk, weighing 20-25 g) were purchased from the Chinese Academy of Military Science (Beijing, China). All animal procedures were in adherence to the EC Directive 86/609/EEC for animal experiments, and the Policy of Animal Care and Use Committee of Tianjin Medical University. The mice were quarantined and housed for one week before being randomly divided into 4 groups: Sham, CCI, CCI + DMSO and CCI + Ac-YVAD- cmk (CCI +YVAD). Ac-YVAD-cmk (Invivogen, San Diego, CA, USA) was dissolved in DMSO. It was dosed in
mice (8 mg/kg, i.p.) at 1 h post-CCI, and every 2 days until 14 d post-CCI (Zhang et al., 2014). Equal volume of DMSO was administrated in mice of the CCI + DMSO group at the same time points. A schematic diagram of the experimental design was showed in supplementary Figure 1.

4.2 CCI mice model. The mice were subjected to CCI after anesthesia with 10% chloride hydrate (3.0 mL/kg, i.p.). They were then positioned in a stereotaxic device using ear bars. Following a midline scalp incision, a 3.0-mm craniotomy was performed centrally over the right parietal bone. The impounder tip of the injury device (eCCI model 6.3, American Instruments, Richmond, VA, USA) was then extended to its full impact distance, positioned on the surface of the exposed dura mater, and reset to impact its surface. For moderate-TBI induction, the impact parameters (velocity: 4.5 m/s, depth: 2.0 mm, dwell time: 200 ms) were set as we previously reported (Xu et al., 2017; Ge et al., 2018). Any mice with a herniation of dura mater were eliminated from the group. After the impact, the craniotomy was closed with bone wax and the scalp was sutured. The mice were placed in a well-ventilated cage at 37 °C until they regained consciousness. Sham-operated mice underwent the same procedures except for the cortical impact. To determine the degree of brain injury, H&E staining was performed on brain sections at 72 h post-CCI.

4.3 Tissue preparation. For immunofluorescence staining, the mice were sacrificed by transcardiac perfusion with cold PBS followed by 2% paraformaldehyde, 10% sodium periodate and 2% L-lysine (70mM) at 72 h post-CCI. The brains were then dissected on the ice and post-fixed in the same solution for 1 h, and incubated in 30% sucrose overnight. After fixation, they were embedded in the optimum cutting temperature medium (Sakura, Torrance, CA, USA) on dry ice. Coronal sectioning at the injured area was performed on a cryostat at -20°C. For western blotting and quantitative RT-PCR, the mice were sacrificed by transcardiac perfusion with cold PBS at 72 h post-CCI. The brains were dissected on the ice, and the injured cerebral cortex was isolated immediately. To collect the microvessels, the brain tissue was placed in 6-ml 30% dextran solution within 20mM NaHCO₃, 10mM Glucose, 1mM NaPyruvate and 0.5% bovine serum albumin (BSA). The mixture was spun at 6,000 × g for 15 min at 4°C. Next, the supernatant was removed, and the remaining pellet was passed through a 100-μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA). The filtrate was then passed through a 40-μm nylon mesh, and the microvessels could be harvested on the mesh (Zacharek et al., 2006).

4.4 Double Immunofluorescence staining. The sections were first treated with 3% BSA for 30 min at 37 °C to block nonspecific staining. They were then incubated overnight at 4 °C with combined primary antibodies of mouse anti-CD31 (1:200; ab24590; Abcam, Cambridge, Cambridgeshire, UK) with rabbit anti-NLRP1 (1:100; ab98181; Abcam), rabbit anti-NLRP3 (1:50; PA5-20838; ThermoFisher Scientific, Waltham, MA, USA), rabbit anti-NLRC4 (1:500; 06-1125; MilliporeSigma, Burlington, MA, USA), or rabbit anti-AIM2 (1:500; ab180665; Abcam). In the next day, the sections were rinsed by PBS, followed by incubated with a mixture of secondary antibodies (goat anti-mouse FITC and goat anti-rabbit TRITC) for 1 h at room temperature. The nuclei were counterstained with DAPI (MilliporeSigma). The sections with five randomly selected fields from injured cerebral cortex were digitized under 20× objective using a 3-CCD color video camera (Sony DXC-970MD, Japan) with an immunofluorescence microscope (Olympus IX81, Japan).

