Exploring the Role of Chemokine Receptor 1 (CX3CR1) after Traumatic Brain Injury

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Introduction

Every year in the United States, approximately 1.5 to 2 million people die or experience permanent disabilities from traumatic brain injury (TBI). TBI remains a major health challenge throughout the world, especially for military personnel and auto accident victims. TBI leads to variety of immune responses in the central nervous system (CNS), which includes the brain and spinal cord. After the injury, the brain is subjected to direct tissue damage and to impairment in regulation of cerebral blood flow and metabolism. Currently, no drugs have proven successful for clinical treatment of TBI. Therefore, it is imperative to discover a drug or a drug target that can introduce an effective therapeutic treatment for TBI.

CX3CR1 is categorized under the chemokine family and is a sole member of CX3C chemokine class, which exists in both membrane bound and soluble forms. Chemokine (C-X3-C motif) ligand 1 (CX3CL1)/CX3C chemokine receptor 1 (CX3CR1) signaling plays an important role in communication between neurons and microglia/macrophages in the central nervous system. Microglia is a type of glial cell that is located throughout CNS, and it plays an active defense role in CNS. Microglial activation is usually regulated by CX3CL1 and CX3CR1. Lately, CX3CR1 deficiency is reported to be associated with reduced activation and neurotoxicity of microglia/macrophages and improved functional recovery after brain ischemia and spinal cord injury. Tang et al. investigated the effects of CX3CR1 deficiency after ischemic stroke. They compared wild-type and CX3CR1-deficient mice after transient middle cerebral artery occlusion (MCAO) and reperfusion, and found that CX3CR1 signaling deficiencies exhibited neuroprotective function by reducing the recruitment of peripheral macrophages and activation of CNS mi-
croglia and macrophages in experimental ischemic stroke. Donnelly et al. investigated the effects of CX3CR1 deficiencies after the spinal cord injury. They found that CX3CR1 signaling deficiencies in intraspinal microglia and monocyte-derived macrophages (MDMs) decreased their synthesis and secretion of inflammatory cytokines and oxidative metabolites, and reducing CX3CR1 signaling promoted neuroprotection by decreasing inflammatory signaling in microglia and MDMs. However, its effect after TBI has not been currently investigated in preclinical models. In this study, we examined whether CX3CR1 deficiencies can protect the brain from injury in mice subjected to the controlled cortical impact (CCI) model of TBI and investigated the underlying mechanisms.
Materials and Methods

Animals

8-10 weeks (20-26 g) male C57BL/6 wild-type (WT), CX3CR1+/−, and CX3CR1−/− mice were used in this study. We housed the mice in the cages, which had free access to food and water throughout the study, under the 12-hour light/dark cycle in a pathogen-free environment. CX3CR1−/− knockout mice were genotyped by PCR amplification of genomic DNA extracted from tail snips. All experimental protocols in this study followed the guidelines and had an approval of Johns Hopkins University Animal Care and Use Committee.

CCI Model of TBI

The mice were anesthetized with isoflurane (3.0% for induction, 1.5% for maintenance) and ventilated with oxygen-enriched air (20%:80%) via a nose cone. We used ear bars and an incisor bar to hold the head in place. After the scalp was incised at the midline, a 5-mm-diameter craniotomy was created approximately midway between the bregma and the lambda on the left side, with the edge of the craniotomy 1 mm lateral to the midline. CCI was carried out with the Benchmark CCI Stereotaxic Impactor (Benchmark Deluxe; MyNeurolab, St. Louis, MO). The impact tip (3 mm in diameter) was directed perpendicular to the brain surface with an impact velocity of 6 m/s, an impact duration of 100 ms, and a depth of 1 mm. Rectal temperature was monitored and maintained at 37.0 °C by an electronic thermostat-controlled warming blanket (Stoelting Co., Wood Dale, IL) throughout the experimental and recovery periods. We followed this CCI model for all mice groups, except for sham group which had scalp incision with intact skull⁸.
**In vivo PI staining**

In vivo PI staining was used to detect cell deaths in the injured brain. 10mg/ml Propidium iodide was diluted in 0.9% NaCl and 0.4 mg/kg was administered 1 hour before sacrificing the mice by intraperitoneal injection. Samples were observed and photographed under a fluorescence microscope using excitation and emission wavelengths at 568 and 585 nm.

**Tissue Processing and Histology**

Mice were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1mol/L phosphate-buffered saline (pH 7.4) on 3 days after TBI. Their brains were removed and kept in 4% paraformaldehyde for 24 hours, and then were immersed in 30% sucrose for 48 hours. Then, brains were cut into 30-mm-thick coronal sections with a cryostat, and these sections were stained with Cresyl Violet. Lesion volumes were quantified using mage J software. The volume of the lesion in cubic millimeters was calculated as the sum of the damaged areas of each section multiplied by the interslice distance⁹.

