Cordycepin Confers long-term neuroprotection via inhibiting neutrophil infiltration and neuroinflammation after traumatic brain injury

Pengju Wei
Fudan University
Yichen Huang
Fudan University
Chen Luo
Fudan University
Dilidaer Misilimu
Fudan University
Huimei Wen
Fudan University
Peng Jin
Fudan University
Chuhua Li
South China Normal University
Wenting Zhang
Fudan University
Ye Gong
Fudan University
Yanqin Gao (yqgao@shmu.edu.cn)
Fudan University https://orcid.org/0000-0002-4915-9819

Research

Keywords: white matter injury, neutrophil, microglia/macrophage, blood brain barrier, traumatic brain injury

Posted Date: January 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-141078/v1

Abstract

Background

Traumatic brain injury (TBI) secondary injury, especially white matter injury (WMI), is highly sensitive to neuroinflammation, which further leads to unfavored long-term outcomes. Although the cross-talk between the three active events, immune cell infiltration, BBB breakdown, and proinflammatory microglial/macrophage polarization, play a role in the vicious cycle, its mechanisms are not fully understood. It has been reported that cordycepin, an extract from *cordyceps militaris*, can inhibit TBI-induced neuroinflammation although the long-term effects of cordycepin remain unknown. Here, we report our investigation of cordycepin's long-term neuroprotective function and its underlying immunological mechanism.

Methods

TBI mice model was established with a controlled cortical impact (CCI) method. Cordycepin was intraperitoneally administered twice daily for a week. Neurological outcomes were assessed by behavioral tests, including grid walking test, cylinder test, wire hang test, and rotarod test. Immunofluorescence staining, transmission electron microscopy, and electrophysiology recording were employed to assess histological and functional lesions. Quantitative-PCR and flow cytometry were used to detect neuroinflammation. The tracers of Sulfo-NHS-biotin and Evans blue were assessed for the Blood-brain barrier (BBB) leakage. Western blot and gelatin zymography were used to analyze protein activity or expression. Neutrophil depletion in vivo was performed via using Ly6G antibody intraperitoneal injection.

Results

Cordycepin administration ameliorated long-term neurological deficits and reduced neuronal tissue loss in TBI mice. Meanwhile, the long-term integrity of white matter was also preserved, which was revealed in multiple dimensions, such as morphology, histology, ultrastructure, and electrical conductivity. Cordycepin administration inhibited microglia/macrophage pro-inflammatory polarization and promoted anti-inflammatory polarization after TBI. BBB breach was attenuated by cordycepin administration at 3d after TBI. Cordycepin suppressed the activities of MMP-2 and MMP-9 and the neutrophil infiltration at 3d after TBI. Moreover, neutrophil depletion provided a cordycepin-like effect, and cordycepin administration united with neutrophil depletion didn't show a benefit of superposition.

Conclusions

The long-term neuroprotective function of cordycepin was via suppressing neutrophil infiltration, thereby preserving BBB integrity and changing microglia/macrophage polarization. These findings provide significant clinical potentials to improve the quality of life for TBI patients.

Introduction

TBI is one of the leading causes of death and disability among all ages worldwide[1]. TBI causes multiple functional deficits in the brain, such as sensory, motor, and cognitive functions, which related to the damaged regions[2]. Neurons and glial cells in damaged tissue release excessive glutamate leading to excitatory toxicity; energy metabolism disorder results in ATP depletion, mitochondrial dysfunction, oxidative stress, and other events leading to neuron death[3–6]. Besides, inflammatory mediators produced by damaged cells recruit cerebral microglia and peripheral immune cells which release inflammatory factors, thus promoting secondary neuronal damage[7]. White matter, which constructs 50% human brain, is mainly consisted of neurite and myelin sheaths, and it has been proved necessary for advanced brain functions[8]. Compared to grey matter (GM), WM is more susceptible to TBI[9]. WMI which includes axonal injury and myelinic degeneration is also responsible for neurological deficits in TBI[10, 11].

WMI in TBI is escalated by altered microglial/macrophage functional state and subsequent neuroinflammation[12]. After TBI, peripheral immune cells are recruited into the brain parenchyma and whereby exacerbate the breakdown of the brain-blood barrier (BBB) and promote microglia/macrophages to polarize toward a pro-inflammatory state[13, 14]. BBB disruption can simultaneously cause microglial activation in return, forming a feed-forward loop[15, 16]. In this way, three active events - immune cell infiltration, BBB disruption, and proinflammatory microglial/macrophage state - reinforce one other, creating a vicious cycle that aggravates brain injury continuously. Although the cross-talk between blood-derived immune cells and cerebral microglia plays a role in the vicious cycle, the mechanisms are not fully understood yet.

Cordycepin, a natural bioactive substance extracted from *Cordyceps militaris*, has been reported to have neuroprotective effects against cerebral ischemia and hemorrhage injury[17, 18]. WIB-801C, a cordycepin-enriched extract of *Cordyceps militaris*, ameliorates infiltration of neutrophils and improves functional recovery after spinal cord injury[19]. So far, only one article reported that cordycepin suppressed neuroinflammation and BBB leakage and reduced neurological severity scores in TBI rats within 24 h post-TBI[20]. However, whether cordycepin has long-term neuroprotective effects and the underlying mechanism are not clear.

In this study, we used a CCI-induced TBI mice model to investigate the neuroprotective effects of cordycepin and the underlying mechanism. We demonstrated that cordycepin has long-term neuroprotection against traumatic brain injury. Furthermore, we found cordycepin specifically inhibits neutrophil infiltration to ameliorate BBB disruption and pro-inflammatory microglia/macrophage polarization, and consequently improve neurological function.

Materials And Methods

Animals

Mile C57BL/6 mice, 9–10 weeks old (25 ~ 28 g), were purchased from Shanghai JieSiJie Laboratory Animal Co., Ltd. Mice were housed at constant humidity and temperature with a 12 h light/dark cycle with food *ad libitum*. All animal experiments in the study were approved by the Animal Care and Use Committee of Shanghai Medical College, Fudan University.

TBI model and cordycepin administration

TBI was conducted using a CCI device (TBI 0310, Precision Systems and Instrumentation). The procedure was described previously[21]. Briefly, mice were anesthetized with 3% isoflurane in 70% N_2/30% O_2 mixture and fixed on stereotaxic apparatus. A midline incision was made to expose the skull. An approximate 4-mm craniotomy was performed over the right parietotemporal cortex using a motorized drill. The CCI was centered 2.5 mm lateral to the midline, 0.5 mm anterior to bregma and was conducted with a 3-mm flat-tip impounder (velocity, 3.5 m/s; duration, 150 ms; depth, 1.5 mm). After CCI, the scalp incision was sutured, and the mice were placed on a heating pad until anesthesia recovery. Sham mice received all these procedures, except for CCI.

Cordycepin was provided by Guangdong Provincial Key Lab of Biotechnology for Plant Development, South China Normal University. Mice were randomly assigned to the injection of Cordycepin (dissolved in saline, 10 mg/kg) intraperitoneally or an equal volume of saline (vehicle) 2 h post-TBI and twice daily for 7 successive days.

Behavioral tests

Grid walking test, cylinder test, wire hang test, and rotarod test were carried out as previously described[22, 23].

The grid walking test was performed on a 40cm × 20 cm flat stainless-steel grid with a mesh size of 2cm × 2 cm 35 cm above the ground. The movement of each mouse was recorded by a camera. A foot-fault step was defined as a step that the foot didn't place on the grid correctly. The data were presented as a percentage of foot-fault steps in total steps in 1 min.

The cylinder test was carried out in a transparent cylinder (9 cm in diameter and 38 cm in height). A camera was placed over the cylinder to for 10 min recording after a mouse was placed in. Forepaw (left/right/both) touches on the wall of the cylinder were recorded from the video. The data was calculated based on the formula: left/(left + right + both) × 100%.

The *wire hang test* apparatus was a 50 cm long 2 mm in diameter steel wire 40 cm over the ground with two platforms at each end. Mice were placed on the middle of the wire with the two forepaws and were observed for 30 s in 3 ~ 4 trials. The mice were scored according to the following criteria: 0, fell off; 1, hung onto the wire with 2 forepaws; 2, hung onto the wire with added attempts to climb onto the bar; 3,

hung onto the wire with 2 forepaws and 1 or both hind paws; 4, hung onto the wire with all 4 paws and with the tail wrapped around the wire; 5, escaped to one of the platforms.

The rotarod test was performed with 47650 Mouse Rota-Rod (UGO BASILE). After the mice were placed on the rods, the rods began to rotate at a speed of 5r/min and accelerated to 40r/min within 300 s and went on rotating at the constant speed for 200 s (total 500 s). The latency to fall off the rotating rod was recorded. All animals were tested 3 times with a break of at least 10 min between tests. Data were presented as the mean value from 3 tests.

All the behavioral tests above were pre-trained for 3 days before TBI, and were performed in a blind manner.

Immunofluorescence staining

After anesthetized deeply, the mice were perfused intracardially with ice-cold saline and 4% paraformaldehyde. The brain was removed and immersed in 4% paraformaldehyde, 20% sucrose, 30% sucrose in sequence to complete after-fixing and dehydration. 25 μm coronal section was collected using the freezing microtome (HM525NX, ThermoFisher).

After washed in PBS and PBS + 0.3% Triton brain sections were incubated in PBS + 1% Triton to break the cell membrane and then were blocked with 10% goat serum or donkey serum for 1 h. M.O.M. Kit was used to block for mouse antibody. Brain sections were incubated with primary antibodies overnight at 4℃. After washing, the sections were incubated for 2 h at room temperature with secondary antibodies conjugated with Alexa Fluor-488/594/647. DAPI-Fluoromount-G was added and cover the sections with a piece of clear coverslip.

10 serial sections with an interval of 11 sections were stained to calculate the volume (mm^3) of tissue loss (= volume of left hemisphere - volume of right hemisphere).

Transmission electron microscopy

Fresh brain tissue (thickness < 1 mm) was fixed with 2.5% glutaraldehyde at 4℃. After dehydrated in graded ethanol and acetone, brain tissue was embedded in epoxy resin and made into 50 nm sections. The prepared ultrathin sections were stained with uranyl acetate and lead citrate, and then observed under Philips CM120 transmission electron microscope and photographed.

Electrophysiology

Compound action potentials (CAPs) in the external capsule were recorded as previously described[24]. Mice were anesthetized with isoflurane and were decapitated to remove the brains rapidly. 350 μm brain slices coronal were made using a vibratome (1200s, Leica). Slices were transferred in artificial cerebrospinal fluid (aCSF) saturated with 95% O_2 + 5% CO_2 mixture at 32℃ for 0.5 h and at room temperature for 1 h subsequently for recovery. The aCSF contained 124 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, 24 mM $NaHCO_3$, 1.3 mM $MgSO_4$, and 10 mM D-glucose. A concentric stimulating

electrode and a glass microelectrode (5 ~ 8MΩ) were used for stimulating and recording. CAPs were induced by monophase square waves (0.1 ms duration) with the stimulus generator (STG 4002, Multichannel). Signals evoked were amplified by Axoclamp 700B (Molecular Devices), and digitized Axon Digidata 1440A (Molecular Devices).