4.5 EB dye extravasation assay. The 2% EB solution (MilliporeSigma) was injected into mice through the caudal vein at a dose of 20 mg/kg at 72 h post-CCI. After 3 h, the mice were sacrificed by transcardiac perfusion with normal saline. The injured cerebral hemisphere was then dissected, weighed, and incubated in N, N-Dimethylformamide at 37°C immediately for 72 h. Next, the tissue was
centrifuged at 1000 g for 15 min. The absorbance of the supernatant was detected by a spectrophotometer (Flow Laboratories, McLean, VA, USA) at the wavelength of 632 nm. According to the standard curve, the quantity of extravasated EB solution was quantified, and expressed using the unit – ug/g tissue. It represents the weight of Evans Blue dye (μg) in one gram of acquired brain tissue.

4.6 Brain water content measurement. The brain water content was measured using the wet-dry weight method as we previously reported (Ge et al., 2015). Briefly, the mice were sacrificed at 72 h post-CCI without transcardiac perfusion. The injured cerebral hemisphere was dissected and weighed (wet weight) immediately, followed by drying in an electro-thermostatic blast oven at 80 °C for 72 h. After that, the brain tissue was weighed (dry weight) again. The percentage of water content was calculated using the equation: \[\text{Percentage of water content} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\%\.

4.7 Western Blotting. The SDS/PAGE and immunoblotting were performed as we previously reported (Han et al., 2015; Ge et al., 2016). Briefly, 8% SDS-acrylamide gel was used for detecting NLRP1 (1:1000; ab98181; Abcam), NLRP3 (1:250; PA5-20838; ThermoFisher Scientific), NLRC4 (1:1000; 06-1125; MilliporeSigma) and ZO-1 (1:500; ab216880; Abcam). 10% SDS-acrylamide gel was used for detecting AIM2 (1:1000; ab180665; Abcam), GSDMD (1:1000; ab209845; Abcam), Occludin (1:1000; ab216327; Abcam), NF-kB (1:1000; 8242; CST, Danvers, MA, USA), p-IκBα (1:1000; 2859; CST) and IκBα (1:1000; 4814; CST). 12% SDS-acrylamide gel was used for detecting ASC (1:1000; sc-22514-R; Santa Cruz Biotechnology, Dallas, TX, USA), cleaved Caspase-1 (1:1000; 67314; CST), IL-1β (1:1000; 12242; CST), IL-18 (1:1000; ab71495; Abcam) and Claudin-5 (1:500; ab15106; Abcam). GAPDH (1:1000; 2118; CST) and α-tubulin (1:1000; 2144; CST) were used as the internal control. For densitometry, the ChemiDoc™ XRS+ Imaging System (Bio-Rad) was employed. Mean pixel density of each band was detected using the Quantity One software (Bio-Rad).

4.8 Quantitative RT-PCR. RT-PCR detection for the mRNA levels of inflammatory mediators was conducted as we previously reported (Huang et al., 2018). Briefly, total RNA was extracted from the microvessels isolated from injured hemispheres using TRIzol reagent (ThermoFisher Scientific). The RNA concentration and quality were evaluated by Nanodrop Spectrophotometer (ND-2000, ThermoFisher Scientific). Reverse transcription and qRT-PCR were performed using Hairpin-it™ mRNA (TNF-α, IL-6 AND IL-10) RT-PCR Quantitation kit (GenePharma, Shanghai, China) with corresponding primers (Table 1). All PCR reactions were performed using standard PCR conditions. GAPDH was used as the internal control. The cycle threshold (Ct value) was detected by a CFX ConnectTM RT-PCR system (Bio-Rad, Hercules, CA USA). The data were analyzed using the 2^ΔΔCt formula.

4.9 mNSS test. The modified neurological severity score, which includes motor, sensory, reflex and balance tests, was evaluated by an observer who was blinded to the experimental conditions and treatments as reported (Chen et al., 2001). The test was performed at 1, 3, 7 and 14 days post-CCI.

