

# CX3CL1/CX3CR1 Axis Plays a Key Role in Ischemia-Induced Oligodendrocyte Injury via p38MAPK Signaling Pathway

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**Abstract** Based on current knowledge on the role of the CX3CL1/CX3CR1 axis in the regulation of microglial activation and on the involvement of activated microglia in damaging oligodendrocytes, we hypothesized that CX3CL1/CX3CR1 axis is associated with the development of ischemic oligodendrocyte and white matter injury. We investigated the effects of CX3CL1, CX3CR1 shRNA, and p38MAPK inhibitor on the apoptosis, proliferation, and myelin proteolipid protein (PLP) expression in oligodendrocytes in co-cultures with BV2 microglia under ischemia. We demonstrated that CX3CL1 markedly increased the numbers of apoptotic oligodendrocytes, decreased PLP expression in oligodendrocytes, and inhibited the increased proliferation of oligodendrocytes induced by ischemia in co-cultures. All these effects of CX3CL1 were suppressed by pre-treatment of BV2 microglia with CX3CR1 shRNA to silence CX3CR1 expression or SB203580 to inhibit p38MAPK pathway. Our findings support that CX3CL1/CX3CR1 axis plays a key role in the

development of ischemia-induced oligodendrocyte injury via p38MAPK signaling pathway.

**Keywords** CX3CL1/CX3CR1 axis · Microglial activation · Oligodendrocyte · BV2 microglia · Ischemia · p38MAPK pathway

## Abbreviations

CX3CL1	Fractalkine
CX3CR1	CX3CL1 receptor
IL-1 $\beta$	Interleukin-1 $\beta$
OGD	Oxygen and glucose deprivation
PLP	Myelin proteolipid protein
TNF- $\alpha$	Tumor necrosis factor alpha

## Introduction

White matter exclusively contains axons and glial cells including astrocytes, oligodendrocytes and microglia. This brain region receives disproportionately less circulation than gray matter and is therefore highly vulnerable to reduced blood supply [1, 2]. Damage of white matter is a major cause of functional disability in cerebrovascular disease, and the majority of ischemic strokes involve both white matter and gray matter [3–5]. Animal studies have indicated that white matter can be damaged by even brief focal ischemia [6].

Oligodendrocyte is one of the targets of white matter injury in ischemic strokes. Compared with other brain cells, oligodendrocytes have higher lipid and iron contents. These might be the reasons for the high vulnerability of the cells [7]. Also, oligodendrocytes have low supplies of the cellular antioxidant enzymes and limited substrates [8, 9]. It is probably why

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oligodendrocytes have a lower ability to cope with an increased generation of reactive oxygen species, which may be another key reason for the cells to be highly sensitive to ischemic injury in the brain [7].

Several mechanisms including oxidative stress, excitatory amino acids, trophic factor deprivation, and activation of apoptotic pathways have been shown to initiate oligodendrocyte injury under ischemic conditions [2, 7, 10]. In addition, microglia activation has been thought to be an important mechanism for damaging oligodendrocyte precursor cells/oligodendrocytes under ischemic white matter injury [11–14] and also implicated in the pathogenesis of other white matter disorders, such as periventricular leukomalacia and multiple sclerosis [15–17]. However, the mechanism of how activated microglia kills oligodendrocytes has not been conclusively determined.

Microglial activation is usually regulated by the chemokine CX3CL1 (also called fractalkine) and its receptor, CX3CR1 [18]. In the brain, CX3CL1 is a unique chemokine, being constitutively expressed by neurons where it is tethered to the extracellular membrane by a mucin stalk [19, 20]. A number of studies have been conducted to investigate the role of CX3CL1/CX3CR1 axis in brain ischemic injury; however, the relevant findings are controversial. The destructive [21–25] and/or beneficial [18, 26] roles of CX3CL1/CX3CR1 axis in ischemic brain injury have been reported. These conflicting data to date do not provide a coherent conclusion on the role of CX3CL1/CX3CR1 axis in ischemic brain [18, 23] and also oligodendrocyte injury.