**Neurologic Deficit Score**

On days 1, 3, and 7, post-TBI, we tested for neurologic deficits on each mouse. We used Neurologic deficit score was based on Clark et al. (table below) which examines seven parameters, including: body symmetry, gait, climbing, circling behavior, front limb symmetry, compulsory circling, and whisker response. Each test was graded from 0 to 4 with maximum deficit score of 28; higher score indicates worse neurologic deficit.
### Table: Neurologic Deficit Score Scale

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td><strong>Body Symmetry</strong></td>
<td>Normal</td>
<td>Slight asymmetry</td>
<td>Moderate asymmetry</td>
<td>Prominent asymmetry</td>
<td>Extreme asymmetry</td>
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<tr>
<td><strong>Gait</strong></td>
<td>Normal</td>
<td>Stiff, inflexible</td>
<td>Limping</td>
<td>Trembling, drifting, falling</td>
<td>Does not walk</td>
</tr>
<tr>
<td><strong>Climbing</strong></td>
<td>Normal</td>
<td>Climbs with strain, limb weakness present</td>
<td>Holds onto slope, does no slip or climb</td>
<td>Slides down slope, unsuccessful effort to prevent fall</td>
<td>Slides immediately, no effort to prevent fall</td>
</tr>
<tr>
<td><strong>Circling Behavior</strong></td>
<td>Not Present</td>
<td>Predominantly one-sided turns</td>
<td>Circles to one side</td>
<td>Circles constantly to one side</td>
<td>Pivoting, swaying, or no Circles constantly to one side movement</td>
</tr>
<tr>
<td><strong>Front Limb Symmetry</strong></td>
<td>Normal</td>
<td>Light asymmetry</td>
<td>Marked asymmetry</td>
<td>Prominent asymmetry</td>
<td>Slight asymmetry, no body/limb movement</td>
</tr>
<tr>
<td><strong>Compulsory Circling</strong></td>
<td>Not Present</td>
<td>Tendency to turn to one side</td>
<td>Circles to one side</td>
<td>Pivot to one side sluggishly</td>
<td>Does not advance</td>
</tr>
<tr>
<td><strong>Whisker</strong></td>
<td>Symmetrical Response</td>
<td>Light asymmetry</td>
<td>Prominent asymmetry</td>
<td>Absent response ipsilaterally, diminished contralaterally</td>
<td>Absent proprioceptive response bilaterally</td>
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**Tail suspension test (TST)**

On 21 days post-TBI, TST was experimented on each mouse. The set-up involved suspending the mice by putting over a piece of adhesive tape (1 cm from the tip, 17 cm long) over the tail from 55 cm above the desk. A camera recorded the mouse movement for 6 minutes. The aim was to examine the duration of immobility, which was calculated by subtracting the duration of total mobility time from 6 minutes.
Forced Swim Test (FST)

On 28 days post-TBI, FST was performed on each mouse to measure the immobility. Mouse was placed individually in cylindrical tanks with a water depth of 15 cm at 23 ± 1°C. Each trial was 6 minutes but started measuring time spent immobile in the last 4 minutes. Similar to TST, the immobility duration was calculated by subtracting the total mobility time from 240 seconds. Mouse was considered immobile if it remained floating in an upright position and made only small movements to keep its head above the water.

Novel Object Recognition Test (NOR)

NOR was performed on mice at 14 days post-TBI, and this test was carried out in a 50 × 25 × 50 cm dimensioned black, open-field box. Initially, each mouse was habituated in the box for 10 min. On the next day, two identical novel objects were placed in the middle of the box, and the mouse was allowed to explore the area for 10 minutes. After 1 hour, one novel object and one old object were placed in the box, and the mouse was allowed to explore for 5 minutes. Its behavior was recorded by camera. This test was to examine the differences in time exploring the old and new objects between treatment and control groups. Normal mouse tends to spend longer on novel object than injured mouse. The exploration time included time in direct contact with the objects and time within the object area; a discrimination index (total time spent with new object/total time devoted to exploration of objects) was also calculated for each mouse.

Y-Maze Test

Y-Maze was performed to test the memory performance of mice, especially their short-term spatial recognition. First, each mouse was placed at the end of one arm and allowed to move freely
through the maze for 8 minutes. For normal mice, they would spend more time exploring the newer arm when introduced. Mice’s alternation behaviors were recorded with camera. The number of alternation was calculated as the ratio of actual to possible alternations, which was operationalized as the total number of arm entries\(^\text{10}\).

**Brain Water Content**

Brain water content shows the extent of brain edema after TBI. It was measured 3 days after TBI. First, mice were sacrificed by decapitation, and their brains were removed and divided into ipsilateral and contralateral striatum and cerebellum. Then, brain samples were weighed, both wet weight and dry weight (dried for 24 hours at 100\(^\circ\)C). Water content was calculated as: Water content (\%) = (wet weight - dry weight)/wet weight\times100%.