Quantitative real-time PCR (q-PCR)

Real-time PCR was performed as described[25]. Briefly, the PCR was performed with the Hieff® qPCR SYBR® Green Master Mix (No Rox) (Shanghai Yeasen, China) in Mastercycler ep realplex (Eppendorf) using corresponding primers (supplementary materials). The cycle time values of the genes of interest were first normalized with glyceraldehyde-3-phosphate dehydrogenase of the same sample and then the gene expression levels were calculated and expressed as fold.

Flow cytometry

Blood was obtained from the heart of anesthetized mice. And then mice were perfused with cold Hank's balanced salt solution (HBSS) to collect the brains. Brains were digested with Neural Tissue Dissociation Kit (Miltenyi) using gentleMACS Octo Dissociator with heaters (Miltenyi). Immune cells were enriched with Percoll (GE Healthcare) and Ficoll (eBioscience) from brain and blood respectively. Cells were blocked with rat anti-mouse CD16/32 (eBioscience), and then were incubated with eFluor 450 conjugated antibody to CD45 (eBioscience), APC-Cy7 conjugated antibody to CD11b (eBioscience), PerCP-Cy5 conjugated antibody to CD11c (eBioscience), BV605 conjugated antibody to Ly6C (BD), PE conjugated antibody to Ly6G (eBioscience), APC conjugated antibody to CD3 (eBioscience), FITC conjugated antibody to CD19 (eBioscience). Flow cytometry was performed on Beckman CytoFlex. Data were analyzed with FlowJo software.

BBB Permeability Assay

Evans blue and sulfo-NHS-biotin were used to determined BBB permeability. Briefly, mice were injected with 4% Evans blue (200 mg/kg) from the femoral vein and perfused at 2 h after injection. Brains were dissected into 1 mm-thick slices. Blue areas on each slice were added up and multiplied by the thickness to calculated leakage volume.

Sulfo-NHS-biotin (7 mg/kg) was dissolved in saline and injected in the femoral vein of the mice. Mice were perfused at 1 h after injection and fixed with 4% paraformaldehyde for the freezing Sect. 10 serial sections same as preceding text were stained with streptavidin conjugated Cy3. The volume of Sulfo-NHS-biotin leakage was obtained by accumulating the area of Sulfo-NHS-biotin in each section and multiplying by 0.3 mm.

Western Blot

Total protein was extracted with RIPA lysis buffer (Shanghai Beyotime, China) containing protease inhibitor cocktails (Roche). Equal amounts of protein were run on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. 3% BSA was used for blocking. The membranes were incubated

with primary antibodies, rabbit anti-ZO-1 (1:250 61-7300, Invitrogen), rabbit anti-MMP-2 (1:1000 ab97779, Abcam), rabbit anti-MMP-9 (1:1000 ab38898, Abcam), overnight at 4℃, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Blots were imaged using ChemiDoc MP (Bio-rad).

Gelatin zymography

As described previously[26], undenatured protein was mixed with loading buffer free of beta-mercaptoethanol and loaded on SDS-PAGE gel containing 1% gelatin. After electrophoresis, gel was washed with 2.5% TritonX-100 and ddH_2O, and then incubated in medium (50 mM Tris, 150 mM NaCl, 10 mM CaCl2, 1‰ TritonX-100, pH7.5) for 42 h at 37℃. At last, the gel was stained with 0.5% Coomassie blue staining solution and was imaged using ChemiDoc MP (Bio-rad) with a white tray.

Neutrophils depletion

As described[27], to depletion neutrophils, each mouse received twice intraperitoneal administration of 100 μg monoclonal anti-mouse Ly6G (clone 1A8, BE0075-1, BioXcell) at 24 h before TBI and 24 h after TBI. Each control mouse received 100ug isotype control antibody rat IgG2a in the same manner.

Image processing and Statistical analysis

All images were quantitated with Image J software. 3D images were made with Imaris 9. The data were analyzed using GraphPad Prism 8. All values are presented as mean ± SE. Multiple comparisons were analyzed using one-way ANOVA and post hoc Bonferroni test. Multiple comparisons over time were analyzed using two-way ANOVA. When comparing two groups, unpaired Student's t-test was used. $p < 0.05$ was considered be statistically significant.

Results

Cordycepin administration ameliorates neurological deficits after TBI, and reduces both GMI and WMI.

Since TBI causes multiple sensorimotor function defects, we performed the cylinder test, grid walking test, wire hanging test, and the rotarod test to evaluate the effect of cordycepin on neurological function after TBI (Fig. 1a). No obvious difference was observed between cordycepin and saline administration in sham mice. However, one-week tests after TBI showed that cordycepin reduced forelimb asymmetry in the cylinder test, decreased foot fault rate in the grid walking test, improved scores in the wire hanging test. Rotarod test was continued to 28d after TBI for tracking long-term outcomes. Cordycepin prolonged duration on rotarod even on 28d after TBI, suggesting a long-term pro-recovery effect of cordycepin. These data indicated that cordycepin administration demonstrated superior sensorimotor functional recovery after TBI compared with vehicle mice.

Immunohistochemical staining of MAP2 on the serial coronary sections was conducted for assessing the neuronal tissue loss (Fig. 1b). Cordycepin significantly decreased the volume of neuronal tissue loss compared with vehicle mice (**Fig. c**).

Besides grey matter, white matter is also the main target of TBI closely linked to neurological impairment[28, 29]. Firstly, MBP and NF-200 were stained to observe myelin and neurofilament changes in the striatum after TBI (Fig. 1d). The fluorescence intensity of NF-200 significantly reduced after TBI, whereas cordycepin increased the NF-200 fluorescence intensity significantly (Fig. 1e, up-left panel). White matter fiber tracts in the striatum were perpendicular to the coronal sections we chose, constructing bundles in the sections. Although no significant difference was found between the groups by measuring the fluorescence intensity of MBP staining (Fig. 1e, **down-left panel)**, we found that the bundles became loose and the space among the bundles decreased in perilesional striatum after TBI. Further analysis revealed that the area proportion of MBP staining bundles in vehicle mice significantly increased because of the atrophy of striatum, while cordycepin reversed the increase (Fig. 1e, **down-right panel**). The data manifested the effect of cordycepin on maintaining white matter structure.

It was reported that TBI led to axonal degeneration and the myelin debris could exist for a long time[30]. So MBP staining may be difficult to reflect the factual conditions of myelin. Secondly, regions of corpus callosum (CC) were dissected from mice in all groups on 35 days after TBI to examine the ultrastructure of axon and myelin via transmission electron microscope (Fig. 1f). After TBI, no substantial variety were observed in the myelinated axon number (**Fig. S1a**) or degenerating myelinated axon number (**Fig. S1b**). But the number of nonmyelinated axons were significantly decreased by TBI and restrained by cordycepin significantly (**Fig. h left panel**). Cordycepin administration tendentiously abolished the TBI-induced degenerating nonmyelinated axons (Fig. 1h, **right panel**, $p = 0.0507$). Interestingly, although no obvious changes were observed in the two types of myelinated axon numbers, g-ratio of myelinated axons was reduced by TBI, whereas cordycepin ameliorated the reduction (Fig. 1g). These data indicated cordycepin protecting the nonmyelinated axons in number and the myelin in structure.

CAPs were usually measured to assess the conductive capacity of white matter fibers. Due to the saltatory conduction of nervous impulse between Ranvier nodes, the nerve conductive velocity on myelinated axons is faster than nonmyelinated axons. The difference of conductive velocity can be presented on the N1 and N2 peaks in CAPs. Thirdly, mice were sacrificed 35d after TBI and sections were prepared to record CAPs in CC with electrophysiology technique (**Fig. S1c**). The amplitudes of N1 and N2 were both deceased by TBI (**Fig. S1d**, Fig. 1i), while only N2 amplitude was significantly rescued by cordycepin (Fig. 1i). The results suggested cordycepin improved nerve conductive capacity of nonmyelinated fibers.

Taken together, cordycepin administration ameliorated TBI-induced neurological deficits and brain tissues injury, including grey matter and white matter injury.

Cordycepin administration inhibits pro-inflammatory microglia/macrophage and promotes anti-inflammatory microglia/macrophage

Neuroinflammation is one of the main causes for secondary WMI in TBI. Our previous study has proved the close correlation between microglia/macrophage polarization mediated neuroinflammation and WMI[31]. According to the temporal characteristic of microglia/macrophage polarization, we double stained CD16/Iba1 (pro-inflammatory) and CD206/Iba1 (anti-inflammatory) on 3d and 7d after TBI (Fig. 2a). Cordycepin inhibited pro-inflammatory microglia/macrophage both in the cortex and striatum on 3d and 7d (Fig. 2b), and promoted anti-inflammatory microglia/macrophage in the striatum on 7d after TBI (Fig. 2c). Besides, the polarization markers were detected on 7d after TBI using q-PCR. Markers related to pro-inflammatory microglia/macrophage such as CD16 and IL17a were significantly decreased by cordycepin (Fig. 2d). Meanwhile, markers related to anti-inflammatory microglia/macrophage such as CD206 and IL-10 were increased in the cordycepin administration group (Fig. 2e). The results demonstrated cordycepin inhibited pro-inflammatory microglia/macrophage and promoted anti-inflammatory microglia/macrophage polarization after TBI.

Cordycepin administration protects BBB integrity after TBI.

Normally, BBB prevents toxic substances in the blood from leaking into the brain parenchyma to maintain homeostasis in the brain. Preservation of BBB integrity contributes to reducing pro-inflammatory microglia/macrophage activation[32]. Therefore, we investigated the effects of cordycepin on BBB integrity in TBI mice on 3d after TBI. Firstly, the tracers of sulfo-NHS-biotin (MW 443.4 Da) and Evans blue (Evans blue-Albumin, MW 68,500 Da)[33] were injected into the femoral vein to examine the BBB permeability. Compared with TBI + vehicle group, sulfo-NHS-biotin or Evans blue leakage volume were significantly reduced by cordycepin (Fig. 3a-b, **Fig. S2**). The data collaboratively indicated that cordycepin reduced the leakage of both large and small molecules in TBI mice.

Endogenous IgG in the blood can leak into brain parenchyma through dysfunctional BBB, which makes IgG an important endogenous biomarker to assess the BBB leakage[34]. We stained 3d-post-TBI frozen section with anti-mouse IgG antibody to detect the leakage of endogenous substance. IgG positive area was remarkably reduced in the cordycepin administration group, compared with the vehicle TBI mice (Fig. 3c **and d, up panel**). Additionally, cordycepin reduced fluorescence intensity of IgG staining in the striatum suggesting the reduction of IgG leakage in amount (Fig. 3d, **down panel**).

Tight junction proteins, such as ZO-1, are the main components of tight junctions between cerebrovascular endothelial cells supporting the BBB integrity. TBI distinctly reduced ZO-1 expression detected by western blot, whereas, cordycepin reduced ZO-1 protein loss after TBI (Fig. 3e-f). We also observed the ultrastructure of BBB with a transmission electron microscope (Fig. 3g). TBI led to edema in astrocyte end-feet, basement membrane thickening, and loss in tight junction proteins, while cordycepin reversed these degenerations.

