TNF α -Mediated Necroptosis in BBB Endothelia as a Potential Mechanism of Increased Seizure Susceptibility in Mice Following Systemic Inflammation

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Abstract

Background

Systemic inflammation is a potent contributor to increased seizure susceptibility. However, less is known about the effects of systemic inflammation on blood-brain barrier (BBB) that affect neuron excitability. Necroptosis and inflammation are intimately associated in various neurological diseases. We hypothesized that necroptosis is involved in the mechanism underlying sepsis-associated neuronal excitability in BBB components.

Methods

Systemic inflammation was induced by LPS. Seizure susceptibility of mice was measured by kainic acid intraperitoneal injection. Pharmacological inhibitors (C87 and GSK872) were used to block signaling of TNF α receptors and necroptosis. To identify the features of sepsis-associated response in the BBB and CNS, brain tissues of mice were obtained for assays of the necroptosis-related protein expression, and immunofluorescence staining for morphological changes of endothelia and glia. Microdialysis assay was also used to evaluate the changes of extracellular potassium and glutamate levels in brain.

Results

Significant findings including induced increased seizure susceptibility and BBB endothelia necroptosis and leakage, Kir4.1 dysfunction, and microglia activation were observed in mice following LPS injection. Inhibition of TNF α receptor inhibitor C87 significantly attenuated increased kainic acid-induced seizure susceptibility and endothelia necroptosis and microglia activation, and restored kir4.1 protein expression, compared with those in controls. GSK872 (a RIP3 inhibitor) treatment, like C87, had consistent effects on these changes following LPS.

Conclusions

Our results showed that TNF α -mediated necroptosis in BBB endothelia damage contributes to the development of increased seizure susceptibility in mice after systemic inflammation. Pharmacologic inhibition targeting this necroptosis pathway may provide a promising therapeutic approach to reduce sepsis-associated BBB dysfunction, astrocyte ion channel dysfunction, and subsequent neuronal excitability.

Introduction

Seizure is a common acute complication of sepsis and systemic inflammation [1–3]. Seizure is provoked by the hyper-excitability of circuits within the brain, due to an imbalance between neuronal excitatory and inhibitory activities. Sepsis leading to the excessive production of proinflammatory factors in the peripheral system and brain is believed to be an important factor in the pathogenesis of neuronal hyper-excitation, leading to an enhanced predisposition to seizure, and subsequent changes of neuroplasticity,

which may evolve into a chronic seizure syndrome [3–6]. Our previous study showed that a single intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) in mice causes increased susceptibility to pentylenetetrazole-induced seizures [7]. Similar observations have been made in postnatal and adult animals [3, 6], including alterations to the number of neuronal receptors and changes to neuronal plasticity [8]. This evidence indicates that inflammatory processes initially from the peripheral system to brain are a common and crucial mechanism in the pathophysiology of seizures and epilepsy. However, the way in which unbalanced regulation of systemic inflammation contributes to seizure development is still unclear.

The blood–brain barrier (BBB) is a major part of the interface between the peripheral and central nervous systems (CNS). The BBB manages the exchange of blood and compounds between the brain and the circulation and maintains a stable CNS environment. It has long been proposed that dysfunction of the BBB may affect neuronal excitability or firing [3, 9, 10]. The BBB is composed of endothelial cells, tight junctions, basal lamina, and associated cells, including astrocytic end-feet and pericytes. The continuous non-fenestrated endothelial cells contribute in part to the restrictive permeability and control of leukocyte transmigration of the BBB via their inherent low pinocytotic activity and their high concentration of efflux transporters [11]. A number of preclinical studies *in vitro* and *in vivo* have shown that inflammatory challenge results in an increase in the permeability of the BBB. The administration of cytokines, such as interleukin (IL)-1, tumor necrosis factor- α (TNF α), and IL-6, increases endothelial permeability [12, 13]. Astrocytes also play a critical role in maintaining the homeostasis of the BBB with respect to neuroglial cells. Astrocyte end-feet contain several channel proteins, such as the inwardly rectifying potassium (Kir) channel subunit Kir4.1, one of the integral parts of the orthogonal arrays of particles, which are required to provide optimal BBB properties [11]. Kir 4.1 channels, specifically expressed in astrocytes, play a key role in controlling spatial K⁺ concentration and regulating extracellular glutamate concentration at tripartite synapses [14, 15]. Astrocytes, via channels such as Kir4.1, directly affect neural excitability and have been implicated in the pathogenesis of seizures and the development of epilepsy [14].

Necroptosis is a regulated necrosis, different from apoptosis and necrosis, and is mediated by death receptors such as TNF α receptor 1, receptor-interacting protein kinase (RIP)1, and RIP3, which activate the phosphorylation of mixed lineage kinase domain-like (MLKL) protein, disturbing the integrity of the cells [16, 17]. The necroptosis pathway has been reported to serve a vital role in many pathologies that involve inflammatory processes, including sepsis, inflammatory bowel disease, and neurodegenerative diseases [18]. The necroptosis mechanism has attracted considerable research attention because of its importance in endothelial damage and BBB leakage after stroke [19, 20]. However, there have been only a few reports that necroptotic cell death signaling occurs in these BBB components following systemic inflammation.

