Maraviroc promotes recovery from traumatic brain injury in mice by suppression of neuroinflammation and activation of neurotoxic reactive astrocytes

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Abstract

Neuroinflammation and the NACHT, LRR, and PYD domains-containing protein 3 inflammasome play crucial roles in secondary tissue damage following an initial insult in patients with traumatic brain injury (TBI). Maraviroc, a C-C chemokine receptor type 5 antagonist, has been viewed as a new therapeutic strategy for many neuroinflammatory diseases. We studied the effect of maraviroc on TBI-induced neuroinflammation. A moderate-TBI mouse model was subjected to a controlled cortical impact device. Maraviroc or vehicle was injected intraperitoneally 1 hour after TBI and then once per day for 3 consecutive days. Western blot, immunohistochemistry, and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) analyses were performed to evaluate the molecular mechanisms of maraviroc at 3 days post-TBI. Our results suggest that maraviroc administration reduced NACHT, LRR, and PYD domains-containing protein 3 inflammasome activation, modulated microglial polarization from M1 to M2, decreased neutrophil and macrophage infiltration, and inhibited the release of inflammatory factors after TBI. Moreover, maraviroc treatment decreased the activation of neurotoxic reactive astrocytes, which, in turn, exacerbated neuronal cell death. Additionally, we confirmed the neuroprotective effect of maraviroc using the modified neurological severity score, rotarod test, Morris water maze test, and lesion volume measurements. In summary, our findings indicate that maraviroc might be a desirable pharmacotherapeutic strategy for TBI, and C-C chemokine receptor type 5 might be a promising pharmacotherapeutic target to improve recovery after TBI.

Key Words: C-C chemokine receptor type 5 (CCR5) antagonist; high mobility group box protein B1 (HMBG1); maraviroc; M1 microglia; nuclear factor-κB pathway; NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome; neuroinflammation; neurological function; neurotoxic reactive astrocytes; traumatic brain injury

Introduction

Traumatic brain injury (TBI) can result in neurological disorders or death and remains a heavy burden to families and society worldwide. However, there are currently no effective treatment guidelines to mitigate the brain damage caused by TBI (Maas et al., 2017). The primary injury occurs immediately after the initial insult and can lead to cerebral contusion, cranial hematoma, and axonal injury. A variety of secondary events follow the initial insult and comprise oxidative stress, blood-brain barrier (BBB) disruption, neuroinflammation, and apoptosis (Corps et al., 2015; Johnson et al., 2018; Ismail et al., 2020). The pathogenesis of TBI is regulated by the immune system and neuroinflammation, including innate and adaptive immunity, resident microglial activation, cytokine release, and inflammasome activation (Jassam et al., 2017).

Microglia and peripheral immunocytes move to the core of the initial insult to defend against pathogens and therefore contribute to secondary injury after TBI (Corps et al., 2015). Microglia polarize into two phenotypes: classically activated M1 microglia and alternatively activated M2 microglia (Jassam et al., 2017). M1 phenotype promotes proinflammatory mediators that aggravate brain tissue damage. In contrast, alternatively activated M2 microglia aid brain recovery by secreting anti-inflammatory factors (Wang et al., 2013; Hu et al., 2015). In addition, it is well established that nucleotide-binding oligomerization domain-like receptor family activation...
is a trigger of cell pyroptosis and leads to poor outcomes after brain injury (O’Brien et al., 2020). NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) has been studied in a number of acute central nervous system (CNS) disorders (Chen et al., 2018; Sun et al., 2019; Chen et al., 2020). The NLRP3 inflammasome consists of the sensor protein NLRP3, the apoptosis-associated speck-like protein adapter, and the precursor enzyme pro-caspase-1. Interleukin (IL)-1β, IL-18, and the amino terminus of gasdermin D are activated by caspase-1 and cleaved by caspase-1 to their active forms. These proteins ultimately lead to cell disruption accompanied by rapid secretion of proinflammatory cytokines (Franke et al., 2021). Thus, reduction of activation of the NLRP3 inflammasome and microglia might be a promising therapeutic strategy for TBI.

Recent studies reported that CNS injury and disease could stimulate polarization of astrocytes into two different phenotypes, termed neurotrophic and reactive astrogliosis. Whether NLRP3 inflammasome activation and inflammatory responses are involved in the development of reactive astrogliosis remains to be clarified. In this study, we used C57BL/6 mice to test the hypothesis that the NLRP3 inflammasome contributes to reactive astrogliosis after TBI.