MMPs are responsible for tight junction protein degradation in brain injury. Our previous study showed knocking out or pharmacological inhibition for MMPs reduced BBB permeability after brain injury[35]. MMP-2 and MMP-9 are the main two MMPs involved in BBB disorder, and they mediate early reversible and late irreversible BBB dysfunction respectively[36]. Accordingly, we observed the effects of cordycepin

on MMP-9 and MMP-2 at 24 h and on 3d after TBI. Western blot showed that cordycepin significantly reduced the protein level of MMP-9 on 3d after TBI, but not MMP-2 (Fig. 3h-i**)**. Many factors participate in MMPs post-transcriptional control. For example, endogenous tissue inhibitors of metalloproteinases (TIMPs) inhibit MMPs activity by high-affinity binding to the catalytic structural domain of MMPs in a non-selective manner[37]. Consequently, it's necessary to pay attention to the activity when evaluating the effects of MMPs. Based on the properties of MMP-9 and MMP-2 to catalytic degrade gelatin, we employed gelatin zymography to assess their activity. Zymography results showed that the activity of MMP-9 and MMP-2 was both reduced by cordycepin on 3d after TBI (Fig. 3j-k**)**.

Cordycepin administration inhibits neutrophil infiltration, without affect peripheral immune system

The massive influx of circulating leukocytes is the biggest contributors to MMPs surging in the early stage of brain injury, especially for neutrophil, which has been proved to be the main source of MMP-9[38, 39]. The main kinds of immune cells, including microglia, macrophage and its inflammatory subset, neutrophil, T cell, B cell, and dendritic cell, were analyzed in the brain on 3d after TBI by flow cytometry (Fig. 4a). The proportion of microglia in total cells decreased, while the proportions of macrophage, inflammatory macrophage, and neutrophil increased after TBI. Cordycepin made no significant differences in the amounts of microglia, macrophage, and inflammatory macrophage in TBI mice. However, the proportion of neutrophils was decreased by cordycepin administration. The proportion of T cell, B cell, and dendritic cell didn't change obviously in each group (Fig. 4b).

Furthermore, immunofluorescent staining of Ly6G showed that the neutrophils adhered to blood vessels or went deep in the brain parenchyma indicating that neutrophils did infiltrate after TBI (Fig. 4c). Most infiltrated neutrophils surrounded the lesion, whereas few were found in other places (Fig. 4d). The number of neutrophils were remarkably reduced by cordycepin in the total or only the cortex (Fig. 4e, **up panel**) and had a downtrend in the striatum (Fig. 4e, **down-left panel**, $p = 0.0501$). More interestingly, we noticed that the number of neutrophils was significantly associated with the number of pro-inflammatory microglia/macrophage (Fig. 4e **down-right panel)** suggesting that infiltrated neutrophils might promote microglia/macrophage polarizing to pro-inflammatory type.

The immune cells in the blood were also detected by flow cytometry (Fig. 5). However, macrophages, inflammatory macrophages, neutrophils, T cells, B cells, and dendritic cells in the blood weren't significantly affected by TBI or cordycepin at 3d after TBI. The results indicated cordycepin did not aggravate immunosuppression induced by brain injury obviously, moreover, its inhibition to neutrophils was more likely due to the chemotaxis in CNS.

Inhibiting of neutrophil infiltration by cordycepin ameliorates BBB disruption and microglia/macrophage pro-inflammatory polarization

To investigate the relationships among three events (neutrophil infiltration, BBB disruption, and microglia/macrophage polarization), anti-Ly6G monoclonal antibody (anti-Ly6G) was intraperitoneal injected twice for neutrophil depletion at 24 h pre- and post-TBI. By flow cytometry analyzing the blood, anti-Ly6G specifically depleted neutrophils without significant effect on macrophages (**Fig. S3**, Fig. 6a-b). Neutrophil depletion remarkably reduced neutrophils (Fig. 6c-d), IgG leakage (Fig. 6e-f), pro-inflammatory microglia/macrophage (Fig. 6g-h) in the brain, while cordycepin combined with neutrophil depletion didn't exert a further influence on them (Fig. 6c-h). Moreover, neutrophil depletion significantly reduced the foot-fault rate on the grid test and prolonged the duration on rotarod at 3 day after TBI, whereas neutrophil depletion and cordycepin-combined group did not show further effect on neurological deficits (Fig. 6i). Interesting, the foot fault rate showed a significant positive correlation with neutrophil in both the cortex and the striatum (Fig. 6j).

These data manifested that neutrophil infiltration contributed to BBB disruption and microglia/macrophage pro-inflammatory polarization. Neutrophil infiltration might have an upstream position among the three events in the effect pathway of cordycepin.

Cordycepin inhibits adenosine A2a receptor expression and ameliorates inflammatory cytokines expression after TBI.

Due to the remarkably similar molecular structure to adenosine (Fig. 7a), cordycepin was considered to probably act on adenosine receptors. And it has been proved that cordycepin has interactions with adenosine A1, A2a receptors[40–42]. After TBI, adenosine receptors begin to work within dozens of minutes[43]. Furthermore, we examined the changes of adenosine A1, A2a receptors at 24 h after TBI. Firstly, their mRNAs were detected by qPCR. A1 receptor mRNA expression had no substantial difference between each group (Fig. 7b, **left panel**), but TBI remarkably upregulated mRNA of A2a receptor, which was inhibited by cordycepin (Fig. 7b, **right panel**). The expression of adenosine A2a receptor by immunofluorescence staining showed a consistent phenomenon (Fig. 7c-d).

It was reported that adenosine A2a receptor knockout reduced the expression of pro-inflammatory cytokines and chemokines after TBI[44]. Like adenosine A2a receptor knockout, cordycepin administration also significantly reduced the expression of TNF-α, IL-1β and CCL3 mRNA in TBI mice (Fig. 7f). These data suggested that cordycepin might work through inhibiting adenosine A2a receptor.

Discussion

TBI, a common disease in departments of neurology, often leads to disability or even threats to lives. In our study, we investigated the anti-inflammatory properties of cordycepin on TBI mice. Our data showed that cordycepin ameliorated neurological deficits and reduced neuronal damage and WMI, exhibiting long-term neuroprotection. Pro-inflammatory microglia/macrophage polarization was inhibited while anti-inflammatory microglia/macrophage polarization was promoted in TBI mice administered with cordycepin. Cordycepin also attenuated neutrophil infiltration and BBB disruption. Although there are complex interrelationships among microglia/macrophage polarization, neutrophil infiltration, and BBB

breakdown, neutrophil depletion indicated cordycepin was specific to neutrophil infiltration. We also observed that cordycepin reversed TBI-induced adenosine A2a receptor high expression, as well as cytokines and chemokines, hinting a connection between adenosine A2a receptor and neutrophil infiltration.

TBI has complicated secondary injury pathways which cause long-term progressive brain jury even lasting for years[45]. Therefore, long-term neuroprotection of a candidate drug is essential for clinical translation. In addition to short-term behaviors, we also observed long-term behaviors and neuronal tissue loss, providing long-term neuroprotection evidences for cordycepin. TBI-induced WMI is positively correlated with neurological deficits, reducing WMI helps improve neurological functions[46]. We observed that cordycepin reserved the morphology of white matter and neurofilament on 35d after TBI. MBP expression didn't change at the statistical level, however, it was consistent with the previous finding that the residual myelin debris can exist for a long time after axonal fracture and degeneration caused by TBI[30]. We solidified the results by observing the ultrastructure of white matter using a transmission electron microscope which provided evidence for cordycepin's ameliorations on axon and myelin damage. Moreover, CAPs recording offered proofs for functional improvement of white matter. Our data not only manifested the long-term neuroprotection of cordycepin but also illustrated the protective pathway of white matter.

Neuroinflammation is an important cause for secondary injury in TBI, especially for secondary WMI[47, 48]. Microglia is the resident immune cell in CNS, which plays a central role in CNS inflammation[49]. The infiltrated macrophages have functional overlap with microglia post TBI[50]. Classical activated microglia/macrophage produces pro-inflammatory factors presenting pro-inflammatory function that promotes tissue damage and hinders damaged tissue remodeling, while alternative activated microglia/macrophage clears up cell debris and produces anti-inflammatory factors presenting anti-inflammatory function that promoting tissue repair[51, 52]. Microglia/macrophage functional polarization is a promising target of pharmacological intervention in TBI[46, 53, 54]. We observed that pro-inflammatory microglia/macrophage had a huge surge in cell number from 3d to 7d after TBI, while anti-inflammatory microglia/macrophage had minute fluctuation between these days. Besides, the pro-inflammatory microglia/macrophage were far more than anti-inflammatory microglia/macrophage on 7d after TBI. These observations were consistent with the findings of Wang et al generally[31], indicating that although endogenous repair mechanism had been activated, it was finally defeated by pro-inflammatory microglia/macrophage over time, resulting in out-of-control neuroinflammation. Cordycepin administration significantly reduced pro-inflammatory microglia/macrophage at both 3d and 7d after TBI and increased anti-inflammatory microglia/macrophage on 7d after TBI. The expressions of their markers corroborated cordycepin's effect on microglia/macrophage. The data provided confirmed evidence for the anti-inflammatory properties of cordycepin in TBI.

Under normal conditions, BBB blocks the interference of blood cells and substances for CNS[55]. When BBB are damaged, peripheral immune cells and toxic substances leak into the brain parenchyma that can further exacerbate neuroinflammation, including microglia/macrophage pro-inflammatory

polarization[15, 32]. We found that cordycepin reduced BBB leakage through injecting tracers into the femoral vein and labeling endogenous IgG. Tight junction protein ZO-1 expression was upregulated in TBI mice treated with cordycepin that provided a foundation for cordycepin protecting the structure and function integrity of BBB. MMPs degrades vascular ECM proteins and tight junction proteins, leading to BBB breakdown under pathological conditions[56]. MMP-2 and MMP-9 are the main two MMPs involved in BBB break down and inhibition of the two MMPs contributes to BBB protection[57, 58]. Cordycepin decreased MMP-9 expression and inhibited the activity of MMP-2 and MMP-9, suggesting cordycepin protected BBB via MMP-2/9 pathway. More interestingly, neutrophils are the main source of MMP-9 in acute brain injury[59, 60]. So, we paid our attention to neutrophil, as expected, neutrophil infiltration was inhibited significantly by cordycepin administration. And we found neutrophil infiltration related with microglia/macrophage pro-inflammatory polarization. But up to this point, all of what we have seen were mere phenomena. There were no proofs to illustrate the relationships among microglia/macrophage polarization, BBB breakdown, and neutrophil infiltration. Therefore, we explored their relationships by neutrophil depletion. Neutrophil depletion exhibited cordycepin-like effects, such as reducing neutrophil infiltration, decreasing BBB leakage, inhibiting microglia/macrophage pro-inflammation polarization, and improving neurological deficits. Moreover, cordycepin administration combined with neutrophil depletion didn’t show additive effects. These results indicated that cordycepin was specifically effective to neutrophil infiltration, and neutrophil infiltration was upstream to BBB breakdown and microglia/macrophage pro-inflammation polarization. Early neutrophil infiltration has been proved critical for the neuroinflammation induced by acute brain injury and promotes the injury[61, 62]. Neutrophils peak at 24 h and are predominant within 72 h post-TBI among peripheral immune cells infiltration, whereas microglia/macrophage polarization stands out after 72 h post-TBI[31, 63]. Precedence in time and neutrophil-produced MMPs mentioned above might be the reasons why neutrophil could be the point to break the vicious cycle of the three events. Our study also indicated that neutrophil infiltration might be a target to intervene TBI-induced inflammatory cascade reaction.