4.10 MWM test. The MWM test was carried out to evaluate cognitive outcome as we previously reported (Ge et al., 2014). For the spatial acquisition trial, the mice were placed in a pool (105 cm diameter) filled with room temperature water and allowed up to 90 s to locate a submerged platform. The mice performed 4 trials a day with a 30-min inter-trial interval for 4 consecutive days (14-17 d). They were introduced in varying quadrants (northwest, northeast, southwest and southeast) of the pool for each trial, but the location of the platform was fixed. The latency-time to reach the platform was recorded, and the 4 trials were averaged. The probe trial was conducted on day 18 (24 h after the last spatial acquisition trial). In this test, the platform was removed and the mice were allowed to swim...
freely for 60 seconds. The percent of the time spent in goal quadrant was measured. In addition, Ac-YVAD-cmk treatment was not conducted during the MWM test.

4.11 Novel object recognition test. The test was performed on 14 d post-CCI as reported (Leger et al., 2013). Briefly, the mice were administered to freely explore a 50 × 50 × 50 cm open-field box (CleverSys, Reston, VA, USA) for 10 min before experimental sessions. During the first session (familiarization session), they were allowed to freely explore two similar objects. During the second session (test session), one of the two objects was replaced by a novel object. The amount of time that the mice spent on exploring each object was recorded, and the index of exploring time on the novel object over the total exploring time was finally calculated.

4.12 Statistical analysis. All data are based on at least 3 independent experiments. The data are expressed as mean ± SD, except for that of the spatial acquisition trials of the MWM test, which is expressed as mean ± SEM. Data of the mNSS test and the spatial acquisition trials of the MWM test were analyzed using two-way ANOVA followed by LSD post hoc analysis. For other data, statistical comparisons were analyzed using one-way ANOVA followed by LSD post hoc analysis or Student’s t-test. A P-value < 0.05 was considered significant.

Author Contributions

Ping Lei and Jianning Zhang were responsible for study design. Xintong Ge developed methodology. Xintong Ge, Wenzhu Li, Shan Huang, Zhenyu Yin and Xin Xu carried out the experiments. Fanglian Chen and Xiaodong Kong provided technical support. Xintong Ge and Wenzhu Li interpreted the results, performed data analysis, and prepared the figures and tables. Xintong Ge wrote the manuscript. Haichen Wang reviewed the manuscript. Ping Lei supervised the study.

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Conflict of interest statement

The authors declare no competing financial interests.

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Figure Legends

Figure 1. TBI led to NLRs and AIM2 inflammasome-mediated pyroptosis in BMVECs from injured cerebral cortex. (A) The H&E staining of injured mice brain at 72 h post-CCI. Scale bar: 250 μm. (B) Double immunofluorescence staining for BMVECs (CD31) with NLRs or AIM2 at 72 h post-CCI. The white arrow indicated representative double-immunostained cells. Note that NLRP1, NLRP3, NLRC4 and AIM2 were largely expressed in BMVECs from injured cerebral cortex. Scale bars: 100 μm. (C) The immunoblot of other inflammasome subunits and downstream pro-inflammatory cytokines that mediated pyroptosis in injured BMVECs at 72 h post-CCI. Note that abundant expression of cleaved Caspase-1, IL-1β and IL-18 in injured BMVECs was induced by CCI. Taken together, TBI could lead to NLRs and AIM2 inflammasome-mediated pyroptosis in injured BMVECs. n = 6.