Methods

Animals

All experimental animal protocols were reviewed and approved by the Animal Care and Use Committee of Tianjin Medical University General Hospital, Tianjin, China, on January 20, 2020 (approval No. IRB2010-DW-19) and conformed to the institutional standards and National Institutes of Health policies, including the Guide for the Care and Use of Laboratory Animals (8th ed., 2011). Male specific-pathogen-free C57BL/6j mice (8–10 weeks old) were purchased from the Institute of Laboratory Animal Technology Co., Ltd., Beijing, China (license No. SCXK Jing 2021-0006). The mice were housed in the animal facilities at 20 ± 2°C temperature and 55% humidity under a 12:12 h light/dark cycle with food and water ad libitum for 1 week before the lesion and in groups at random using the random number table method (n = 20/group); one group was sham operated, and the other two groups were subjected to TBI. Then, the mice were placed in a stereotactic apparatus (RWD Life Science Co., Ltd.) and tested using the rotarod protocol (Sacks et al., 2018). The mice were tested for 30 minutes at 15 seconds each day with a speed of 4 to 40 r/min to record baseline latency on the day before TBI. Data were collected at 1, 3, 7, and 14 days post-TBI (n = 10/group). Lower scores imply better neurological outcomes.

Rotarod test

Morris water maze

The spatial learning and memory abilities of the mice were measured using a Morris water maze (MWM) (O’Brien et al., 2020). The MWM pool consisted of a stainless steel cylindrical pool (122 cm in diameter and 51 cm in depth) with a submerged hidden platform (10 cm in diameter). The MWM apparatus was filled with water 22 ± 2°C and dyed white with nontoxic paint. The experiment was separated into two consecutive phases: a training phase of 15–20 days and a spatial memory test phase of 21 days. In the probe phase, the mice were allowed to swim for 90 seconds in the platform location where they received the platform test to evaluate reflexes, alertness, coordination, and motor abilities. The mNSS was used to evaluate neurological function at 1, 3, 7, and 14 days post-TBI (n = 10/group). Lower scores imply better neurological outcomes.

Immunohistochemical staining

Immunofluorescence

At 3 days post-CCI, the mice (n = 5/group) were euthanized for sampling. Tissue samples were collected and stored at -80°C until analysis. Brain sections (thickness, 20 μm) were cut using a cryostat and then mounted onto a slide. The sections were washed in PBS for 5 minutes and incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. The slides were washed in PBS three times and then incubated for 1 hour at room temperature with 5% normal bovine serum in PBS and then exposed to primary antibodies. The sections were incubated with secondary antibodies for 1 hour at room temperature. Afterward, the sections were washed with PBS three times and then mounted with Fluoroshield (Invitrogen) containing DAPI. The slides were visualized using a confocal microscope (Zeiss). The expression level was determined by ImageJ software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA; Schneider et al., 2012) and was standardized to that of β-actin.

Immunohistochemical staining

At 3 days post-CCI, the mice (n = 5/group) were deeply anesthetized and killed by transcardiac perfusion with PBS followed by 4% paraformaldehyde. The brain samples were then removed and stored in 20% sucrose formaldehyde for 2–3 days. The brains were then cut into 40-μm coronal sections using a cryostat microtome (CM1950, Leica Biosystems, Nussloch, Germany) and were rinsed twice with 0.1% PBS to remove the optimal cutting compound temperature medium and then were permeabilized and blocked with 0.2% Triton X-100 (MilliporeSigma) and 3% dimethyl sulfoxide, 40% polyethylene glycol 300, and 5% Tween 80 in saline, and 20 mg/kg maraviroc was injected intraperitoneally 1 hour after CCI and daily for the next 3 days. Mice receiving vehicle received identical proportions of dimethyl sulfoxide, polyethylene glycol 300, and Tween 80 in saline by intraperitoneal injection 1 hour after CCI or the sham operation. The dose of maraviroc (20 mg/kg) used in the study was selected in accordance with previous reports (Joy et al., 2019; Friedman-Levi et al., 2021).
bovine serum albumin for 1.5 hours. The sections were incubated overnight at 4°C with the primary antibodies shown in Table 1. After washing with PBS, the sections were immersed in the corresponding Alexa Fluor-conjugated IgG (1:500 dilution from Thermo Fisher Scientific) for 1 hour at room temperature. The following secondary antibodies were used: donkey anti-rabbit IgG, Alexa Fluor 488 (Cat# A-21200), donkey anti-rabbit IgG, Alexa Fluor 555 (Cat# A-31572), donkey anti-mouse IgG, Alexa Fluor 488 (Cat# A-22288), donkey anti-mouse IgG, Alexa Fluor 555 (Cat# A-31570), donkey anti-rat IgG, Alexa Fluor 488 (Cat# A-21208), donkey anti-goat IgG, Alexa Fluor Plus 555 (Cat# A-38216). Finally, 4′,6-diamidino-2-phenylindole (Abcam, Cambridge, UK) was applied to counterstain the nuclei. A fluorescence microscope (IX73, Olympus Corporation, Tokyo, Japan) was used to take micrographs of each slice. We captured five fields of view for every section and for each sample in the same regions of the pericontusional cortex. The number of cells and the fluorescence intensity were determined using ImageJ software (Schneider et al., 2012).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining

We determined apoptosis of neurons in the brain tissue sections at 3 days post-TBI using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland). The mouse brain slices (n = 5/group) were rinsed with PBS and incubated with 0.2% Triton X-100 and 3% bovine serum albumin for 1.5 hours. Each section was stained with rabbit anti-neuronal nuclear protein antibody shown in Table 1 overnight at 4°C. The sections were warmed to room temperature for 30 minutes and subsequently washed with PBS three times. Then, the sections were incubated with Alexa Fluor 555 donkey anti-rabbit IgG (Cat# A-31572, Thermo Fisher Scientific) and fixed with TUNEL reaction solution for 60 minutes at 37°C. The counterstaining of nuclei was conducted with 4′,6-diamidino-2-phenylindole for 5 minutes.

Hematoxlin and eosin staining and measurement of lesion volume

Lesion volume was determined as previously described (Xu et al., 2018a). After the MWM test, the mice (n = 6/group) were euthanized, and the brains were removed and embedded in paraffin, and 5-μm coronal sections were sliced at intervals of 120 μm (approximately 25 sections/brain). For hematoxlin and eosin (H&E) staining, the sections were deparaffinized with xylene and an alcohol gradient, and the sections were counterstained with H&E (Beijing Solarbio Science & Technology Co., Ltd.) for 5 minutes. Then, the sections were dried and mounted with neutral balsam (Beijing Solarbio Science & Technology Co., Ltd.) followed by observation using a light microscope (IX73, Olympus Corporation). The volume of each tissue lesion was calculated by measuring the area of the lesion in the contralateral and ipsilateral hemisphere using Image software. Then, the lesion volume (%) was calculated as follows: (interval distance × lesion volume of each section)/area of the contralateral hemisphere × 100.

Statistical analysis

No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in previous publications (Xu et al., 2018a). All data are based on at least three independent experiments. Measurement data are shown as the mean ± standard deviation (SD). All statistical analyses were performed using SPSS 22.0 software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corporation.). To analyze the neurobehavioral evaluation data, two-way analysis of variance followed by Tukey’s post hoc test was performed. Other multigroup comparisons were performed using one-way analysis of variance followed by Tukey’s post hoc test. P < 0.05 was deemed statistically significant.

Results

Administration of maraviroc improves neurological functions after traumatic brain injury

All experimental designs are shown in Figure 1A. To investigate whether administration of maraviroc is protective in mice with TBI, we conducted mNSS tests, rotarod tests, and MWM tests. In the mNSS tests, animals in the TBI + vehicle group had much higher scores than the sham group at any time point measured. Maraviroc treatment significantly alleviated the impairment of motor abilities between the third and seventh days post-TBI (Figure 1B), and mice in all groups reached full spontaneous recovery 12–14 days post-TBI. The rotarod test results showed that the mice in the TBI + vehicle group had the worst motor coordination and balance, and mice in the TBI + maraviroc group displayed significant improvement on the third and seventh days post-TBI. The vehicle group was more than the sham group, and administration of maraviroc decreased the escape latency compared with the TBI + vehicle group on days 19 and 20 (Figure 1D). There was no significant difference in the swim speed of all groups (Figure 1G). Once we removed the hidden platform at 21 days post-TBI to evaluate the number of crossings, a significant decrease in crossing number was observed in the TBI + maraviroc group compared with the TBI + vehicle group. The TBI + vehicle group spent less time in the target region than the sham group, while maraviroc treatment ameliorated this phenomenon (Figure 1F and I). Mice in the maraviroc group traveled a shorter distance while searching for the platform compared with those in the TBI + vehicle group (Figure 1H).