Based on the role of the CX3CL1/CX3CR1 axis in the regulation of microglial activation and the involvement of activated microglia in damaging oligodendrocytes, we hypothesized that the CX3CL1/CX3CR1 axis is associated with the development of oligodendrocyte and white matter injury under ischemia although its role is unknown. In the present study, we investigated the effects of the addition of CX3CL1, silencing CX3CR1 expression by CX3CR1 shRNA and inhibition of p38MAPK using SB203580 on the apoptosis, proliferation, and expression of myelin proteolipid protein (PLP) in oligodendrocytes co-cultured with BV2 microglia under ischemia.

## Materials and Methods

### Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA. Primary anti-myelin PLP antibody was purchased from Abcam, MA, USA, and goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody from Li-Cor, Lincoln, NE, USA. The monoclonal antibody against BrdU was bought from Invitrogen, Carlsbad, CA, USA, and Alexa 546-conjugated secondary

antibody from Millipore, Billerica, MA, USA. A porous polycarbonate transwell membrane obtained from Corning, Corning, NY, USA, and Bradford assay kit from Bio-Rad, Hercules, CA, USA. CX3CR1 shRNA (against CX3CR1 retrovirus) and negative-shRNA (negative control) were obtained from Genechem Co. Ltd., Shanghai, China. Human oligodendroglia cells (a cell line derived from fetal human oligodendrocytes) were kindly supplied by Professor Peng Xie of the Department of Neurology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China.

### Transwell Co-Culture of Oligodendrocytes with BV2 Microglia

Transwell co-culture of oligodendrocytes with BV2 microglia was conducted as described by Rodriguez-Crespo et al. [27] and Yao et al. [28]. BV2 microglia were cultured [29] and then seeded on the lower surface of a porous polycarbonate transwell membrane (0.4- $\mu$ m pore size,  $2.0 \pm 0.2 \times 10^6$ /cm<sup>2</sup> pore density, 24-mm membrane thickness). The 0.4- $\mu$ m pore size prevents direct cell–cell interactions but allows the diffusion of soluble factors through the membrane.

### Oxygen and Glucose Deprivation

Ischemia in co-cultures was induced by oxygen and glucose deprivation (OGD) as previously described [30]. In brief, the co-cultures were exposed to the medium of serum-free DMEM without glucose (Invitrogen-Life Technologies) and then put in a hypoxic incubator (INVIVO2, RUSKINN) with 1 % O<sub>2</sub>, 94 % N<sub>2</sub>, and 5 % CO<sub>2</sub> at 37 °C for 4 h.

### Hoechst 33342 Staining

Oligodendrocytic apoptosis was observed by fluorescence microscopy with Hoechst 33342 staining [31]. Rounded cells with condensed or fragmented nuclei were considered apoptotic. Briefly, oligodendrocytes in chambers were washed by PBS after OGD, fixed with 4 % paraformaldehyde for 1 h and then stained with Hoechst 33342 (5  $\mu$ mol/L) for 1 h at 4 °C. After washing three times, the cells were observed under a fluorescent microscope (Leica, Wetzlar, Germany) equipped with a 350-nm excitation laser and a digital camera (Canon, Japan).

### Cell Proliferation Assay

Cell proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) assay. BrdU was added to the culture medium at a final concentration of 10  $\mu$ M and maintained for 24 h. Oligodendrocytes were fixed in 4 % paraformaldehyde for 15 min at room temperature and treated with 2 N HCl for 30 min at 37 °C to denature DNA, and then incubated with a monoclonal antibody against BrdU (1:500) for 2 h followed by Alexa

546-conjugated secondary antibody (1:1000). Subsequently, the slides were washed three times with PBS and the red fluorescein-labeled nuclei were visualized by using a Leica TCS SP8 confocal microscope (Leica, Buffalo Grove, IL). The percentages of nuclei displaying red fluorescence were calculated as a percent in total cells viewed in the same field. Five individual experiments were conducted, and at least five slides per group were used.