Figure 2. NLRs and AIM2 inflammasome-mediated pyroptosis in injured BMVECs after TBI could be inhibited by Caspase-1 inhibitor, Ac-YVAD-cmk. (A) The immunoblot and (B, C) quantitative data of cleaved Caspase-1, downstream pro-inflammatory cytokines and GSDMD in injured BMVECs at 72 h post-CCI. Ac-YVAD-cmk suppressed the increased expression on essential inflammasome subunit – Caspase-1, and pivotal downstream pro-inflammatory cytokines – IL-1β and IL-18 after TBI. Besides, it also hindered the cleavage of GSDMD after TBI, characterized by increased expression of uncleaved GSDMD and decreased expression of cleaved GSDMD. (F) The immunoblot and (G) quantitative data of ASC at 72 h post-CCI. The expression of ASC monomers was increased in injured BMVECs, and was not suppressed by Ac-YVAD-cmk treatment. In contrast, the increased ASC dimers and oligomers after TBI could be inhibited by Ac-YVAD-cmk treatment, suggesting that the oligomerization of ASC was blocked. (F) The immunoblot and (G) quantitative data of NLRs, and AIM2 at 72 h post-CCI. Note that the increased expression on NLRs and AIM2 in injured BMVECs after TBI was not inhibited by Ac-YVAD-cmk treatment. n = 6. (**P<0.01, ***P<0.001, N.S.: no significance)

Figure 3. The impact of pyroptosis on regulating BBB damage after TBI. (A) The coronal view and (B) quantitative data of EB extravastion from injured hemisphere at 72 h post-CCI. Note that the leakage of EB solution after TBI was alleviated by Ac-YVAD-cmk treatment. (C) The quantitative data of brain water content measurement for injured hemisphere at 72 h post-injury. Note that the percentage of brain water content in injured brain was significantly decreased by Ac-YVAD-cmk treatment. (D) The immunoblot and (E) quantitative data of tight junction proteins (Occludin, Claudin-5 and ZO-1) in injured BMVECs at 72 h post-CCI. Note that the expression of tight junction proteins was inhibited by Ac-YVAD-cmk treatment. Thus, inhibiting pyroptosis could alleviate BBB damage after TBI. n = 6. (*P<0.05, **P<0.01, ***P<0.001)

Figure 4. The impact of pyroptosis on regulating the inflammatory response in injured BMVECs after TBI. (A) The expression levels of inflammatory mediators (TNF-α, IL-6 and IL-10) in injured BMVECs at 72 h post-CCI detected by quantitative RT-PCR. Note that CCI induced inflammation in BMVECs, characterized by increased expression on pro-inflammatory TNF-α and IL-6,
and decreased expression on anti-inflammatory IL-10. Ac-YVAD-cmk treatment could reverse the expression changes on inflammatory mediators, thus exerted an anti-inflammatory effect. (B) The immunoblot and (C) quantitative data of NF-κB signaling factors in injured BMVECs at 72 h post-CCI. Ac-YVAD-cmk treatment suppressed the activation of NF-κB signaling after TBI by inhibiting the expression of NF-κB and phosphorylated IκBα (p-IκBα), and promoting that of total IκBα. Thus, inhibiting pyroptosis could suppress the inflammatory response in injured BMVECs after TBI. n = 6. (*P<0.05, **P<0.01, ***P<0.001)

Figure 5. Inhibiting pyroptosis could improve the neurological outcome after TBI. The neurological function of CCI mice was evaluated by (A) mNSS test, (B, C) MWM test and (D) novel object recognition test. (A) Ac-YVAD-cmk treatment decreased the neurological score at 3 d, 7 d and 14 d post-CCI. (B) Escape latency for the spatial acquisition trail was shortened, and (C) time spent in the goal quadrant for the probe trail was improved by Ac-YVAD-cmk treatment. (D) The index of exploring time on the novel object over the total exploring time was increased by Ac-YVAD-cmk treatment. Taken together, inhibiting pyroptosis improved the neurological outcome of CCI mice. n = 7-10. (*P<0.05, **P<0.01, ***P<0.001)
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Highlights

- TBI leads to NLRs and AIM2 inflammasome-mediated pyroptosis in injured BMVECs
- Ac-YVAD-cmk inhibits NLRs and AIM2 inflammasome-mediated pyroptosis in injured BMVECs
- Inhibiting pyroptosis by Ac-YVAD-cmk treatment could alleviate BBB damage after TBI
- Ac-YVAD-cmk treatment could suppress the inflammatory response in injured BMVECs
- Ac-YVAD-cmk treatment could improve the neurological outcome after TBI