Administration of maraviroc enhances tissue preservation after traumatic brain injury

At 21 days post-TBI, H&E staining of brain tissue showed that the sham group had no gross lesion to the cortex, while noticeable damage was observed in the TBI + vehicle group. The TBI + maraviroc group had more tissue preservation than the TBI + vehicle group (Figure 1J and K).

Administration of maraviroc regulates microglial polarization and reduces neutrophil and macrophage infiltration after traumatic brain injury

Microglia convert from the resting type into the M1 and M2 phenotypes after TBI; this process plays a vital role in the neuroinflammatory response (Long et al., 2020). To determine the effect of maraviroc on microglial polarization, immunofluorescence staining of a panmicroglial marker (ionized calcium-binding adapter molecule 1 [Iba-1]), an M1 microglial marker (inducible nitric oxide synthase [iNOS]), and an M2 microglial marker (macrophage mannose receptor 1 [CD206]) was performed to determine the shifts in microglial polarization 3 days post-TBI. Maraviroc administration remarkably decreased the number of Iba-1-positive cells that also expressed iNOS and increased the expression of CD206 in the perilesional area, indicating an M1-to-M2 transition (Figure 2A–D). Furthermore, western blot analysis at 3 days post-TBI in the lesioned cortex showed that iNOS expression was inhibited, and CD206 expression was significantly increased after maraviroc administration. However, the protein expression level of Iba-1 in tissues from mice in the maraviroc treatment group did not significantly differ from that in mice in the vehicle treatment group (Figure 2E–H). In addition, immunofluorescence staining showed the accumulation of adhesion G protein-coupled receptor E1 (cell surface glycoprotein F4/80 [F4/80])-positive and lymphocyte antigen 6G (Ly-6G)-positive cells in the pericontusional region in the TBI + vehicle group compared with that in the TBI + maraviroc group at 3 days post-TBI (Figure 3A–D).
Figure 1 | Neuroprotective effect of maraviroc on neurological function and cortical lesion size after traumatic brain injury. (A) Experimental design. The black boxes in the H&E-stained illustration show the perilesional cortex, and the red region shows the lesioned area. (B–G) Neurological performance was assessed by the mNSS score (B), rotarod tests (C), and MWM tests (D–G). Representative heatmap of swimming traces (H) and thermal imaging of the probe trial (I) at 21 days post-TBI. The red circle in Figure 1H indicates the location of the platform in the MWM test. (J) Representative H&E-stained images of the brain slices of each group, and (K) quantitative analysis of the lesioned area volume at 21 days post-TBI. *P* values shown in B–D represent the statistical significance between the TBI + maraviroc and TBI + vehicle groups. Two-way analysis of variance followed by Tukey’s post hoc test was performed to analyze the neurobehavioral evaluation data in B–G. One-way analysis of variance followed by Tukey’s post hoc test was used in K. Data are shown as the mean ± SD. The sample size was n = 10/group for neurological function assessment and n = 6/group for H&E staining. All experiments were repeated at least three times. H&E: Hematoxylin and eosin; IHC: immunohistochemistry; i.p.: intraperitoneally; mNSS: modified neurological severity score; MWM: Morris water maze; TBI: traumatic brain injury.