Sepsis and system inflammation induce the excessive production of proinflammatory factors and rapidly corresponding neuroinflammation [7], which may lead to BBB damage and subsequent leakage [13]. Necroptosis and inflammation are intimately associated. Therefore, we hypothesized that necroptosis links systemic inflammation inflammatory mediators such as TNF α , changes in BBB components such

as endothelial cell and astrocytes, and BBB dysfunction, contributing to alteration in susceptibility to seizure. By using an LPS-induced systemic inflammation mouse model, we found for role of TNF α -mediated necroptosis in BBB changes, including endothelial damage and astrocytic Kir4.1 dysregulation, related to an increase in susceptibility to seizures in mice, following systemic inflammation.

Materials And Methods

Animals and the LPS-induced systemic inflammation model

Eight- to nine-week-old male C57BL/6J mice were purchased from the National Lab Animal Center (Taiwan). The animals were housed in a specific pathogen-free room at 21°C under a 12-h light/12-h dark artificial lighting cycle, with free access to feed. Age- and weight-matched animals were used for the present experiments. All procedures were approved by the Animal Care and Use Committee of Changhua Christian Hospital. The mouse model of systemic inflammation was induced by an i.p. injection of 4 mg/kg LPS (*Escherichia coli*, strain O111:B4, Calbiochem, San Diego, CA, USA), as previously described [7].

Determination of susceptibility to seizure using kainic acid

To assess susceptibility to seizure, mice were injected i.p. with 3 or 20 mg/kg kainic acid (KA) 72 h after treatment with LPS (Sigma, St. Louis, MO, USA) or vehicle (normal saline). KA is a common proconvulsant agent used for the induction of seizures. Seizure activity was recorded on video during an observation period of 2 h after KA injection. Behavioral seizures were scored according to a previously defined scale [21] as follows: stage 0, no response; stage 1, body position lowering and hypoactivity; stage 2, body automatic shaking, whisker twitching, and sudden muscle or tail contraction; stage 3, repetitive scratching, head bobbing, or circling; stage 4, forelimb clonus and rearing and falling; stage 5, repetitive behavior of stage 4; and stage 6, severe generalized tonic-clonic seizure. In the behavior test, we recorded the severity score according to the aforementioned criterion for each 5 min over a 120-min period.

Plasma levels of TNF α after peritoneal administration of LPS

To assess the acute effects of LPS on systemic inflammation with and without pretreatment with TNF α receptor inhibitor C87 and RIP3 kinase inhibitor GSK872, blood samples were obtained from the cheek of the mice 1 h after the i.p. injection of 4 mg/kg LPS. The plasma samples were stored at -80°C until they were assayed for TNF α concentrations using Duo set kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Western blotting analysis

For protein lysate extraction, the dissected hippocampus was homogenized and lysed in ice-cold modified radioimmunoprecipitation assay buffer containing 150 mM NaCl; 50 mM Tris-HCl (pH 7.4); 1 mM phenylmethylsulfonyl fluoride; 1% Nonidet P-40; 1 mM EDTA; 10 μ g/mL each of

leupeptin, aprotinin, and pepstatin; 1 mM NaF; and 1 mM Na_3VO_4 . Immunoblotting analysis was performed as previously described [7]. Briefly, 20–30 μg of total protein was loaded per lane on 10%–15% polyacrylamide gels. The gels were transferred to polyvinylidene difluoride membranes, which were probed with antibodies against proteins including JNK (1:1000; #9258, Cell Signaling, Danvers, MA, USA), Bax (1:1000; #2772, Cell Signaling), cleaved Caspase 3 (1:500; Abcam Cambridge, UK; ab13847), RIP3 (1:1000; #95702, Cell Signaling), RIP3 (phosphoS232) (1:1000; ab195117, Abcam), MLKL (1:500; ab196436, Abcam), MLKL (Ser125) (1:500; PA5-105677, Invitrogen, CA, USA), TNF α (1:1000; #11948, Cell Signaling), NKCC1 (1:1000; #14581, Cell Signaling), KIR4.1 (1:500; sc-23637, Santa Cruz, CA, USA), and alpha-tubulin (1:1000; GTX628802, GeneTex, CA, USA), and beta-actin (1:2500; sc-47778, Santa Cruz) overnight at 4°C, followed by pairing different secondary antibodies with HRP-conjugated goat anti-mouse and goat anti-rabbit at dilution of 1:10,000. Signals were visualized and quantified using the GeneGnome chemiluminescence imaging system (Syngene, Bengaluru, India).

Immunofluorescence staining

Mice brains were harvested and soaked in 4% paraformaldehyde overnight and then dehydrated in 30% sucrose at 4°C for 48 h. Serial 20- μm cryosections were cut, washed 3 times with PBS, and then treated with sodium citrate buffer at 80°C for 30 min. The cryosections were washed with PBS three times after cool down and permeabilized with 0.15% Triton X-100 (Thermo Fisher Scientific, MA, USA) in commercial blocking buffer at room temperature (23°C–25°C) for 30 min. The cryosections were hybridized with each primary antibody, including those against, Iba1 (1:200; GTX100042, GeneTex), GFAP (1:500; GTX85454, GeneTex), MLKL (Ser125) (1:100; PA5-105677, Invitrogen;), Kir4.1 (1:250; sc-23637; Santa Cruz), CD31 (1:800; ab24590, Abcam), and CD68 (1:300; MAB1435, Merk Millipore, MA, USA), overnight at 4°C and then with different rhodamine- or Alexa-488-conjugated secondary antibodies, as previously described [7].

Sections were counterstained with DAPI to identify the cell nucleus. Microfluorescence images were taken using an Olympus microscope (Olympus DP80® Dual CCD Microscope, Tokyo, Japan).