**Immunofluorescence**

Brain sections were incubated with mouse anti-CD68, CD16/32, iNOS, YM1, CD206 at 4\(^\circ\)C overnight and then with Alexa Fluor 594-conjugated secondary antibody for 1 hour at 25\(^\circ\)C. This staining was followed by examining the sections under a microscope. On 15 randomly selected locations (5 fields per section x 3 sections per mice), positive area was counted around the lesion by Image J software.

**Quantitative assessment of microglia and astrocytes**

A computer-based tracing system, Neurolucida software, was used at a 40x lens magnification to analyze somas and dendrites of microglia and astrocytes. For each immunofluorescence images, cell body area, cell volume, number of trees, total number, length and surface of dendrite were measured.
Stereological measurement

Stereological analyses were used to quantify neurons. Coded slides were used to blind this experiment. With Cavalieri's estimator, the total reference volume of the ipsilateral injury area was obtained.

Statistical Analyses

Statistical data presented in this paper are represented in mean ± SD. Differences between experimental groups and control groups were tested with the Student’s t-test, and the criterion for statistical significance was \( p < 0.05 \). Two-way ANOVA was used for all behavioral tests for the analysis. One-way or two-way ANOVA were used for anatomical and biochemical studies.
Results

Figure 1: 3 days post-TBI

Figure 1a compares three different groups of mice: wild-type, CX3CR1\(^{+/−}\), and CX3CR1\(^{−/−}\). The study found that there was no difference in lesion volume between wild-type and CX3CR1\(^{+/−}\) mice, but CX3CR1\(^{−/−}\) showed statistically significant decrease in lesion volume. For brain water content, there were no differences between CX3CR1\(^{+/−}\), and CX3CR1\(^{−/−}\). Figure 1b compares CX3CR1\(^{+/−}\) and CX3CR1\(^{−/−}\) on 1) number of neurons survived and 2) number of neurons dead. Top image was produced from Cresyl Violet staining and stereology that quantified the survived neurons. CX3CR1\(^{−/−}\) showed significantly increased numbers of survived neurons. On the other hand, bottom image was produced from in-vivo PI staining where red fluorescence is higher when there is higher cell death. CX3CR1\(^{−/−}\) group exhibited fewer dead neurons compared to the CX3CR1\(^{+/−}\) group.
Figures 2a and 2b compare three groups of mice on days 1, 3, and 7 after TBI. Figure 2a examined neurological deficit in which CX3CR1−/− group received significantly lower score only on day 3, where lower score indicates better motor function. Given that only on day 3 did CX3CR1−/− exhibited better function, the result could be due to a small sample. Figure 2b was a corner test to examine motor functions. CX3CR1−/− group had significant increase in number of left turns on both day 1 and day 7 after TBI. (Mice usually turn right after the injury). Figure 2c was from a novel object recognition (NOR) test on 14 days after TBI. Normal mouse for tends to spend more time on newer object, while injured mouse spend more time on older objects than normal due to problems in memory. CX3CR1−/− group performed better than other groups, however, there were no statistically significant differences, which may be due to low sample size. Figure 2d was a Y-maze test on 14 days after TBI to look at memory function. This test is similar to
NOR, in which normal mouse would spend more time exploring the new object or path. CX3CR1−/− group spent significantly more time in the new arms of Y-maze, compared to other groups. Figures 2e and 2f were tail suspension test (TST) on 21 days and forced swim test on 28 days post-TBI, respectively. There were no statistically significant differences among three groups for both TST and forced swim test.
Figure 3: (a) Microglia and (b) Astrocyte

Figure 3 was obtained from image tracing software which examined cell body (soma) and dendrites of microglia and astrocytes. First row is an image of cells with tracing. There were four features that were analyzed: cell body area, cell body roundness, number of dendrites, and total length of dendrites. Second row is data for microglia and third row for astrocytes. Data analysis showed that only microglia’s number of dendrites was statistically significant, where CX3CR1−/− had lower number of dendrites.
Figure 4: 5 Different Markers to detect 1) All active microglial 2) M1-microglia 3) M2-microglia

Figure 4 shows five different markers: CD68, CD16/32, iNOS, YM1, CD206. CD68 shows all of the active microglial. CD16/32 and iNOS show M1-microglia which secretes pro-inflammation cytokine that is deleterious for brain. YM1 and CD206 show M2-microglia which secretes pre-inflammation cytokine that has protective function for brain injury. The data shows that CX3CR1−/− group of mice had statistically significant decrease in active microglial activities as...
well as number of M1-microglia, while had an increase in M2-microglia. The data suggests that CX3CR1 knockout may have protective function for the brain after TBI.
Conclusion

This project provided valuable and novel information regarding the role of CX3CR1 after traumatic brain injury. The results indicate that CX3CR1\(^{-/-}\) mice had better histologic and functional outcomes than CX3CR1\(^{+/+}\) mice. There was a decrease in M1 and increase in M2-microglia, which suggests that knocking out CX3CR1 may aid in protecting the brain after injury. The insights gained are essential for planning more detailed preclinical studies and future clinical trials on CX3CR1 antagonists for treating traumatic brain injury.
References