Figure 2 | Effect of maraviroc on microglial activation and polarization in the perilesional cortex area of mice after traumatic brain injury. (A, B) Representative immunofluorescence staining photographs of iNOS (green, A) and CD206 (green, B) in Iba-1-positive cells (red) in the perilesional cortex on the third day post-TBI. Scale bar: 50 µm. (C, D) Quantitative data corresponding to A and B. Maraviroc administration significantly decreased the number of iNOS-positive M1 microglia (*P* = 0.0039) and increased the expression of CD206-positive microglia (*P* = 0.0381) in the perilesional area compared with those in the TBI + vehicle group. The black boxes in the hematoyxin and eosin-stained illustration show the perilesional cortex. (E–H) Representative western blot results of Iba-1 (F), iNOS (G), and CD206 (H) at 3 days post-TBI. Administration of maraviroc decreased the expression level of iNOS (*P* = 0.0387) and increased the expression level of CD206 (*P* = 0.0339) but did not alter the protein level of Iba-1. One-way analysis of variance followed by the Tukey’s post hoc test was used. Data are expressed as the mean ± SD (n = 5/group). All experiments were repeated at least three times. DAPI: 4′,6-Diamidino-2-phenylindole; iNOS: inducible nitric oxide synthase; TBI: traumatic brain injury.
Figure 4 | Effect of maraviroc on neutrophil and macrophage infiltration after traumatic brain injury.
(A–D) Representative immunofluorescence staining micrographs of Ly-6G (green, A) and F4/80 (green, C) in the perilesional cortex at 3 days post-TBI. Scale bars: 50 µm. (B, D) Maraviroc decreased the number of Ly-6G-positive (P = 0.01) (B) and F4/80-positive (P = 0.0083) (D) cells after traumatic brain injury. The black boxes in the hematoxylin and eosin-stained illustration show the perilesional cortex. One-way analysis of variance followed by Tukey's post hoc test was used. Data are expressed as the mean ± SD (n = 5/group). All experiments were repeated at least three times. DAPI: 4′,6-Diamidino-2-phenylindole; F4/80: adhesion G protein-coupled receptor E1; cell surface glycoprotein F4/80; Ly-6G; lymphocyte antigen 6G.

Administration of maraviroc suppressed NLRP3 inflammasome activation after traumatic brain injury
Western blotting and immunofluorescence were conducted to determine the NLRP3 inflammasome expression levels among different groups. The TBI + vehicle group had elevated levels of NLRP3, caspase-1 p20, apoptotic speck-containing protein, IL-18, IL-1β, and gasdermin-D compared with the sham group (Figure 5A–G). The TBI + maraviroc group had significantly decreased protein levels of the NLRP3 inflammasome compared with the TBI + vehicle group. In addition, immunofluorescence analysis revealed that the elevated caspase-1 p20 immunoreactivity in the pericontusional cortex was greatly alleviated by maraviroc administration compared with vehicle administration (Figure 5H and I).

Figure 3 | Effect of maraviroc on neutrophil and macrophage infiltration after traumatic brain injury.
(A–D) Representative immunofluorescence staining micrographs of Ly-6G (green, A) and F4/80 (green, C) in the perilesional cortex at 3 days post-TBI. Scale bars: 50 µm.

Administration of maraviroc inhibits the HMGB1/NF-κB pathway and alters the inflammatory response in the pericontusional cortex after traumatic brain injury
High mobility group protein B1 (HMGB1) translocation and release have been shown to activate microglia and exacerbate neuroinflammation induced by TBI (Paudel et al., 2020). The nuclear factor kappa B (NF-κB) pathway is related to high expression levels of HMGB1 and the subsequent release of inflammatory factors. The HMGB1/NF-κB pathway may play a critical role in the pathological process of TBI (Chen et al., 2018). Western blot analysis illustrated that the expression levels of HMGB1 and NF-κB p65 in the lesioned cortex were significantly increased at 3 days after TBI (Figure 4A, E, and F). In contrast, administration of maraviroc effectively decreased HMGB1 and NF-κB p65 protein expression compared with vehicle treatment after TBI. Moreover, a western blot assay of proinflammatory cytokine levels showed that maraviroc treatment significantly inhibited the expression of these inflammatory factors compared with vehicle treatment after TBI (Figure 4A–D). Immunofluorescence staining further demonstrated that the TBI + maraviroc group had a significantly reduced percentage of cells with nuclei stained positive for NF-κB p65 compared with that in the TBI + vehicle group (Figure 4G and H).

Administration of maraviroc decreased the activation of neurotoxic reactive astrocytes
We estimated whether maraviroc treatment suppressed neurotoxic reactive astrocytes using immunohistochemical and western blot assays. The expression levels of glial fibrillary acidic protein (GFAP) were significantly increased in the TBI + vehicle group in the ipsilateral hemisphere compared with the sham group (P = 0.0091), but there was no difference between the TBI + vehicle group and the TBI + maraviroc group (P = 0.3392; Figure 6A and B). However, we observed that the protein levels of the neurotoxic reactive...
Enlarged Vehicle). Coimmunofluorescence revealed that the maraviroc treatment group exhibited a notable decline in the number of C3-positive and GFAP-positive astrocytes compared with the vehicle treatment group in the lesioned cortex 3 days post-TBI (Figure 6D and E).