Immunofluorescence images were used to analyze the staining density in the CA3 subregions using ImageJ software (NIH, Bethesda, MD, USA)[22]. The number of activated microglia and reactive astrocytes were counted using a stereological approach from three sections of the CA3 subregion in each hippocampus ($n = 3$ mice per group), employing an Olympus DP80® Dual CCD Microscope, as previously described [23]. Briefly, images were captured from the CA3 subregion at –2.18 to –2.54 mm from the bregma. The coordinates for the CA3 were taken from the 2.0- to 3.0-mm medial to lateral regions and the 2.0- to 3.2-mm dorsal to ventral regions. Activated microglia were distinguished from resting microglia by their amoeboid appearance and significant enlargement. Reactive astrocytes had a hypertrophic morphology distinct from that of resting astrocytes. A single experimenter, who was blind to each animal's treatment, performed the quantification of cells of interest using images taken at 200 $\times$ magnification in selected rectangular regions.

Brain interstitial fluid microdialysis

Eight- to nine-week-old male C57BL/6J mice were used for brain microdialysis assays, using procedures modified from previous studies [24, 25]. Mice were anesthetized with isoflurane for stereotaxic surgery to place a guide cannula (Plastics One, Roanoke, VA, USA) into the right hippocampus (anterior–posterior, –2.3 mm; medial–lateral, 2.0 mm; and dorsal–ventral, –2.0 mm relative to the bregma). One day post-operatively, mice (n = 36) were treated with either vehicle (0.25% DMSO) or 2 mg/kg GSK872, and 1 h later treated with saline or 4 mg/kg LPS. Eighteen of these mice had CMA/12 probes (CMA/12 Elite, lengths 2 mm, CMA, Stockholm, Sweden) inserted 2 h before GSK872 treatment and were placed in a Plexiglas dialysis chamber. Then, the CMA/12 probes were immediately perfused with Ringer's solution (147 mM Na⁺, 2.2 mM Ca⁺², 4 mM K⁺, pH 7.0) at a flow rate of 2 µL/min, set to collect 60 µL of dialysate every 30 min for 6 h. Four days post-operatively, 18 more mice that had been treated with GSK872 and LPS had probes inserted into the placed guide cannulas to collect the dialysates once every 30 min for 2 h in each mouse. The extracellular K⁺ concentrations of these dialysates were measured using a flame atomic absorption spectrometer (FAAS, Z6100 Hitachi, Japan). K⁺ levels were calculated from a standard curve prepared from the standard solutions (Merck, Darmstadt, Germany). Glutamate concentrations were measured using an enzymatic colorimetric method using a microdialysis analyzer (CMA/600, Carnegie Medicin, Stockholm, Sweden).

Statistical analysis

The means of two groups were compared using Student's *t*-tests. The means of more than two groups were compared using one-way ANOVA, followed by Bonferroni *post hoc* tests. Differences in KA-induced seizure severity among treated mouse groups and extracellular potassium and glutamate changes among treated GSK872 mouse groups were assessed using two-way repeated measures ANOVA, adjusted using Bonferroni *post hoc* tests. Statistical analysis was performed using the GraphPad Prism version 7 software (GraphPad Software, San Diego, CA, USA, www.graphpad.com). All values are presented as mean ± standard error of the mean (SEM). Differences were considered significant at *p* < 0.05.

Results

LPS-induced systemic inflammation increased susceptibility to KA-induced seizure in mice

To explore the mechanisms underlying the onset and development of seizures following systemic inflammation, our established animal model was used with a single 4-mg/kg dose of LPS via intraperitoneal injection to induce systemic inflammation in 8- to 9-week-old male mice [7]. Seizure susceptibility was subsequently determined by scoring the severity and duration of the KA-induced seizure every 5 min for a 2-h period (Fig. 1A). The mortality rate among the mice was approximately 12% within 3 days after LPS injection (Fig. 1B). During a 2-h period after KA injection, one of 13 saline-treated mice administered 20 mg/kg KA and 2 out of 13 LPS-treated mice administered 3 mg/kg KA died from severe seizures. Among the groups treated with saline and either 3 or 20 mg/kg KA and the group treated with LPS and 3 mg/kg KA, two-way repeated measures ANOVA revealed that the main effect for these

three groups yielded an F ratio value of $F(2, 900) = 395.89$, $p < 0.0001$ (Fig. 1C), indicating a significant difference in susceptibility to KA-induced seizure between these three groups. Bonferroni *post hoc* tests further revealed that there was a significant difference between the 3-mg/kg-KA-saline-treated group and 20-mg/kg-KA-saline-treated group ($F(1, 600) = 860.18$; $p < 0.0001$), but no difference between the 20-mg/kg-KA-saline-treated group and 3-mg/kg-KA- and LPS-treated groups ($F(1, 600) = 1.17$, $p = 0.285$). These results indicated increased susceptibility to 3-mg/kg-KA in LPS-treated mice was similar to that in saline- and 20-mg/kg-KA-treated mice (Fig. 1C). The latency to initial seizure onset that was defined as seizure score stage 4 (i.e., tonic with or without clonic convulsion) or more after KA administration was significantly decreased in LPS-treated mice given 3 mg/kg KA, compared with that in saline-treated mice given 20 mg/kg KA (Fig. 1D). All saline-treated mice administered 3 mg/kg were not observed to reach tonic with or without clonic convulsion. We measured the duration of stage 4–6 seizure among the mice receiving LPS or vehicle control with KA. The seizure duration was 40.00 ± 5.31 min (mean $\pm$ SEM) for the saline- and 20-mg/kg-KA-treated groups and 57.69 ± 6.69 min for the LPS- and 3-mg/kg-KA-treated groups (Fig. 1E). The LPS-treated group had increased susceptibility to 3 mg/kg KA-induced seizures in the latency of initial seizure onset and seizure-behavior duration, compared with the 20-mg/kg-KA-treated mice without LPS injection.