The analysis of immune cells in the blood showed no obvious difference made by cordycepin administration. The results suggested that cordycepin didn’t disturb systematic immunity, on the other hand, inhibited neutrophil infiltration by decreasing CNS chemotaxis rather than reduced neutrophils in the blood. Thus, we went back to CNS to find the reason for neutrophil inhibition. Due to the striking similarity between cordycepin and adenosine in structure, cordycepin was linked to adenosine receptors by many researchers. And our previous work demonstrated cordycepin could function through adenosine A1, A2a receptors[40–42]. We examined the expressions of the two adenosine receptors after TBI, and we observed that cordycepin reversed TBI-induced high expression of adenosine A2a receptor. Similar to adenosine A2a receptor knocking out and pharmacological inhibition[43, 64], cordycepin also inhibited TNF-α and IL-1β upregulations after TBI. Besides, we also observed that CCL3 was also inhibited by cordycepin. CCL3 recruits neutrophils through binding to the CCR1 on the neutrophil surface, neutrophil recruitment in mice with CCL3 deficiency significantly decreased as reported[65, 66]. Moreover, TNF-α and IL-1β mobilize neutrophils and induce adhesion molecule expression on cerebral endothelial and glial cells, thereby promoting neutrophil accumulation and migration into CNS[65, 67, 68]. These data

suggested that cordycepin might inhibit neutrophil infiltration by decreasing adenosine A2a receptor-mediated chemotaxis.

Although we did a series of works to illustrate the underlying mechanism of cordycepin's neuroprotection, some key links in evidence are still missing, such as how infiltrated neutrophil affects microglia/macrophage pro-inflammation polarization, and what is the detailed adenosine A2a receptor pathway of cordycepin. For next-step work, we will additionally perform in vitro experiments to complete the evidence chain.

Conclusion

We proved that the long-term neuroprotective function of cordycepin was via inhibiting neutrophil infiltration, thus preserving BBB and inhibiting microglia/macrophage pro-inflammation polarization. These findings provide a basis for cordycepin clinical translation in the future.

Abbreviations

aCSF
Artificial cerebrospinal fluid
BBB
Brain-blood barrier
CAPs
Compound action potentials
CC
Corpus callosum
CCI
Controlled cortical impact
CCL
Chemotactic cytokine ligand
CCR
Chemotactic cytokine receptor
CD
Cluster of differentiation
CNS
Central nervous system
Cor
Cordycepin
dpi
Days post injury
ECM
Extra cellular matrix

GMI
Grey matter injury
HBSS
Hank's balanced salt solution
IL
Interleukin
iNOS
Inducible nitric oxide synthase
MAP2
Microtubule-associated protein 2
MBP
Myelin basic protein
WMI
White matter injury
MMPs
Matrix metalloproteinases
MW
Molecular weight
NF-200
Neurofilament-200
PBS
Phosphate-buffered saline
q-PCR
Quantitative real-time PCR
TBI
Traumatic brain injury
TGF
Transforming growth factor
TIMPs
Endogenous tissue inhibitors of metalloproteinases
TNF
Tumor necrosis factor
veh
Vehcle
ZO-1
Zonula occludens-1

Declarations

Ethics approval and consent to participate

All animal experiments in the study were approved by the Animal Care and Use Committee of Shanghai Medical College, Fudan University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This project was supported by the Chinese Key R&D Plan of the State Ministry of Science and Technology (2017YFC1308403, 2018YFC1312604), National Natural Science Foundation of China grants (81870971, 82071311, 81571285, 81771419), and Project Supported by Shanghai Municipal Science and Technology Major Project (No.2018SHZDZX01) and ZJLab.

Authors' Contributions

YGao designed the article. PW, CLuo, YH, DM, HW, PJ and WZ performed the experiments. PW, CLuo and YGao analyzed the data. PW, YC and YGao wrote the manuscript. YGao and YGong critically edited the manuscript. CLi provided the cordycepin.

Acknowledgements

Not applicable.

Author information

Pengju Wei, Yichen Huang, Chen Luo, Dilidaer Misilimu, Huimei Wen, Peng Jin, Wenting Zhang, Ye Gong and Yanqin Gao.

Affiliation

Department of Critical Care Medicine and Neurosurgery of Huashan Hospital, State Key Laboratory of Medical Neurobiology, MOE Frontiers Center for Brain Science, and Institutes of Brain Science, Fudan University, Shanghai 200032, China.

Chuhua Li.

Affiliation

School of Life Science, South China Normal University, Guangzhou 510631, China.

Corresponding authors

Correspondence to Dr. Yanqin Gao, State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai 200032. China. E-mail address: yqgao@shmu.edu.cn.

Or Dr. Ye Gong, Department of Intensive Care Unit and Department of Neurosurgery of HuaShan Hospital, Fudan University, Shanghai, 200040, China. E-mail address: gong_ye@fudan.edu.cn

References

1. Injury GBDTB, Spinal Cord Injury C: **Global, regional, and national burden of traumatic brain injury and spinal cord injury, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016.** *Lancet Neurol* 2019, **18:**56-87.
2. Stocchetti N, Carbonara M, Citerio G, Ercole A, Skrifvars MB, Smielewski P, Zoerle T, Menon DK: **Severe traumatic brain injury: targeted management in the intensive care unit.** *Lancet Neurol* 2017, **16:**452-464.
3. Rana A, Singh S, Sharma R, Kumar A: **Traumatic Brain Injury Altered Normal Brain Signaling Pathways: Implications for Novel Therapeutics Approaches.** *Curr Neuropharmacol* 2019, **17:**614-629.
4. Zhou Z, Austin GL, Young LEA, Johnson LA, Sun R: **Mitochondrial Metabolism in Major Neurological Diseases.** *Cells* 2018, **7**.
5. Khatri N, Thakur M, Pareek V, Kumar S, Sharma S, Datusalia AK: **Oxidative Stress: Major Threat in Traumatic Brain Injury.** *CNS Neurol Disord Drug Targets* 2018, **17:**689-695.
6. Ng SY, Lee AYW: **Traumatic Brain Injuries: Pathophysiology and Potential Therapeutic Targets.** *Front Cell Neurosci* 2019, **13:**528.
7. Needham EJ, Helmy A, Zanier ER, Jones JL, Coles AJ, Menon DK: **The immunological response to traumatic brain injury.** *J Neuroimmunol* 2019, **332:**112-125.
8. Lorenzini L, Fernandez M, Baldassarro VA, Bighinati A, Giuliani A, Calza L, Giardino L: **White Matter and Neuroprotection in Alzheimer's Dementia.** *Molecules* 2020, **25**.
9. Armstrong RC, Mierzwa AJ, Marion CM, Sullivan GM: **White matter involvement after TBI: Clues to axon and myelin repair capacity.** *Exp Neurol* 2016, **275 Pt 3:**328-333.
10. McDonald S, Dalton KI, Rushby JA, Landin-Romero R: **Loss of white matter connections after severe traumatic brain injury (TBI) and its relationship to social cognition.** *Brain Imaging Behav* 2019, **13:**819-829.
11. Filley CM, Kelly JP: **White Matter and Cognition in Traumatic Brain Injury.** *J Alzheimers Dis* 2018, **65:**345-362.

12. Lee J, Hamanaka G, Lo EH, Arai K: **Heterogeneity of microglia and their differential roles in white matter pathology.** *Cns Neuroscience & Therapeutics* 2019, **25:**1290-1298.
13. Alam A, Thelin EP, Tajsic T, Khan DZ, Khellaf A, Patani R, Helmy A: **Cellular infiltration in traumatic brain injury.** *J Neuroinflammation* 2020, **17:**328.
14. Xu X, Gao W, Cheng S, Yin D, Li F, Wu Y, Sun D, Zhou S, Wang D, Zhang Y, Jiang R, Zhang J: **Anti-inflammatory and immunomodulatory mechanisms of atorvastatin in a murine model of traumatic brain injury.** *J Neuroinflammation* 2017, **14:**167.
15. Abrahamson EE, Ikonomovic MD: **Brain injury-induced dysfunction of the blood brain barrier as a risk for dementia.** *Exp Neurol* 2020, **328:**113257.
16. Haruwaka K, Ikegami A, Tachibana Y, Ohno N, Konishi H, Hashimoto A, Matsumoto M, Kato D, Ono R, Kiyama H, Moorhouse AJ, Nabekura J, Wake H: **Dual microglia effects on blood brain barrier permeability induced by systemic inflammation.** *Nat Commun* 2019, **10:**5816.
17. Cai ZL, Wang CY, Jiang ZJ, Li HH, Liu WX, Gong LW, Xiao P, Li CH: **Effects of cordycepin on Y-maze learning task in mice.** *Eur J Pharmacol* 2013, **714:**249-253.
18. Cheng Y, Wei Y, Yang W, Song Y, Shang H, Cai Y, Wu Z, Zhao W: **Cordycepin confers neuroprotection in mice models of intracerebral hemorrhage via suppressing NLRP3 inflammasome activation.** *Metab Brain Dis* 2017, **32:**1133-1145.
19. Lee JY, Choi HY, Baik HH, Ju BG, Kim WK, Yune TY: **Cordycepin-enriched WIB-801C from Cordyceps militaris improves functional recovery by attenuating blood-spinal cord barrier disruption after spinal cord injury.** *J Ethnopharmacol* 2017, **203:**90-100.
20. Yuan J, Wang A, He Y, Si Z, Xu S, Zhang S, Wang K, Wang D, Liu Y: **Cordycepin attenuates traumatic brain injury-induced impairments of blood-brain barrier integrity in rats.** *Brain Res Bull* 2016, **127:**171-176.
21. Wang G, Jiang X, Pu H, Zhang W, An C, Hu X, Liou AK, Leak RK, Gao Y, Chen J: **Scriptaid, a novel histone deacetylase inhibitor, protects against traumatic brain injury via modulation of PTEN and AKT pathway : scriptaid protects against TBI via AKT.** *Neurotherapeutics* 2013, **10:**124-142.
22. Yang H, Ni W, Wei P, Li S, Gao X, Su J, Jiang H, Lei Y, Zhou L, Gu Y: **HDAC inhibition reduces white matter injury after intracerebral hemorrhage.** *J Cereb Blood Flow Metab* 2020**:**271678X20942613.
23. Zhang J, Zhang W, Gao X, Zhao Y, Chen D, Xu N, Pu H, Stetler RA, Gao Y: **Preconditioning with partial caloric restriction confers long-term protection against grey and white matter injury after transient focal ischemia.** *J Cereb Blood Flow Metab* 2019, **39:**1394-1409.
24. Xu MY, Wang YF, Wei PJ, Gao YQ, Zhang WT: **Hypoxic preconditioning improves long-term functional outcomes after neonatal hypoxia-ischemic injury by restoring white matter integrity and brain development.** *CNS Neurosci Ther* 2019, **25:**734-747.
25. Mao L, Sun L, Sun J, Sun B, Gao Y, Shi H: **Ethyl pyruvate improves white matter remodeling in rats after traumatic brain injury.** *CNS Neurosci Ther* 2020.
26. Shi H, Wang HL, Pu HJ, Shi YJ, Zhang J, Zhang WT, Wang GH, Hu XM, Leak RK, Chen J, Gao YQ: **Ethyl pyruvate protects against blood-brain barrier damage and improves long-term neurological**

outcomes in a rat model of traumatic brain injury. *CNS Neurosci Ther* 2015, **21:**374-384.