## ELISA

TNF- $\alpha$  and IL-1 $\beta$  concentrations in culture medium of BV2 microglia were measured using commercially available ELISA kits according to the manufacturer's instruction (R&D Systems). The optical density (OD) at 450 nm was read by using an ELX-800 microplate assay reader (Elx800, Bio-tek, USA). The average absorbance values for each set of standards and samples were calculated from the standard curve [32].

## Western Blot Analysis

Oligodendrocytes receiving different treatments were washed with ice-cold PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 % SDS, 1 % Nonide P-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 5 % b-mercaptoethanol, 400  $\mu$ M phenylmethylsulfonyl fluoride, 2 mg/ml each of pepstatin, aprotinin, and eupeptin). After centrifugation at 12,000g for 10 min at 4 °C, the supernatant was collected, and protein concentration in the extracts was determined by Bradford assay kit. Aliquots of the cell extract containing equal amounts of protein were boiled in a protein loading buffer for 5 min, separated on a 10 % SDS-polyacrylamide gels, and transferred to PVDF membranes. The membranes were blocked with 5 % non-fat milk in Tris-buffered saline for 1 h and incubated in primary anti-myelin PLP antibody (1:500) overnight at 4 °C. After three washes with TBS-T, the membrane was incubated with goat anti-rabbit IRDye 800 CW IgG (1:10,000) for 1 h at room temperature. The intensities of the specific bands were detected and analyzed by Odyssey infrared imaging system at a resolution of 169  $\mu$ m (Li-Cor Biosciences, Nebraska, USA) [33]. To ensure even loading of the samples, the same membrane was probed with rabbit anti-rat  $\beta$ -actin polyclonal antibody at a 1:2000 dilution.

## Statistical Analysis

All data were presented as mean $\pm$ standard error (SEM). Statistical analyses were performed using SPSS software for Windows (version 13.0) (SPSS, Inc., Chicago, IL). The differences between the means were all determined by two-way analysis of variance (ANOVA). A probability value of  $P < 0.05$  was taken to be statistically significant.

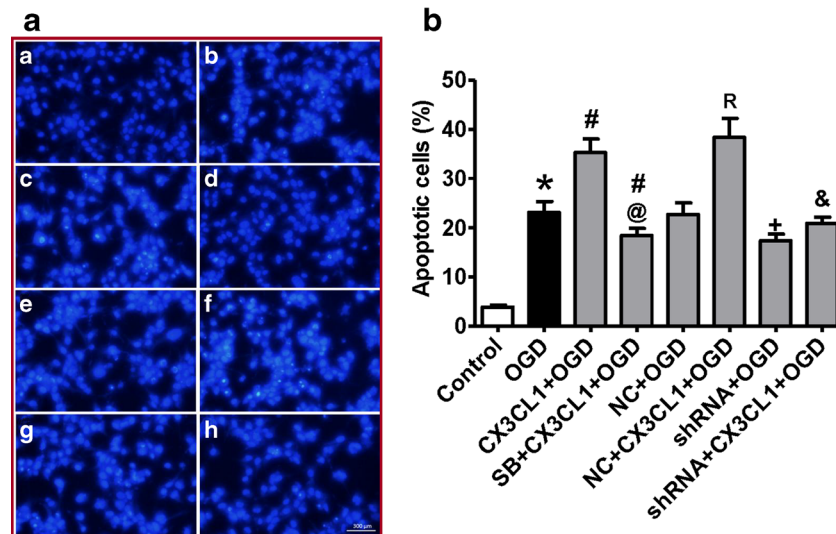
## Results

### Effects of CX3CL1/CX3CR1 Axis and p38MAPK on Apoptosis of Oligodendrocytes Co-Cultured with BV2 Microglia Under Ischemia