LPS-induced systemic inflammation induced programmed necroptosis and Kir4.1 dysregulation in the hippocampus

We determined the role of several signaling pathways that could be involved in the mechanisms of neuronal hyper-excitability following systemic inflammation, including pathways involved in apoptosis, necroptosis, and ion channel changes [26, 27]. Seventy-two hours after injection with vehicle (saline) or 4 mg/kg LPS, the mice were intraperitoneally administered vehicle (H_2O) or 3 mg/kg KA. Two hours later, the hippocampus was obtained for the assessment of the expression of proteins involved in the apoptosis and necroptosis pathways, as well as astrocytic ion channels ($n = 3$ mice per group) using Western blotting. Among these four groups of saline- or LPS-treated mice administered vehicle or KA, we found the protein levels of JNK, Bax, and cCaspase 3, which are part of the apoptosis pathway, were significantly increased by KA treatment, but no difference was found in mice treated with LPS alone (Fig. 2A–2D). The protein levels of phosphated RIP3 and phosphated MLKL, from the necroptosis pathway, were increased in LPS-treated mice, enhanced by KA treatment, compared with those in vehicle-treated mice (Fig. 2A, 2E and 2F). The levels of phosphated MLKL and TNF α were markedly enhanced after treatment with 3 mg/kg KA (Fig. 2A and 2G). The protein levels of Kir4.1, but not NKCC1, were significantly decreased in LPS-treated mice, compared with those in vehicle-treated mice (Fig. 2H and 2I).

Inhibition of either TNF α receptor or RIP3 attenuated the increased susceptibility to KA-induced seizure in mice following LPS injection

TNF α is rapidly released and is one of the most abundant mediators of inflammation in the peripheral blood after infection or exposure to LPS [7]. TNF α has been implicated in the pathogenesis of several inflammation-related diseases, such as vascular leaks, via different signaling pathways [28]. On the basis

of the aforementioned evidence (Fig. 2), TNF α -dependent necroptosis appeared to be involved in the increase of LPS-induced susceptibility to seizure. Therefore, the TNF α receptor inhibitor, C87, and the RIP3 inhibitor, GSK872, were used to explore the role of TNF α -dependent necroptosis on LPS-associated susceptibility to seizure. Mice were injected intraperitoneally with 2 doses of C87 (2 mg/kg, i.p.) at 1 and 24 h, or one dose of GSK872 (2 mg/kg, i.p.) at 1 h, before LPS injection (Fig. 3A). The mortality rate of the mice was approximately 12% within 3 days after the administration of LPS only, and no deaths were recorded in mice receiving either C87 or GSK872 with LPS treatment. Seventy-two hours after LPS injection, 3 mg/kg KA was administered to evaluate the susceptibility to seizure of these mice ($n = 7-10$ mice per group) by scoring once every 5 min for 2 h. During a 2-h period after KA treatment, one of the 10 LPS-treated mice given KA died because of severe seizures; no death was observed in the LPS-treated groups given C87 ($n = 10$), or GSK872 ($n = 10$), as well as in saline-treated group ($n = 7$). Two-way repeated measures ANOVA revealed that the main effect for these four groups yielded an F ratio of $F(3, 825) = 192.65, p < 0.0001$ (Fig. 3B). Bonferroni post-tests analysis further revealed that there was a significant difference between the C87-treated group ($F(1, 450) = 273.49, p < 0.0001$) and the GSK872-treated group ($F(1, 450) = 117.60, p < 0.0001$) from vehicle-treated mice following LPS injection. These results indicated that either C87 treatment or GSK872 treatment attenuated the susceptibility to KA-induced seizure following LPS injection. There was a significant difference between C87-treated mice and GSK872-treated mice ($F(1, 450) = 25.56, p < 0.0001$), indicating that C87 treatment was better than GSK872 treatment for decreasing seizure susceptibility in the LPS-treated mice. The latency to initial seizure onset (Fig. 3C) and seizure duration during a 2-h period (Fig. 3D) after KA administration (stage 4 or more) were significantly attenuated by either C87 or GSK872 treatment in LPS-treated mice, compared with those in vehicle-treated mice. Half of the mice treated with C87 and GSK872 were not observed to have tonic with or without clonic seizure (i.e., stage 4).