27. Kang L, Yu H, Yang X, Zhu Y, Bai X, Wang R, Cao Y, Xu H, Luo H, Lu L, Shi MJ, Tian Y, Fan W, Zhao BQ: **Neutrophil extracellular traps released by neutrophils impair revascularization and vascular remodeling after stroke.** *Nat Commun* 2020, **11:**2488.
28. Vakhtin AA, Zhang Y, Wintermark M, Massaband P, Robinson MT, Ashford JW, Furst AJ: **White Matter Asymmetry: A Reflection of Pathology in Traumatic Brain Injury.** *J Neurotrauma* 2020, **37:**373-381.
29. Kim E, Seo HG, Lee HH, Lee SH, Choi SH, Cho WS, Wagner AK, Oh BM: **Altered White Matter Integrity after Mild to Moderate Traumatic Brain Injury.** *J Clin Med* 2019, **8**.
30. Mierzwa AJ, Marion CM, Sullivan GM, McDaniel DP, Armstrong RC: **Components of myelin damage and repair in the progression of white matter pathology after mild traumatic brain injury.** *J Neuropathol Exp Neurol* 2015, **74:**218-232.
31. Wang G, Zhang J, Hu X, Zhang L, Mao L, Jiang X, Liou AK, Leak RK, Gao Y, Chen J: **Microglia/macrophage polarization dynamics in white matter after traumatic brain injury.** *J Cereb Blood Flow Metab* 2013, **33:**1864-1874.
32. Bernstein DL, Zuluaga-Ramirez V, Gajghate S, Reichenbach NL, Polyak B, Persidsky Y, Rom S: **miR-98 reduces endothelial dysfunction by protecting blood-brain barrier (BBB) and improves neurological outcomes in mouse ischemia/reperfusion stroke model.** *J Cereb Blood Flow Metab* 2020, **40:**1953-1965.
33. Goldim MPS, Della Giustina A, Petronilho F: **Using Evans Blue Dye to Determine Blood-Brain Barrier Integrity in Rodents.** *Curr Protoc Immunol* 2019, **126:**e83.
34. Edwards DN, Salmeron K, Lukins DE, Trout AL, Fraser JF, Bix GJ: **Integrin alpha5beta1 inhibition by ATN-161 reduces neuroinflammation and is neuroprotective in ischemic stroke.** *J Cereb Blood Flow Metab* 2020, **40:**1695-1708.
35. Shi Y, Zhang L, Pu H, Mao L, Hu X, Jiang X, Xu N, Stetler RA, Zhang F, Liu X, Leak RK, Keep RF, Ji X, Chen J: **Rapid endothelial cytoskeletal reorganization enables early blood-brain barrier disruption and long-term ischaemic reperfusion brain injury.** *Nat Commun* 2016, **7:**10523.
36. Yang Y, Rosenberg GA: **Matrix metalloproteinases as therapeutic targets for stroke.** *Brain Research* 2015, **1623:**30-38.
37. Li K, Tay FR, Yiu CKY: **The past, present and future perspectives of matrix metalloproteinase inhibitors.** *Pharmacol Ther* 2020, **207:**107465.
38. Li P, Gan Y, Sun BL, Zhang F, Lu B, Gao Y, Liang W, Thomson AW, Chen J, Hu X: **Adoptive regulatory T-cell therapy protects against cerebral ischemia.** *Ann Neurol* 2013, **74:**458-471.
39. Kumari R, Bettermann K, Willing L, Sinha K, Simpson IA: **The role of neutrophils in mediating stroke injury in the diabetic db/db mouse brain following hypoxia-ischemia.** *Neurochem Int* 2020, **139:**104790.
40. Han YY, Chen ZH, Shang YJ, Yan WW, Wu BY, Li CH: **Cordycepin improves behavioral-LTP and dendritic structure in hippocampal CA1 area of rats.** *J Neurochem* 2019, **151:**79-90.

41. Cao ZP, Dai D, Wei PJ, Han YY, Guan YQ, Li HH, Liu WX, Xiao P, Li CH: **Effects of cordycepin on spontaneous alternation behavior and adenosine receptors expression in hippocampus.** *Physiol Behav* 2018, **184:**135-142.
42. Dong ZS, Cao ZP, Shang YJ, Liu QY, Wu BY, Liu WX, Li CH: **Neuroprotection of cordycepin in NMDA-induced excitotoxicity by modulating adenosine A1 receptors.** *Eur J Pharmacol* 2019, **853:**325-335.
43. Dai SS, Zhou YG, Li W, An JH, Li P, Yang N, Chen XY, Xiong RP, Liu P, Zhao Y, Shen HY, Zhu PF, Chen JF: **Local glutamate level dictates adenosine A2A receptor regulation of neuroinflammation and traumatic brain injury.** *J Neurosci* 2010, **30:**5802-5810.
44. Ning YL, Yang N, Chen X, Xiong RP, Zhang XZ, Li P, Zhao Y, Chen XY, Liu P, Peng Y, Wang ZG, Chen JF, Zhou YG: **Adenosine A2A receptor deficiency alleviates blast-induced cognitive dysfunction.** *J Cereb Blood Flow Metab* 2013, **33:**1789-1798.
45. Sandsmark DK, Bashir A, Wellington CL, Diaz-Arrastia R: **Cerebral Microvascular Injury: A Potentially Treatable Endophenotype of Traumatic Brain Injury-Induced Neurodegeneration.** *Neuron* 2019, **103:**367-379.
46. Xia Y, Pu H, Leak RK, Shi Y, Mu H, Hu X, Lu Z, Foley LM, Hitchens TK, Dixon CE, Bennett MVL, Chen J: **Tissue plasminogen activator promotes white matter integrity and functional recovery in a murine model of traumatic brain injury.** *Proc Natl Acad Sci U S A* 2018, **115:**E9230-E9238.
47. Shi K, Zhang J, Dong JF, Shi FD: **Dissemination of brain inflammation in traumatic brain injury.** *Cell Mol Immunol* 2019, **16:**523-530.
48. Sen T, Saha P, Gupta R, Foley LM, Jiang T, Abakumova OS, Hitchens TK, Sen N: **Aberrant ER Stress Induced Neuronal-IFN beta Elicits White Matter Injury Due to Microglial Activation and T-Cell Infiltration after TBI.** *Journal of Neuroscience* 2020, **40:**424-446.
49. Eyolfson E, Khan A, Mychasiuk R, Lohman AW: **Microglia dynamics in adolescent traumatic brain injury.** *J Neuroinflammation* 2020, **17:**326.
50. Abe N, Choudhury ME, Watanabe M, Kawasaki S, Nishihara T, Yano H, Matsumoto S, Kunieda T, Kumon Y, Yorozuya T, Tanaka J: **Comparison of the detrimental features of microglia and infiltrated macrophages in traumatic brain injury: A study using a hypnotic bromovalerylurea.** *Glia* 2018, **66:**2158-2173.
51. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, Chen J: **Microglial and macrophage polarization-new prospects for brain repair.** *Nat Rev Neurol* 2015, **11:**56-64.
52. Lloyd AF, Davies CL, Holloway RK, Labrak Y, Ireland G, Carradori D, Dillenburg A, Borger E, Soong D, Richardson JC, Kuhlmann T, Williams A, Pollard JW, des Rieux A, Priller J, Miron VE: **Central nervous system regeneration is driven by microglia necroptosis and repopulation.** *Nature Neuroscience* 2019, **22:**1046-+.
53. Pu H, Jiang X, Wei Z, Hong D, Hassan S, Zhang W, Liu J, Meng H, Shi Y, Chen L, Chen J: **Repetitive and Prolonged Omega-3 Fatty Acid Treatment After Traumatic Brain Injury Enhances Long-Term Tissue Restoration and Cognitive Recovery.** *Cell Transplant* 2017, **26:**555-569.

54. Xu H, Wang Z, Li J, Wu H, Peng Y, Fan L, Chen J, Gu C, Yan F, Wang L, Chen G: **The Polarization States of Microglia in TBI: A New Paradigm for Pharmacological Intervention.** *Neural Plast* 2017, **2017:**5405104.
55. Ubogu EE: **Biology of the human blood-nerve barrier in health and disease.** *Exp Neurol* 2020, **328:**113272.
56. Rohlwink UK, Walker NF, Ordonez AA, Li YJ, Tucker EW, Elkington PT, Wilkinson RJ, Wilkinson KA: **Matrix Metalloproteinases in Pulmonary and Central Nervous System Tuberculosis-A Review.** *Int J Mol Sci* 2019, **20**.
57. Li XF, Zhang XJ, Zhang C, Wang LN, Li YR, Zhang Y, He TT, Zhu XY, Cui LL, Gao BL: **Ulinastatin protects brain against cerebral ischemia/reperfusion injury through inhibiting MMP-9 and alleviating loss of ZO-1 and occludin proteins in mice.** *Exp Neurol* 2018, **302:**68-74.
58. Rui Q, Ni H, Lin X, Zhu X, Li D, Liu H, Chen G: **Astrocyte-derived fatty acid-binding protein 7 protects blood-brain barrier integrity through a caveolin-1/MMP signaling pathway following traumatic brain injury.** *Exp Neurol* 2019, **322:**113044.
59. Bao Dang Q, Lapergue B, Tran-Dinh A, Diallo D, Moreno JA, Mazighi M, Romero IA, Weksler B, Michel JB, Amarenco P, Meilhac O: **High-density lipoproteins limit neutrophil-induced damage to the blood-brain barrier in vitro.** *J Cereb Blood Flow Metab* 2013, **33:**575-582.
60. Rosell A, Cuadrado E, Ortega-Aznar A, Hernandez-Guillamon M, Lo EH, Montaner J: **MMP-9-positive neutrophil infiltration is associated to blood-brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke.** *Stroke* 2008, **39:**1121-1126.
61. Yao HW, Kuan CY: **Early neutrophil infiltration is critical for inflammation-sensitized hypoxic-ischemic brain injury in newborns.** *Journal of Cerebral Blood Flow and Metabolism* 2020, **40:**2188-2200.
62. Cai W, Wang JL, Hu MY, Chen X, Lu ZQ, Bellanti JA, Zheng SG: **All trans-retinoic acid protects against acute ischemic stroke by modulating neutrophil functions through STAT1 signaling.** *Journal of Neuroinflammation* 2019, **16**.
63. Gao C, Qian Y, Huang J, Wang D, Su W, Wang P, Guo L, Quan W, An S, Zhang J, Jiang R: **A Three-Day Consecutive Fingolimod Administration Improves Neurological Functions and Modulates Multiple Immune Responses of CCI Mice.** *Mol Neurobiol* 2017, **54:**8348-8360.
64. Zhao ZA, Zhao Y, Ning YL, Yang N, Peng Y, Li P, Chen XY, Liu D, Wang H, Chen X, Bai W, Chen JF, Zhou YG: **Adenosine A2A receptor inactivation alleviates early-onset cognitive dysfunction after traumatic brain injury involving an inhibition of tau hyperphosphorylation.** *Transl Psychiatry* 2017, **7:**e1123.
65. Ramos CD, Canetti C, Souto JT, Silva JS, Hogaboam CM, Ferreira SH, Cunha FQ: **MIP-1alpha[CCL3] acting on the CCR1 receptor mediates neutrophil migration in immune inflammation via sequential release of TNF-alpha and LTB4.** *J Leukoc Biol* 2005, **78:**167-177.
66. Pelisch N, Rosas Almanza J, Stehlik KE, Aperi BV, Kroner A: **CCL3 contributes to secondary damage after spinal cord injury.** *J Neuroinflammation* 2020, **17:**362.