Administration of maraviroc protects neurons against traumatic brain injury-induced neuronal apoptosis

Excessively activated inflammatory responses, NLRP3 inflammasomes, and A1 astrocytes are closely related to the prevalence of apoptosis (Liu et al., 2013; Roth et al., 2014; Liddelow et al., 2017; Skelly et al., 2019). We estimated the effect of maraviroc on neuronal cell death at 3 days post-TBI, and western blotting analyses were performed to quantify apoptotic cells. Maraviroc administration decreased the levels of cleaved caspase-3 and the apoptosis regulator BAX compared with vehicle administration (Figure 7A and B). In addition, double staining with the TUNEL assay and neuronal nuclear protein revealed many more apoptotic cells in the vehicle treatment group than in the sham group, but maraviroc treatment significantly decreased the apoptotic index compared with vehicle treatment (Figure 7C–E).

**Figure 5** | Effect of maraviroc on the expression of the NLRP3 inflammasome after traumatic brain injury.

(A–G) Representative western blot bands and quantitative data of NLRP3 (B), ASC (C), cleaved caspase-1 p20 (D), IL-1β (E), IL-18 (F), and cleaved GSDMD (G) in the pericontusional cerebral cortex 3 days post-TBI. Maraviroc treatment alleviated the TBI-induced activation of NLRP3 inflammasome components and substrates at 3 days postinjury compared with the TBI + vehicle group (P = 0.0082 for B, P < 0.05 for C–G). (H–I) Representative immunofluorescence staining micrographs for caspase-1 p20 (green) in the perilesional cortex at 3 days post-TBI. The black boxes in the hematoxylin and eosin-stained illustration show the perilesional cortex. Scale bars: 100 μm. Maraviroc treatment significantly decreased the number of caspase-1 p20-positive cells compared with the TBI + vehicle group (P = 0.0027). One-way analysis of variance followed by Tukey’s post hoc test was used. Data are shown as the mean ± SD (n = 5/group). All experiments were repeated at least three times. ASC: Apoptosis-associated speck-like protein containing a CARD; DAPI: 4′,6-diamidino-2-phenylindole; GFAP: glial acidic fibrillary protein; GSDMD: gasdermin-D; IL: interleukin; NLRP3: NACHT, LRR, and PYD domains-containing protein 3; TBI: traumatic brain injury.

**Figure 6** | Effect of maraviroc on neurotoxic reactive astrocyte activation in the pericontusional cortex of mice with traumatic brain injury.

(A–C) Representative immunoblot bands and quantitative data of GFAP (B) and C3 (C) 3 days post-TBI. Maraviroc administration significantly decreased C3 protein expression after TBI (P = 0.0138). (D–E) Representative double immunofluorescence staining photographs and corresponding quantitative data for complement C3 (green) and GFAP (red) in the perilesional cortex at 3 days post-TBI (P = 0.0157). The black boxes in the hematoxylin and eosin-stained illustration show the perilesional cortex. Scale bars: 50 μm. One-way analysis of variance followed by the Tukey’s post hoc test was used. Data are expressed as the mean ± SD (n = 5/group). All experiments were repeated at least three times. DAPI: 4′,6-Diamidino-2-phenylindole; GFAP: glial acidic fibrillary protein; C3: complement C3; TBI: traumatic brain injury.
Effect of maraviroc on neuronal apoptosis at 3 days after traumatic brain injury. Maraviroc or pharmacologically inhibiting CCR5 suppresses the inflammatory response poststroke (Xiong et al., 2016). Ample evidence indicates that knocking out CCR5 gene, confers relative resistance to HIV infection (Dean et al., 1996). CCR5 inhibitors, such as maraviroc (Xu et al., 2014). Ample evidence has demonstrated that CCR5 receptor inhibition to the HMGB1/toll-like receptor 4 or HMGB1/advanced glycosylation end product-specific receptor activates p38 and NF-κB to amplify the neuroinflammatory response. Recent studies have shown that activation of the HMGB1/toll-like receptor 4 or HMGB1/advanced glycosylation end product-specific pathways results in the activation of NF-κB to exacerbate the inflammatory cascade (Cresw et al., 2013; Jia et al., 2019). In addition, several studies have illustrated that activation of NLRP3 is triggered by ATP, which leads to pyroptosis-mediated cell death in endothelial cells and acute pancreatitis (Jia et al., 2019; Wu et al., 2021). Our study demonstrates that maraviroc mitigates the protein levels of HMGB1 and NF-κB in the perilesional cortex at 3 days post-TBI, which is the first study, to our knowledge, that links CCR5 receptor inhibition to the HMGB1/NF-κB/NLRP3 pathway.