Inhibition of TNF α receptor or RIP3-attenuated BBB leakage, endothelial necroptosis, and astrocytic Kir4.1 down-regulation in the hippocampus of mice treated with LPS

To investigate the effect of the TNF α -dependent necroptosis signal pathway on the integrity of the BBB following LPS injection, we examined the effects of C87 and GSK872 treatment on BBB leakage and neuroinflammation. Mice were dosed with C87 at 24 and 1 h before LPS injection, or one dose of GSK872 at 1 h before LPS, or vehicle and then were sacrificed at 72 h after LPS treatment. Hippocampus sections were prepared for immunostaining with Iba-1 antibody for microglia, CD68 antibody for monocytes, GFAP antibody for astrocytes, and Kir4.1 antibody for channel proteins. Activated microglia were identified by their increased cell size and irregular shape. The percentage of activated microglia (Fig. 4A and 4B) and leaked monocytes (Fig. 4C) in the CA3 regions of the hippocampus was significantly increased in the LPS-treated group, which was attenuated by pretreatment with either C87 or GSK872. Reactive astrocytes indicate the presence of hypertrophic morphology. The percentage of active astrocytes was significantly higher in the LPS-treated mice than in the C87- or GSK-872-treated mice (Fig. 4D and 4E). The proportion of GFAP-positive cells co-localized with Kir4.1 was constant in the control and KA-treated mice but markedly decreased in the LPS-treated mice (Fig. 4F). For determining the MLKL activity of BBB endothelial cells, immunostaining was performed using CD31 antibody for the endothelial cells and p-

MLKL antibody for the bioactivity (Fig. 4G). The proportion of CD31-positive cells co-localized with p-MLKL was lower in control mice, but markedly increased in LPS-treated mice (Fig. 4H). C87 and GSK872 pretreatment of LPS-treated mice reversed the phenomenon of enhanced p-MLKL-positive staining in BBB endothelial cells and decreased Kir4.1-positive staining in astrocytes.

GSK872 suppressed RIP3-mediated necroptosis and restored Kir4.1 protein expression in mice within 3 days after LPS injection

We examined the effects of GSK872 on dynamic changes in RIP3-mediated necroptosis and Kir4.1 protein within 3 days after induction by LPS. Mice were pretreated with GSK872 (2 mg/kg, i.p.) or vehicle (0.25% DMSO) 1 h before LPS was given (Fig. 5A). The protein levels of the hippocampus were measured at 6, 48, and 72 h after LPS injection ($n = 3$ per group for each time point). The protein levels of p-RIP3 and p-MLKL were significantly higher (Fig. 5B–5D) and Kir4.1 protein levels were significantly lower than those given vehicle (Fig. 5B and 5E) at these time points within 3 days after LPS treatment. GSK872 treatment improved the RIP3-mediated necroptosis and the parallel decrease in expression of Kir4.1 ion channel proteins.

GSK872 attenuated increased levels of extracellular potassium and glutamate in the hippocampus within 3 days after LPS injection

Microdialysis was used for continuous measurement of free and unbound analyte levels in the extracellular fluid in mice treated with GSK872 and LPS. One day after the cannula was implanted in the hippocampus, the mice were treated with GSK872 (2 mg/kg, i.p.) or vehicle (0.25% DMSO, i.p.) ($n = 6$ per group) 1 h before the administration of either saline or 4 mg/kg LPS (Fig. 6A). The mean of the first three samples immediately before LPS administration was defined as the basal levels (100%) of extracellular potassium and glutamate in the hippocampus. Within 5 h after GSK872 and LPS injections, we found that the concentrations of extracellular potassium and glutamate had increased (Fig. 6B and 6C). Two-way repeated measures ANOVA revealed that the main effect for these three groups yielded an F ratio of $F(2, 165) = 28.66$, $p < 0.0001$ on potassium levels (Fig. 5B) and $F(2, 165) = 24.31$, $p < 0.0001$ on glutamate levels (Fig. 6C). Bonferroni post-test analysis further revealed that there was a significant difference in potassium ($F(1, 110) = 25.87$, $p < 0.0001$) and glutamate levels ($F(1, 110) = 12.59$, $p = 0.0006$) in the GSK872-treated group compared with that in the vehicle-treated group following LPS injection. These findings indicated that GSK872 treatment attenuated the changes in the levels of these extracellular molecules induced by LPS. Microdialysis experiments were also performed in treated mice ($n = 6$ per group) 72 h after LPS injection, with samples collected once every 30 min for 2 h from each mouse after 1 h stabilization. The mean levels of extracellular potassium (Fig. 6D) and glutamate (Fig. 6E) were significantly higher in the LPS-treated group than in the vehicle-treated group and GSK872-treated group. These results indicated that GSK872 treatment attenuated LPS-induced aggravated potassium and glutamate changes within 3 days after LPS injection.

Discussion

In this study, we consistently observed that sepsis and systemic inflammation is linked to increased seizure susceptibility into corresponding neuro-inflammation such as the activation of microglia and astroglia in the brain [3–5, 7]. We also demonstrated that the TNF α -dependent necroptosis signal pathway governed the changes to the integrity of the BBB, such as endothelial cell damage and astrocytic ion channel Kir4.1 dysregulation following systemic inflammation, leading to neuronal hyper-excitability and the induction of seizures.