67. Kolaczkowska E, Kubes P: **Neutrophil recruitment and function in health and inflammation.** *Nat Rev Immunol* 2013, **13:**159-175.

68. Kong LL, Wang ZY, Han N, Zhuang XM, Wang ZZ, Li H, Chen NH: **Neutralization of chemokine-like factor 1, a novel C-C chemokine, protects against focal cerebral ischemia by inhibiting neutrophil infiltration via MAPK pathways in rats.** *J Neuroinflammation* 2014, **11:**112.

Figures

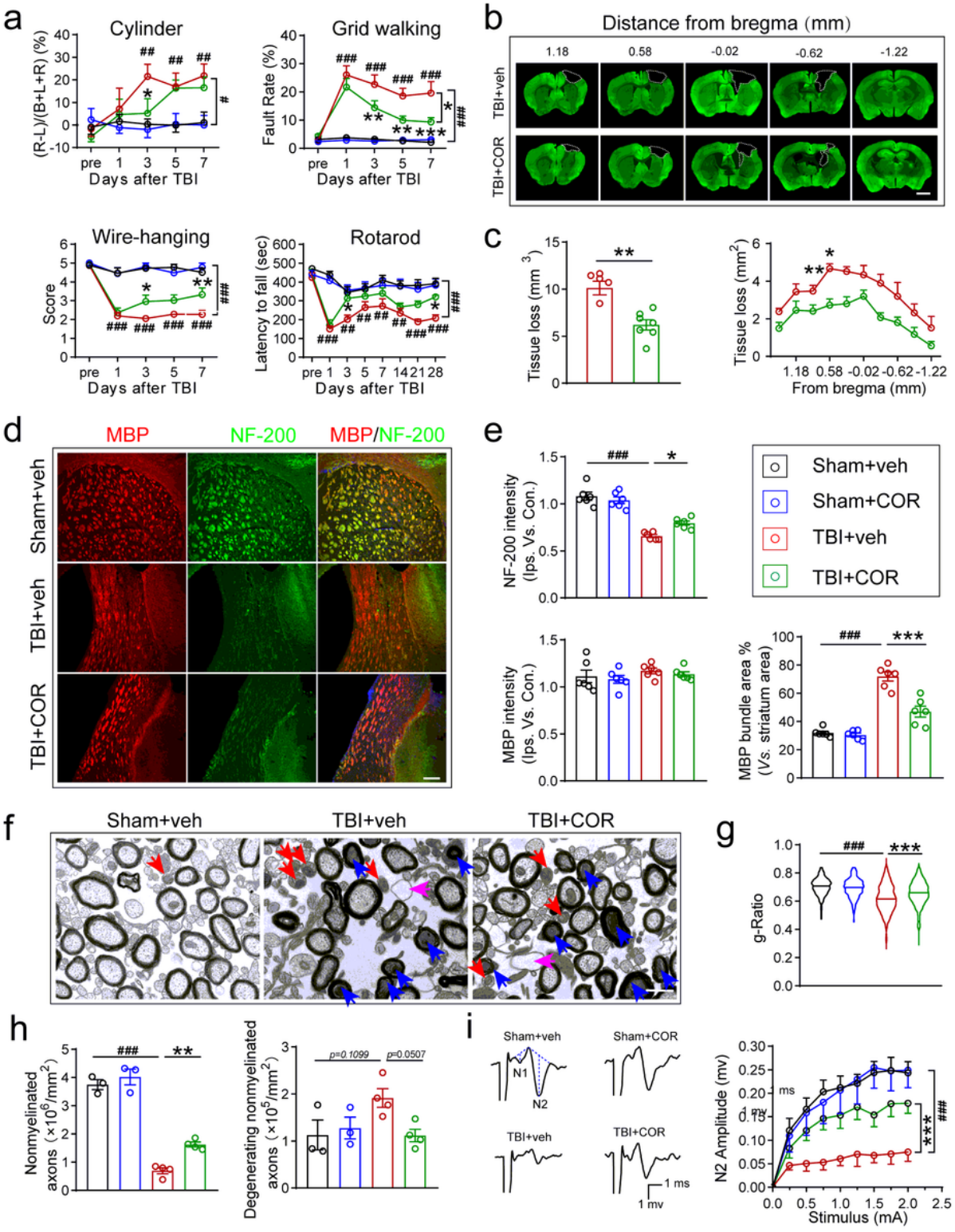

Figure 1

Effect of cordycepin on sensorimotor behavior, GMI and WMI. a Cylinder test, grid walking test, and wire-hanging test conducted for 7d after TBI, and rotarod test up to 28d after TBI. n=9/group. b Representative images of serial coronal sections labeled with MAP2 35d after TBI. Scale bar: 1 mm. c Total tissue loss volume and tissue loss area of each serial section. n=6~7/group. d Representative MBP/NF-200 stained images of the striatum 35d after TBI. Scale bar: 50 μm. e Quantification of NF-200 fluorescence intensity,

MBP fluorescence intensity, and percentage of bundles area in the striatum. n=6/group. f Representative transmission electron microscope images of the corpus callosum 35d after TBI, blue arrows and red arrows indicate degenerating myelinated axons and nonmyelinated axons, respectively. Scale bar: 500 nm. g Quantification of the g-Ratio of myelinated axons (inner diameter/outside diameter), h Quantification of the numbers of nonmyelinated axons, and degenerating nonmyelinated axons. n=3~4/group. Random 100 axons of each mouse were focused for g-Ratio statistics. i Representative CAPs and quantification of N2 amplitude. n=6/group. veh (vehicle), COR (cordycepin), #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. Sham+veh group, *p < 0.05. **p < 0.01 and ***p < 0.001 vs. TBI+veh group.

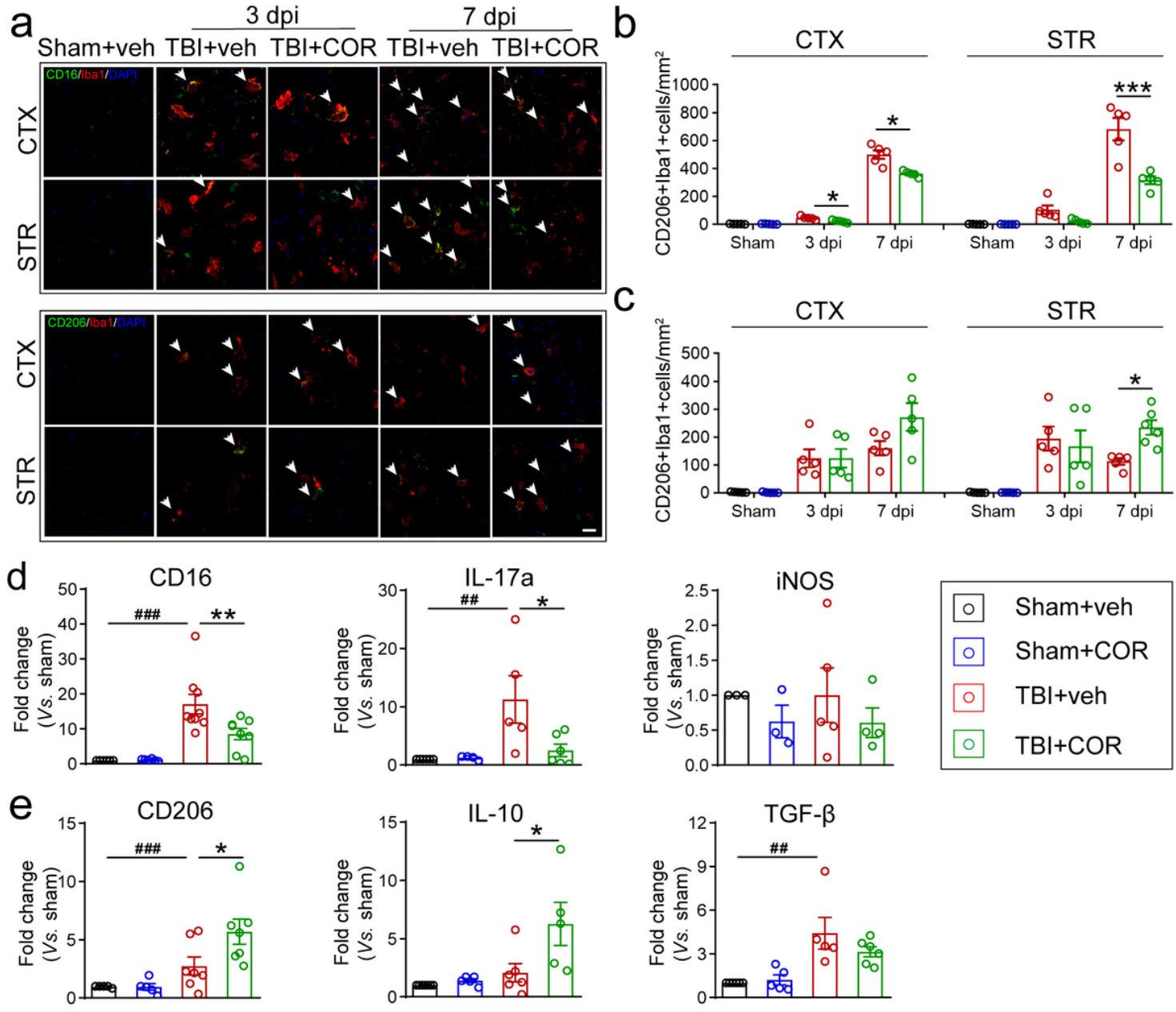

Figure 2

Effect of Cordycepin on microglia/macrophage polarization. a Representative CD16/Iba1 and CD206/Iba1 stained images of cortex (CTX) and striatum (STR) on 3d and 7d after TBI. White arrows

indicate double positive cells. Scale bar: 20 μm. b-c Quantification of the numbers of CD16+Iba1+cells (b)and CD206+Iba1+cells (c) in CTX and STR. d-e qPCR results of pro-inflammatory (d) and anti-inflammatory markers (e) on 7d after TBI. n=3-7/group. ##p < 0.01 and ###p < 0.001 vs. Sham+veh group, *p < 0.05. **p < 0.01 and ***p < 0.001 vs. TBI+veh group.