Astrocytes are widely distributed in the mammalian CNS and perform numerous essential functions. Astrocytes undergo a process called astroglia to become “reactive astrocytes” in reaction to CNS injury (Zamanian et al., 2012). Previous studies reported that reactive astrocytes restrict neuroinflammation, BBB repair, neuronal protection, and neurocognitive function recovery (Sofroniew, 2015; Almad and Maragakis, 2018; Gobbel et al., 2020). However, reactive astrocytes can exert negative effects, such as aggravating inflammation or interfering with axon growth (Silver and Miller, 2004). Recent studies demonstrated that reactive astrocytes were reactive nociceptive astrocytes and neuroprotective reactive astrocytes (A2 astrocytes) in response to neuroinflammation and ischemia, respectively (Liddelow et al., 2017). IL-1α, TNF-α, and complement C1q are released by activated microglia, causing the activation of A1 astrocytes in CNS injuries and diseases, such as Alzheimer’s disease, Parkinson’s disease, stroke, and TBI (Goetzl et al., 2018; Yun et al., 2018; Clark et al., 2019; Cao et al., 2021). A1 astrocytes lose their fundamental functions and exert neurotoxic functions, such as inducing the death of neuronal cells and mature oligodendrocytes (Liddelow et al., 2017). Neurotoxic reactive astrocytes that highly expressed complement C3 play neurotoxic roles in CNS diseases by releasing very-long-chain fatty acid acyl chains and free fatty acids (Escartin et al., 2021). Targeting neurotoxic reactive astrocytes may be a neuroprotective approach to promote the preservation of neuronal cells. In our study, we demonstrated that maraviroc had no effect on the activation of astrocytes or GFAP expression in the ipsilateral hemisphere. However, maraviroc induced a significant reduction in complement C3, which is a neurotoxic reactive astrocyte marker, in the pericontusional cortex 3 days postinjury. Double immunofluorescence staining of GFAP and complement C3 confirmed that maraviroc inhibits A1 astrocyte activation. The NF-κB signaling pathway is involved in pathophysiologic processes after TBI, such as neuroinflammatory reactivity in astrocytes and microglia and cell survival. Furthermore, downregulation of NF-κB and upregulation of phosphatidylinositol 3-kinase/protein kinase B regulates the shift from the A1 to the A2 phenotype (Xu et al., 2014). Notably, results suggest that maraviroc might suppress neurotoxic reactive astrocyte alterations by regulating the NF-κB pathway. To the best of our knowledge, our study illustrates for the first time that maraviroc exerts a neuroprotective role by modulating neurotoxic reactive astrocyte activation and reducing neuronal cell loss.

The major points of our present study are as follows: administration of maraviroc, an U.S. Food and Drug Administration–approved drug, alleviated neurological deficits and resulted in neurological function recovery after TBI; maraviroc treatment enhanced tissue preservation after TBI; maraviroc treatment regulated microglial polarization, reduced neutrophil and macrophage infiltration and NLRP3 inflammasome activation, and inhibited the HMGB1/NF-κB pathway and subsequent release of inflammatory factors after TBI, and maraviroc treatment inhibited neuronal apoptosis and reduced complement C3 and caspase-3 expression levels. Neuroinflammation exerts a vital effect on the physiological process of TBI (Morganti-Kossmann et al., 2019). At the early stage of TBI, resident microglia are activated, and peripheral neutrophils are recruited to the perilesional cortex. Subsequently, chemokine signaling causes the recruitment and infiltration of immune cells into the lesioned cortex (Jassam et al., 2017). Meanwhile, inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are released by these immune cells. Excessive posttraumatic neuroinflammation contributes to secondary brain damage and neuronal cell death in the perilesional cortex and hippocampus and exacerbates neurological dysfunctions (Morganti-Kossmann et al., 2019). Microglia rapidly respond to brain injury and are then recruited to the pericontusional cortex and release inflammatory cytokines, ultimately resulting in axonal injury and neuronal cell death after TBI (Witcher et al., 2015). Moreover, activated microglia polarize from the proinflammatory M1 subtype to the anti-inflammatory M2 subtype to regulate neuroinflammation (Wang et al., 2013; Hu et al., 2015). M1 microglia infiltrate lesioned cortex areas at 7 days poststroke, and M2 microglia are the main subtype present at 3 days poststroke (Kiong et al., 2016). Ample evidence indicates that knocking out or pharmacologically inhibiting CCR5 suppresses the inflammatory response by alleviating leukocyte, T cell, and macrophage infiltration and by promoting M2 macrophage activation (Glass et al., 2005; Rosi et al., 2005; Arberas et al., 2013; Long et al., 2020). Our study demonstrated that maraviroc could decrease neutrophil and macrophage infiltration and prionneuroinflammatory cytokine release. Furthermore, the notion that maraviroc treatment encourages a shift from M1 microglia toward M2 microglia to inhibit progressive inflammation and the destruction of the lesioned cortex 3 days post-TBI.
of the HMGB1/NF-κB/NLRP3 pathway after TBI. Furthermore, maraviroc administration protected neuronal cells against apoptosis by decreasing the expression of caspase-3 and BAX. Maraviroc administration also inhibited neurotoxic reactive astrocyte activation and the caspase-3 pathway to exert antiapoptotic effects.