Our previous study showed increased susceptibility to seizure induced by using the proconvulsant pentylenetetrazole in mice following LPS injection [7]. This phenomenon was further observed that the susceptibility to low-dose (3 mg/kg, i.p.) KA-induced seizure in mice with LPS injection was similar but more severe in initial latency of seizure onset and tonic-clonic seizure duration than those in mice injected with a high dose (20 mg/kg, i.p.) of KA with saline injection (Fig. 1). These results indicate that systemic inflammation increased neuronal excitability, which in turn reduced the threshold at which seizures are initiated by proconvulsants, or induced the onset of seizures. Besides sepsis-induced neuroinflammation and the subsequent production in the brain of proconvulsive cytokines such as TNF α and IL-1 β , which may be involved in neuronal activity changes [7], the results of this study suggest that dysregulation of the ion channel Kir4.1 of astrocytes may play an important role in the underlying mechanism for changes in seizure threshold in LPS-treated mice. The inwardly rectifying K⁺ channels, Kir4.1, are enriched on the processes of astrocytes surrounding the synapses and blood vessels of the BBB but not in neurons and oligodendrocytes in the brain [29]. In neuronal excitation, the astrocytic Kir4.1 channels play a major role in extracellular potassium (K⁺) buffering to maintain the homeostasis of the neuronal microenvironment [30]. Diminished Kir4.1 buffering capabilities, such as pharmacological or genetic inhibition and down-regulation, may induce membrane hypo-polarization coupled to reduced glutamate clearance in astrocytes, leading to neuronal hyper-excitability [30, 31]. Increasing evidence strongly suggests that astrocytic Kir4.1 channels are involved in the development of seizure and epilepsy (epileptogenesis) [14, 32, 33]. The present study found for the first time that down-regulation of Kir4.1 in astrocytes combined with increase of extracellular potassium and glutamate levels may be involved in the LPS-induced decreased seizure threshold in mice. Both *in vitro* and *in vivo* studies suggest that primary mediators of the inflammatory response, such IL-1 β , could have an effect on the down-regulation of Kir4.1 transcription and protein expression in astrocytes [34, 35]. The present study further demonstrated that Kir4.1 dysregulation in astrocytes produced by systemic inflammation could be restored by the inhibition of the TNF α -mediated necroptosis signaling pathway on BBB endothelial cells.

The BBB helps to regulate the reciprocal periphery-to-brain exchange of molecules and immune cells and maintain a tightly stable microenvironment for the CNS. Dysfunction of the BBB destroys hemostasis, leading to the pathological development of seizure disorders and other neurological disorders [36, 37]. During systemic inflammation, the components of the BBB could be changed at histological and/or molecular levels [13]. Endothelial cells are key components of the BBB, and damage to these cells during systemic inflammation may contribute to barrier dysfunction, whereas the other BBB components, including astrocytes, pericytes, and microglia/macrophages, seem to play a little role in the LPS-mediated

disruption of the BBB [38]. In the present study, pretreatment with TNF α receptor inhibitor C87 abolished BBB endothelial necroptosis with a parallel change in monocyte leakage and corresponding neuroinflammation, and Kir4.1 dysregulation. By excluding the possibility that C87 treatment might alleviate LPS-induced peripheral TNF- α levels (see Additional file 1), these results strongly indicate that proinflammatory TNF α is a key factor involved in endothelial cell damage in the BBB and changes in the permeability of the BBB in the event of systemic inflammation.

Upon binding to TNF α receptor 1, TNF α triggers a range of signal pathways to regulate bioactivity in various cell types and tissues. TNF α may therefore impact the BBB endothelial cells and other components [28]. Both *in vitro* and *in vivo* studies show that TNF α may induce endothelial cell injury and death via the apoptosis and necroptosis pathways [39, 40] and disrupt endothelial tight junction barriers via targeting different pathways, such as the NF- κ B pathway [41, 42], increasing endothelial leakage. The present study found that both inhibition of TNF α receptor by C87 and RIP3 necroptosis by GSK872 significantly attenuated the increased seizure susceptibility in LPS-treated mice (Fig. 3), although there were significant differences between these two treatments. This phenomenon could be explained by the possibility that TNF α may more broadly target the components of the BBB. RIP3 inhibition might also affect apoptosis in some situations, due to the possibility of cross-talk between necroptosis and apoptosis [43]. Given that our data showed that LPS did not increase the level of cCasp3 in mouse brains 72 h after injection (Fig. 2A and 2D), the contribution of LPS-triggered apoptotic death of BBB endothelium following necroptosis to the disruption of BBB integrity, if any, could be not significant. Therefore, among the signaling pathways involving TNF α , our data suggest that endothelial necroptosis plays a critical role in changes of seizure susceptibility in the context of TNF α -induced inflammatory events, including astrocytic ionic Kir4.1 channels and BBB dysfunction.

TNF α production peaked in the peripheral circulating blood at 1 h after LPS injection [7] and in the brain at approximately 30 h [44]. Evans Blue-measured BBB leakage was observed early, within 6 h after LPS injection (see Additional file 2), which corresponded to the finding of increased RIP3-mediated necroptosis (Fig. 5) and extracellular K⁺ and glutamate within 4 h after LPS injection (Fig. 6). The results support the contention that systemic TNF α is an early and key peripheral proinflammatory factor causing BBB disruption during systemic inflammation but could not completely exclude the effects of the TNF α derived from microglia [19]. In the CNS, the levels of TNF α consistently returned to the basal levels by 72 h after LPS (Fig. 2G). KA treatment could not raise the brain levels of TNF α but rapidly increased TNF α levels (Fig. 2G) [45, 46] and enhanced the activity of MLKL in necroptosis in mice 72 h after LPS injection (Fig. 2F). Although the detailed mechanisms of this phenomenon remain incompletely understood, severe seizures may induce brain injury via a process of necroptosis involving MLKL [47]. These results suggest that a seizure threshold low enough to easily induce severe seizure in LPS-treated mice and increased levels of TNF α after KA treatment may play a role in additional MLKL-executed necroptosis in the brain (Fig. 2F).