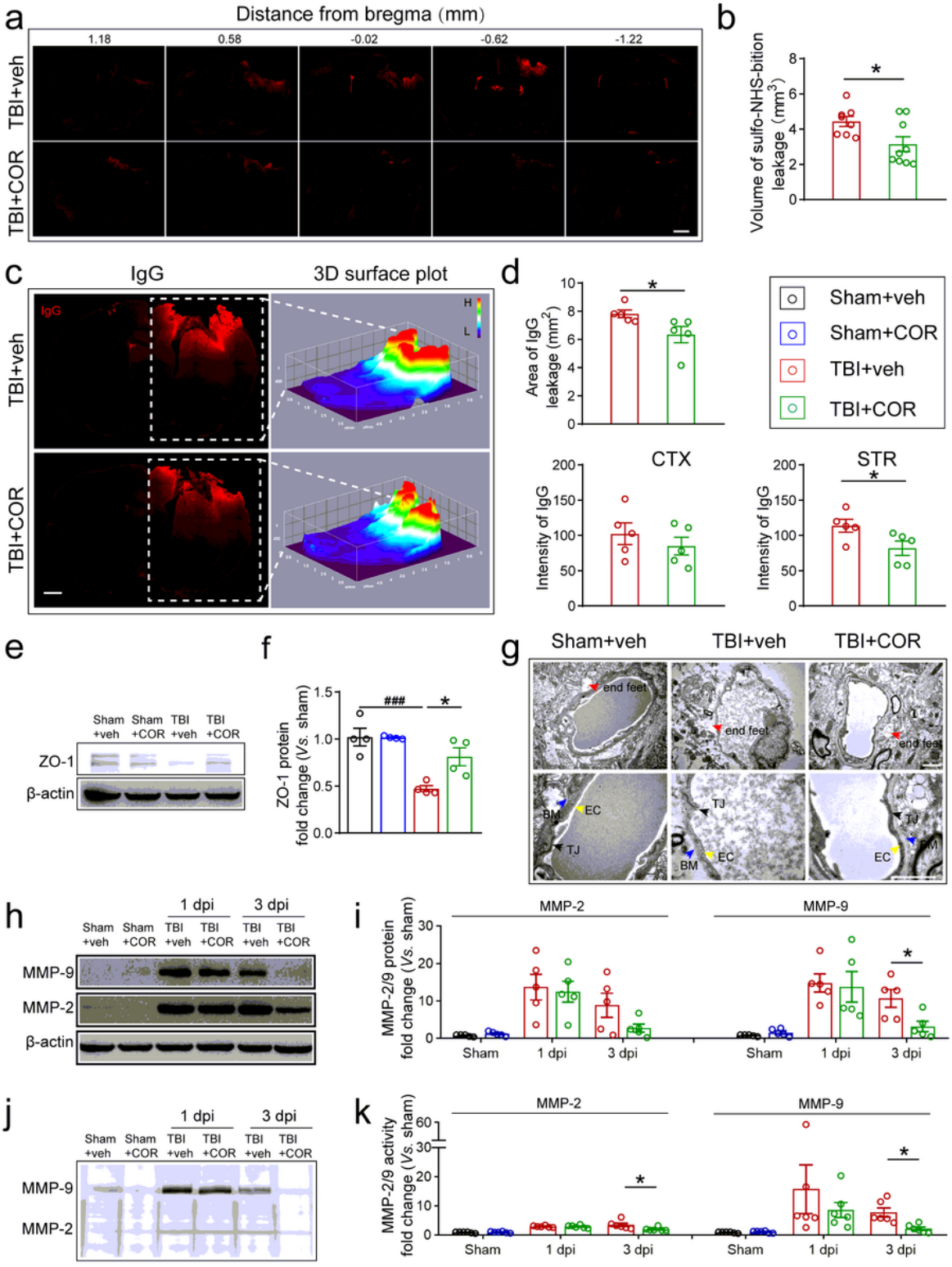

Figure 3

Effect of cordycepin on BBB integrity. a Representative images of serial coronal sections of sulfo-NHS-biotin leakage on 3d after TBI. Scale bar: 1 mm. b Quantification of sulfo-NHS-biotin leakage volume. n=7~9/group. c Representative images and 3D surface plot of IgG leakage on 3d after TBI. Scale bar: 1mm. d Quantification of IgG leakage area, intensity in CTX and STR. n=5/group. e Representative images of ZO-1 blot on 3d after TBI. f Quantification of ZO-1 protein expression. n=4/group. g Representative transmission electron microscope images of BBB structure. Scale bar: 1 μm. h Representative images of MMP-2/9 blot on 24h and 3d after TBI. i Quantification of MM-2/9 protein expression. n=5/group. j Representative images of gelatin zymography. k Quantification of MMP-2/9 activity. n=6/group. ###$p < 0.001$ vs. Sham+veh group, *$p < 0.05$ vs. TBI+veh group.

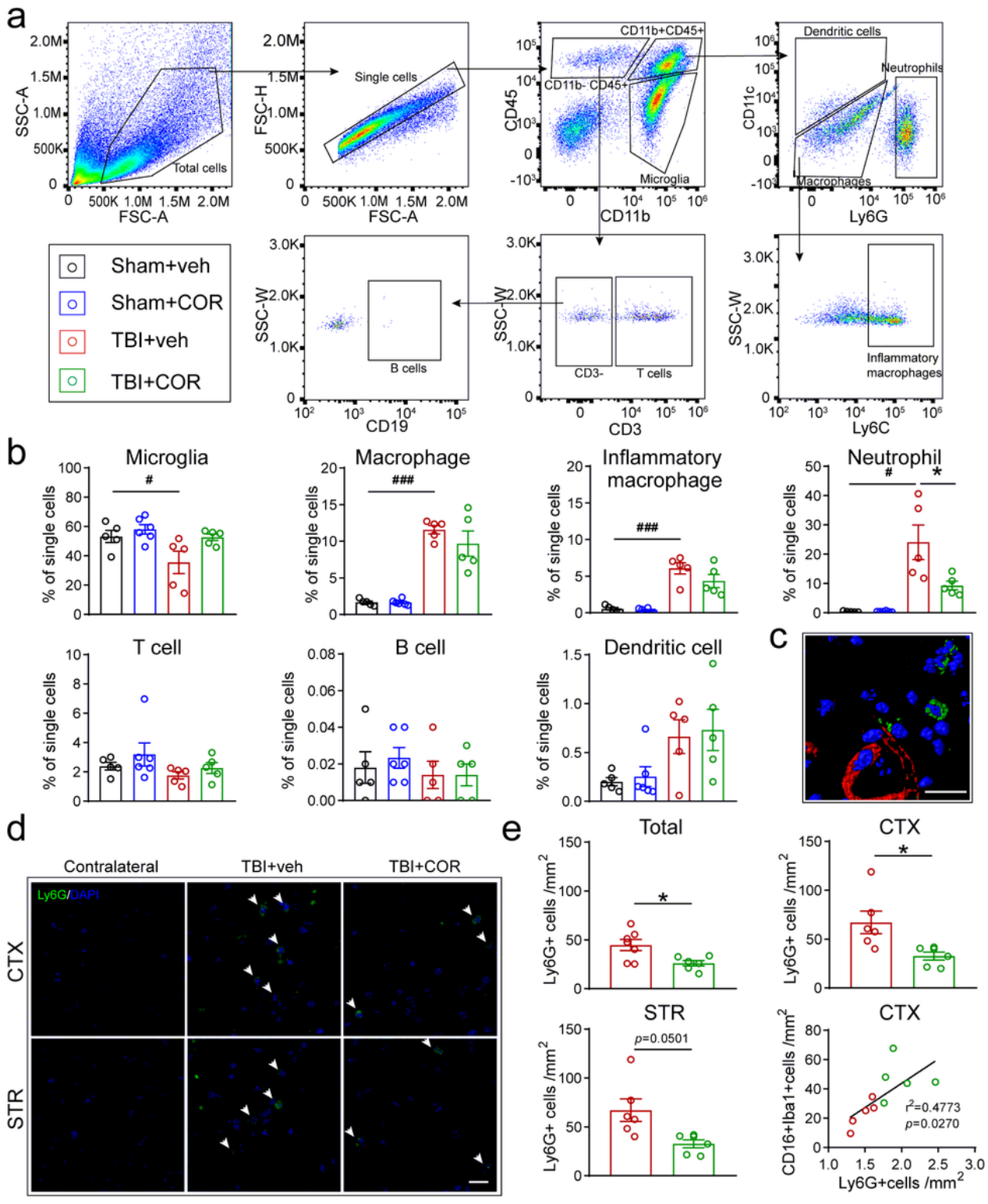

Figure 4

Effect of cordycepin on peripheral immune cells infiltration. a Illustration of flow cytometry analysis of circulating immune cells in the brain on 3d after TBI. b Quantification of the numbers of infiltrating immune cells to the brain. n=5/group. c 3D-reconstructed image of confocal pictures of Ly6G/tomato-lectin straining. Scale bar: 20 μm. d Representative images of Ly6G straining. White arrows indicate Ly6G+cells. Scale bar: 20 μm. e Quantification of numbers of total Ly6G+cells and Ly6G+cells in CTX and

STR, n=6/group. And correlation analysis of Ly6G+cells and CD16+Iba1+cells. n=5/group. #p < 0.05, ###p < 0.001 vs. Sham+veh group, *p < 0.05 vs. TBI+veh group.