Neurologic dysfunction, including short-term neurologic dysfunction and long-term cognitive dysfunction, is common after brain injury, and more than half of patients with TBI experience TBI-induced chronic cognitive impairment (Rabinowitz and Levin, 2014). TBI induces the apoptosis of hippocampal neurons, which is responsible for cognitive deficits in the chronic postinjury phase (Yang et al., 2018). Our findings reveal that maraviroc treatment promotes the survival of neurons by inhibiting neuroinflammation, caspase-3 expression, and neurotoxic reactive astrocyte activation and improves cognitive function recovery after TBI.

Our study had some limitations. We only focused on the potential anti-inflammatory effects of maraviroc without investigating its effects on BBB leakage and endoplasmic reticulum dysfunction following TBI. Further studies are required to determine the effects of maraviroc on BBB function after TBI.

In summary, our study provides compelling evidence that maraviroc could attenuate neuroinflammation and regulate the polarization of microglia and astrocytes after TBI via pharmacological blockade of the CCR5 receptor. Thus, the CCR5 receptor might be a promising pharmacotherapeutic target after TBI.

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Author contributions: JNZ, SZ and YZ designed the experiments. XLL, DDS, MTZ and HHHN carried out the experiments. MTZ and HHHN analyzed the experimental results. XLL and DDS wrote the manuscript. XLL, LZ, YY, ZWW, HTR, JYWW, GLY, XL and FLC took part in the experiments and proposed some suggestions. All authors approved the final version of this paper.

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Additional file: Additional Table 1: Modified Neurological Severity Scoring (mNSS).

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### Additional Table 1 Modified Neurological Severity Scoring (mNSS)

| Tests                        | Score |
|------------------------------|-------|
| **Motor tests**              |       |
| Raising mouse by tail        | 3     |
| Flexion of forelimb          | 1     |
| Flexion of hindlimb          | 1     |
| Head moved >10° to vertical axis within 30s | 1 |
| Placing mouse on floor (normal=0; maximum=3) | 3 |
| Normal walk                  | 0     |
| Inability to walk straight   | 1     |
| Circling toward paretic side | 2     |
| Falls down to paretic side   | 3     |
| **Sensory tests**            |       |
| Placing test (visual and tactile test) | 1 |
| Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles) | 1 |
| Beam balance tests (normal=0; maximum=6) | 6 |
| Balances with steady posture | 0     |
| Grasps side of beam          | 1     |
| Hugs beam and 1 limb falls down from beam | 2 |
| Hugs beam and 2 limb fall down from beam, or spins on beam (>60s) | 3 |
| Attempts to balance on beam but falls off (>40s) | 4 |
| Attempts to balance on beam but falls off (>20s) | 5 |
| Falls off; no attempt to balance or hang on to beam (<20s) | 6 |
| **Reflex absence and abnormal movements** | 4 |
| Pinna reflex (head shake when auditory meatus is touched) | 1 |
| Corneal reflex (eye blink when cornea is lightly touched with cotton) | 1 |
| Startle reflex (motor response to a brief noise from snapping a clipboard paper) | 1 |
| Seizures, myoclonus, myodystonia | 1 |
| **Maximum points**           | 18    |