Necroptosis is a type of programmed cell death with necrosis and is involved in a variety of biological processes, including inflammation and immune responses. Accumulating evidence suggests that the

TNF α -mediated necroptotic pathway may be a potential therapeutic target in the treatment of inflammatory diseases [48, 49]. Over the past years, several types of inhibitors targeting the kinase activity of necroptosis, such as RIP3, have been reported [49, 50]. In the present study, GSK872, an RIP3 inhibitor, reduced the phosphorylation of MLKL and the programmed necrosis of BBB endothelial cells, supporting the suggestion that necroptosis could be a therapeutic target in efforts to prevent BBB damage from systemic inflammation. RIP3 is indispensable in the TNF α -stimulated necroptosis pathway and can also promote non-necroptotic pathways such as inflammation activation and cytokine IL-1 β production through stimulation of Toll-like receptors [48]. Therefore, the effects of RIP3 inhibition on improvement of seizure susceptibility related to systemic inflammation could be partially due to non-necroptotic anti-inflammation, even though MLKL activation in BBB endothelial cells was significantly attenuated in our observations. It will be important to further determine which RIP3 function drives the signaling pathways, such as those of inflammation and necroptosis, in each disease condition. In the future, studies exploiting more specific inhibitors of RIP3 and MLKL kinases may provide crucial insight into the prevention of necroptosis-associated BBB damage and neuroinflammation following sepsis.

Conclusions

Our results showed that TNF α -mediated necroptosis induced BBB endothelial damage and BBB leakage, leading to neuroinflammation and astrocyte Kir4.1 dysregulation and contributing to the development of increased susceptibility to seizure induced by KA in mice, following LPS-induced systemic inflammation. Pharmacologic inhibition targeting elements of the necroptosis pathway, such as TNF α receptor and RIP3 kinases, reduces BBB endothelial cell damage and BBB dysfunction. This evidence may indicate a promising therapeutic approach to reduce sepsis-associated BBB dysfunction, astrocyte ion channel dysfunction, and subsequent neuronal excitability.

Abbreviations

BBB, blood–brain barrier; LPS, lipopolysaccharide; TNF α , tumor necrosis factor- α ; i.p., intraperitoneal; CNS, central nervous systems; IL, interleukin; Kir, inwardly rectifying potassium; RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like; KA, kainic acid.

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Additional files. All material used in this manuscript will be made available to researchers subject to confidentiality.

Authors' contributions

YLL, KHL, HYC, SL and CHL performed the experiments. HMW and KSH designed the study, and WYH analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were approved by the Animal Care and Use Committee of Changhua Christian Hospital, and the experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the United States.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Increased seizure susceptibility to kainic acid in mice following LPS-induced systemic inflammation. (A) The experimental protocol. Mice were injected intraperitoneally with vehicle (normal saline) or 4 mg/kg lipopolysaccharide (LPS). Three days later, seizure susceptibility to kainic acid (KA) administered intraperitoneally was evaluated. (B) The mortality rate of mice within 3 days after LPS or vehicle injection. (C) Seizure susceptibility of these mice was scored once every 5 min over the 2-h period following injection with 3 or 20 mg/kg KA (n = 13 mice per group). Two-way repeated measures ANOVA revealed the main effect for these three groups including the LPS- and 3-mg/kg-KA-treated mice, and saline-treated mice with either 3 or 20 mg/kg KA treatment yielded an F ratio of $F(2, 900) = 395.89$, $p < 0.0001$; Bonferroni post-test analysis further revealed that there was a significant difference between the 3-mg/kg-KA-saline-treated group and 20-mg/kg-KA-saline-treated group ($F(1, 600) = 860.18$; $p < 0.0001$) and between the 20-mg/kg-KA-saline-treated group and 3-mg/kg-KA- and LPS-treated groups ($F(1, 600) = 1.17$, $p = 0.285$). (D) The latency to initial seizure onset of stage 4 (i.e., tonic with or without clonic convulsion) or more after KA injection. Data are presented as mean $\pm$ SEM. Student's t-test; * $p < 0.05$. (E) The total duration (min) of seizure behavior of stage 4 or more. Data are presented as mean $\pm$ SEM. Student's t-test; 3-mg/kg-KA- and LPS-treated group vs. 20-mg/kg-KA- and saline-treated group; * $p < 0.05$.

Figure 2

LPS-induced systemic inflammation induced the programmed necroptosis pathway and Kir4.1 dysregulation in the hippocampus. Three days after LPS or vehicle (saline) injection, 3 mg/kg kainic acid or vehicle (H₂O) was intraperitoneally administrated to the mice. Two hours later, the hippocampus was obtained for assessing the protein expression (n = 3 per group) using Western blots. (A) A representative Western blot showing the specific bands for these proteins. An equal amount of protein sample (30 µg) obtained from the hippocampus homogenate was applied to each lane, and α-tubulin protein was used as an internal control. (B–D) Bar graph showing the densitometric analysis of the molecules JNK, Bax, and cCaspase 3 involved in the apoptotic pathway; (E–G) of the proteins phosphated RIP3, phosphated MLKL, and TNFα in the necroptosis pathway; and (H and I) of the proteins NCKK1 and Kir4.1 in ion channels, normalized to α-tubulin expression. Each bar represents the mean ± SEM. One-way ANOVA; Bonferroni post hoc test among the groups; *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 3