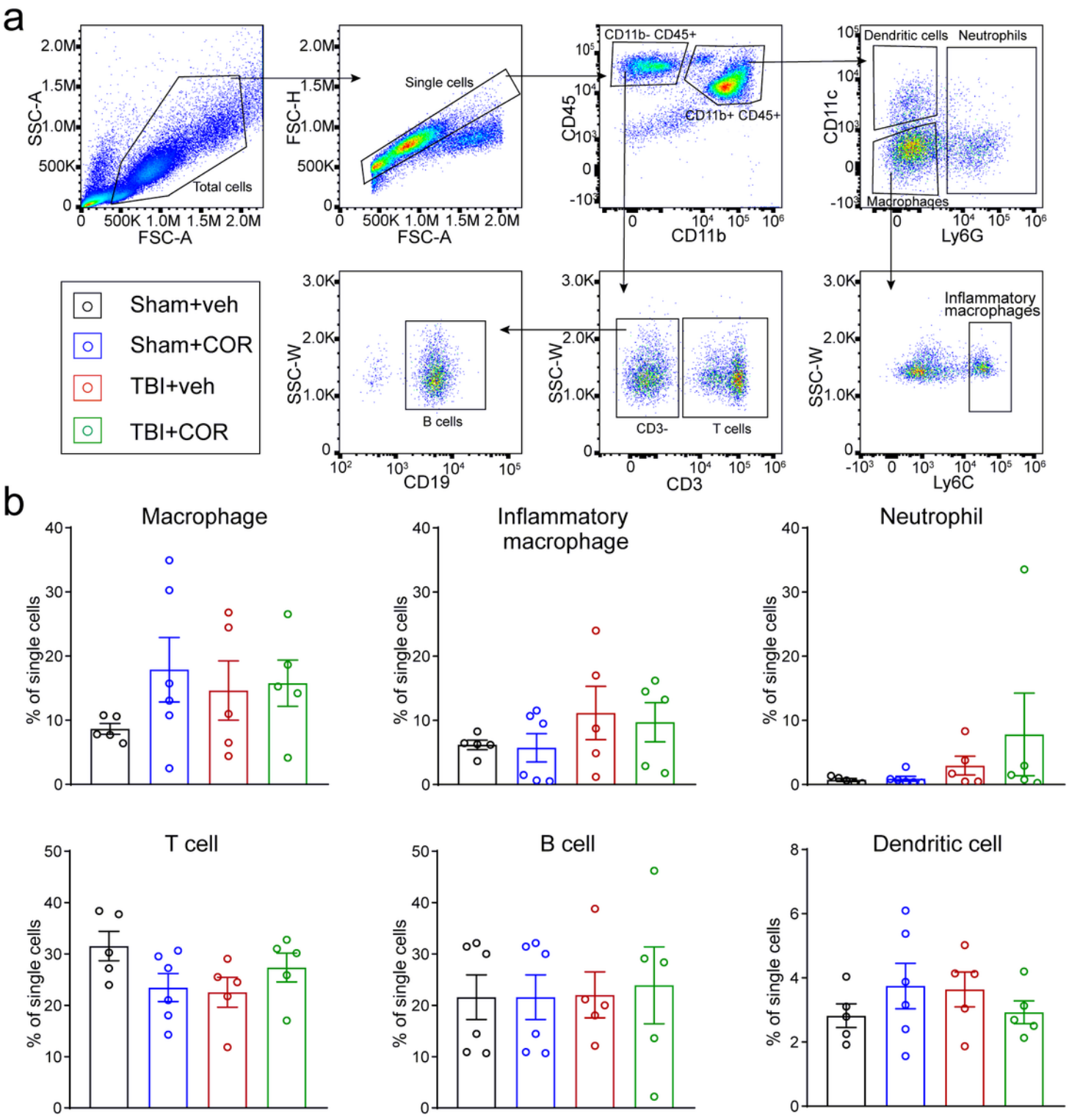

Figure 5

Effect of cordycepin on immune cells infiltration in blood. a Illustration of flow cytometry analysis of immune cells in the blood on 3d after TBI. b Quantification of numbers of immune cells in the blood. n=5/group.

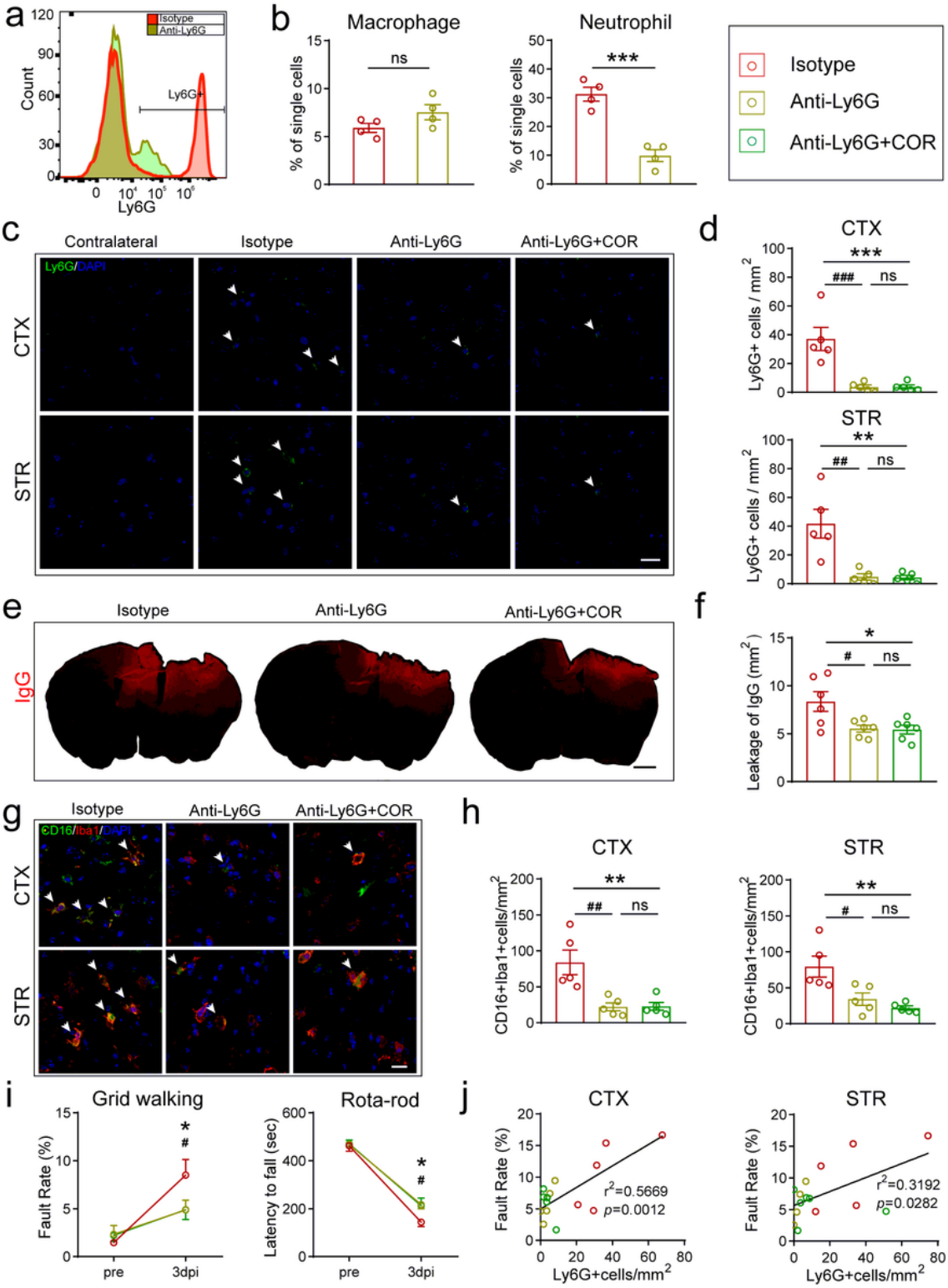

Figure 6

Neutrophil depletion abolished Cordycepin conferred protection. a Representative images of flow cytometry analysis of neutrophils (Ly6G+) in the blood on 3d after TBI. b Quantification of numbers of macrophages or neutrophils in the blood. n=4/group. c Representative images of Ly6G staining, white arrows indicate Ly6G+cells. Scale bar: 20 μm. d Quantification of numbers of Ly6G+cells in CTX and STR. n=5/group. e Representative images of IgG leakage on 3d after TBI. Scale bar: 1mm. f Quantification of

IgG leakage area. n=6 for each group. g Representative CD16/Iba1 stained images in CTX and STR on 3d after TBI, white arrows indicate CD16+Iba1+ cells. Scale bar: 20 μm. h Quantification of numbers of CD16+Iba1+ cells in CTX and STR. n=5/group. i Rotarod test and grid walking test results on 3d after TBI. n=7~9/group. j Foot fault rate in grid walking test was corelated with the number of infiltration neutrophils (Ly6G+) in the brain. n=5/group. #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. Isotype group, *p < 0.05. **p < 0.01 and ***p < 0.001 vs. Anti-Ly6G group.

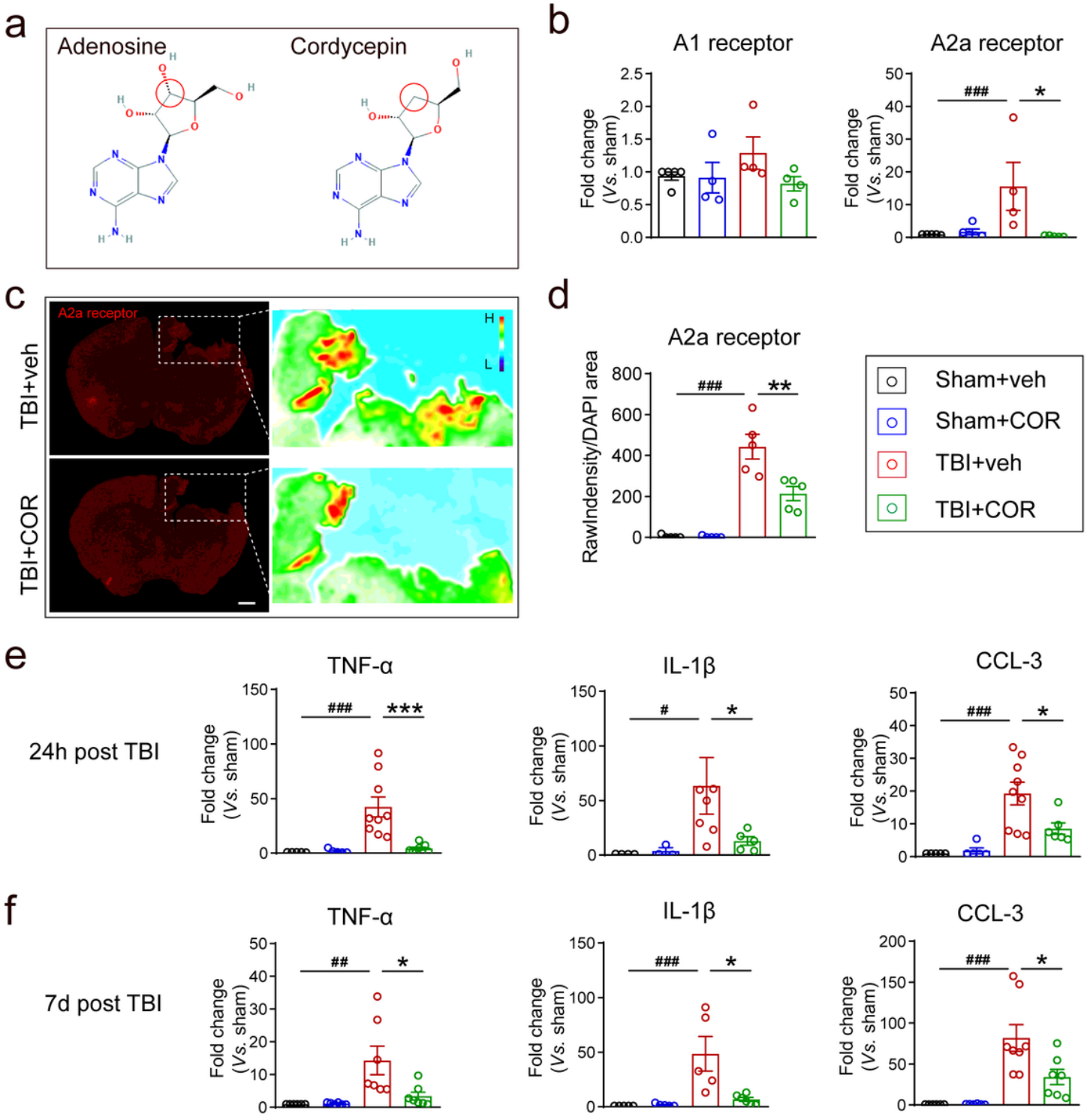

Figure 7

Cordycepin enhances adenosine receptors expression and ameliorates inflammatory cytokines expression after TBI. a Chemical structure of adenosine and cordycepin (from sigma website), the red circles indicate the difference between them. b Quantitative RT-PCR analysis of A1 and A2a receptors. n=4~5/group. c Representative images of A2a receptor expression and their heat maps. Scale bar: 1 mm. d Quantification of A2a receptor intensity surrounding the lesion. n=5/group. e-f Quantitative RT-PCR analysis cytokines on 3d (e) and 7d (f)after TBI. n=5~9/group. #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. Sham+veh group, *p < 0.05. **p < 0.01 and ***p < 0.001 vs. TBI+veh group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 1228SupplementaryMaterials.docx