Inhibition of TNF α receptor and RIP3 attenuated increased kainic acid seizure susceptibility in mice following LPS injection. (A) The experimental protocol. Mice were injected intraperitoneally with 12.5 mg/kg C87 given at 24 and 1 h before 4 mg/kg LPS i.p. injection or with 2 mg/kg GSK872 1 h before LPS injection. Three days later, seizure susceptibility to 3 mg/kg kainic acid (KA) was evaluated ($n = 7-10$ mice per group). (B) Seizure susceptibility of treated mice scored once every 5 min over the 2-h period following KA injection. Using two-way repeated measures ANOVA analysis, Bonferroni post hoc analysis revealed that there was a significant difference between C87-treated mice ($F(1, 450) = 273.49$, $p < 0.0001$) and GSK872-treated mice ($F(1, 450) = 117.60$, $p < 0.0001$) from vehicle-treated mice following LPS injection, and between C87- and GSK872-treated mice ($F(1, 450) = 25.56$, $p < 0.0001$). (C) Latency to initial seizure onset (tonic with or without clonic convulsion) after KA administration. Data are presented as mean $\pm$ SEM. One-way ANOVA; Bonferroni post hoc test vs. vehicle- and LPS-treated groups; * $p < 0.05$, ** $p < 0.01$. (D) The total duration (min) of seizure behavior of stage 4 or more. Data are presented as mean $\pm$ SEM. One-way ANOVA; Bonferroni post hoc test vs. vehicle- and LPS-treated groups; *** $p < 0.001$.

Figure 4

Inhibition of TNF α receptor and RIP3 attenuated BBB leakage and endothelial cell MLKL activity, and down-regulated KIR4.1 expression. Mice were treated with 2 doses of TNF α receptor C87 or vehicle at 24 and 1 h before 4 mg/kg LPS injection (i.p.), or with RIP3 inhibitor GSK872 1 h before LPS was given, and then sacrificed 72 h after LPS administration. Hippocampal tissues of these mice were prepared for immunostaining (n = 3 per group). (A) Representative images of microglia stained with Iba-1 and monocytes stained with CD68 in the CA3 region of the hippocampus. Increased cell size and irregular shape identified as the activated microglia. (B) The proportions of activated microglia and (C) the number/mm² of CD68-positive cells in the CA3 of the hippocampus were estimated. (D) Representative images of astrocytes stained with GFAP and Kir4.1. Hypertrophic morphology identified as the activated astrocytes. (E) The proportions of activated GFAP-positive astrocytes and (F) the densitometric analysis of Kir4.1- and GFAP-positive protein levels in CA3 hippocampus. (G) Representative images of BBB endothelial cells stained with CD31 and p-MLKL in the CA3 regions and (H) densitometric analysis of co-localized CD31- and p-MLKL-positive protein levels. Densitometry was semi-quantified using ImageJ software. Data represent the mean $\pm$ SEM of values from three animals per treatment group. One-way ANOVA; Bonferroni post hoc test vs. vehicle-treated mouse group; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5

GSK872 suppressed RIP3-mediated necroptosis and restored Kir4.1 protein expression in mice within 3 days after LPS injection. (A) The experimental protocol. Mice were treated with GSK872 (2 mg/kg, i.p.) or vehicle (0.25% DMSO) 1 h before 4 mg/kg LPS was given. The hippocampus was obtained to assess the protein levels at 6, 48, and 72 h after LPS injection ($n = 3$ per group, each time point). The expression levels of p-RIP3, RIP3, p-MLKL, and MLKL detected RIP3-mediated necroptosis. (B) A representative Western blot showing the specific bands for these proteins and Kir4.1 protein. An equal amount of protein sample (20 μ g) obtained from the hippocampus homogenate was applied to each lane, and β -actin protein was used as the internal control. (C–E) Bar graphs showing the densitometric analysis of the molecules p-RIP3 and total RIP3 (C), p-MLKL and total MLKL (D), and Kir4.1 (E), normalized to total RIP3, total MLKL or β -actin expression, respectively. Each bar represents the mean $\pm$ SEM. One-way ANOVA; Bonferroni post hoc test vs. saline- and vehicle-treated groups or LPS- and vehicle-treated groups; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 6

GSK872 attenuated the increased extracellular levels of potassium and glutamate in the hippocampus within 72 h after LPS injection. (A) The experimental protocol. One day after the chronic indwelling guide cannula was implanted in the hippocampus, mice were treated with GSK872 (2 mg/kg, i.p.) or vehicle (0.25% DMSO) 1 h before 4 mg/kg LPS was given. Microdialysis experiments were carried out 1 h before GSK872 injection. Samples were collected once every 30 min for 5 h from each mouse after 1 h stabilization, and the first three samples were defined as the basal levels (100%). (B and C) The time course concentrations of extracellular potassium and glutamate in the hippocampus after GSK872 plus LPS treatment ($n = 6$ per group). Data represent percentages (mean $\pm$ standard error of the mean (SEM)) of values. Using two-way repeated measures ANOVA analysis, Bonferroni post hoc analysis revealed that GSK872 significantly inhibited the increase in potassium levels ($F(1, 110) = 25.87$, $p < 0.0001$) and glutamate levels ($F(1, 110) = 12.59$, $p = 0.0006$) induced by LPS given systemically. At 72 h after LPS injection, additional microdialysis experiments were performed in treated mice with sample collection once every 30 min for 2 h from each mouse after 1 h stabilization. (D and E) Mean levels of extracellular potassium and glutamate in the hippocampus 72 h after LPS treatment ($n = 6$ per group).

Data represent the mean $\pm$ SEM of values. One-way ANOVA; Bonferroni post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001.

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