We speculated that ischemia-induced apoptosis of oligodendrocytes in vivo is associated with the enhanced release of a variety of inflammatory and cytotoxic mediators such as TNF- $\alpha$ , IL-1 $\beta$ , and reactive oxygen species from the activated microglia, while the enhanced mediators might be due to the increased expression and release of CX3CL1 in neuron and the activation of CX3CR1 as well as p38MAPK signaling pathway in microglia under ischemia. To test the possibilities, we first investigated the effects of the addition of CX3CL1, silencing CX3CR1 expression and inhibition of p38MAPK on apoptosis of oligodendrocytes in the co-cultures with BV2 microglia under ischemia. BV2 microglia were pre-infected with CX3CR1 shRNA or negative shRNA (MOI=10) for 72 h or pre-treated with 20  $\mu$ M of SB203580 (a p38MAPK inhibitor) for 1 h, and then the co-cultures of oligodendrocytes with BV2 microglia were subjected to OGD for 4 h followed by reperfusion for 20 h in the presence of 0 or 50 ng/ml of CX3CL1. After the treatments, oligodendrocyte apoptosis was detected by Hoechst 33342 staining.

As shown in Fig. 1, ischemia (OGD) induced a significant increase in the numbers of apoptotic cells. The percentage of apoptotic cells in total cells in OGD group is significantly higher than that in control group. In the presence of 50 ng/ml of CX3CL1, OGD treatment led to a further increase in the percentage of apoptotic cells, which was markedly high than that in OGD group. However, the numbers of apoptotic cells in SB+CX3CL1+OGD group were found to be significantly lower than those in CX3CL1+OGD and also OGD groups ( $P < 0.05$ ). This implied that pre-treatment of BV2 microglia with SB before co-cultured with oligodendrocytes could protect oligodendrocytes from OGD and CX3CL1-induced injury by inhibiting p38MAPK signaling.

In addition, we found that pre-infection of BV2 microglia with negative shRNA (NC) has no significant effects on the numbers of apoptotic cells under the conditions of OGD or CX3CL1+OGD treatments. There were no differences in the percentage of apoptotic cells in total cells between OGD and NC+OGD or between CX3CL1+OGD and NC+CX3CL1+OGD groups. However, pre-infection of BV2 microglia with CX3CR1 shRNA induced a significant inhibiting effect on the numbers of apoptotic cells. The numbers of apoptotic cells in shRNA+OGD or shRNA+CX3CL1+OGD groups were significantly lower than those in NC+OGD or NC+CX3CL1+OGD groups, respectively. This suggested that silencing



**Fig. 1** The effects of CX3CL1/CX3CR1 axis and p38MAPK on apoptosis of oligodendrocytes co-cultured with BV2 microglia under ischemia. BV2 microglia were pre-infected with CX3CR1 shRNA (shRNA) or negative shRNA (NC) (MOI=10) for 72 h or pre-treated with 20  $\mu$ M of SB203580 (SB) for 1 h, and then the co-cultures of oligodendrocytes with BV2 microglia were subjected to OGD for 4 h followed by reperfusion for 20 h in the presence of 0 or 50 ng/ml of CX3CL1. After the treatments,

the apoptosis of oligodendrocytes was detected by Hoechst 33342 staining as described in the “Materials and Methods” section. **a** Representative photographs (a-h) of oligodendrocytic nucleus by Hoechst 33342 staining. **b** The percentage of apoptotic cells in total cells. Data were presented as mean  $\pm$  SEM ( $n=7$ ). \* $P<0.05$  vs. control; # $P<0.05$  vs. OGD; @ $P<0.05$  vs. CX3CL1+OGD; <sup>R</sup> $P<0.05$  vs. NC+OGD; <sup>±</sup> $P<0.05$  vs. NC+OGD; & $P<0.05$  vs. NC+CX3CL1+OGD

CX3CR1 expression in BV2 microglia by pre-treatment with CX3CR1 siRNA could also protect oligodendrocytes from OGD and CX3CL1-induced apoptosis (Fig. 1).

### CX3CL1 Reduced Expression of Myelin Proteolipid Protein in Oligodendrocytes Probably via Activation of CX3CR1 and p38MAPK Pathway

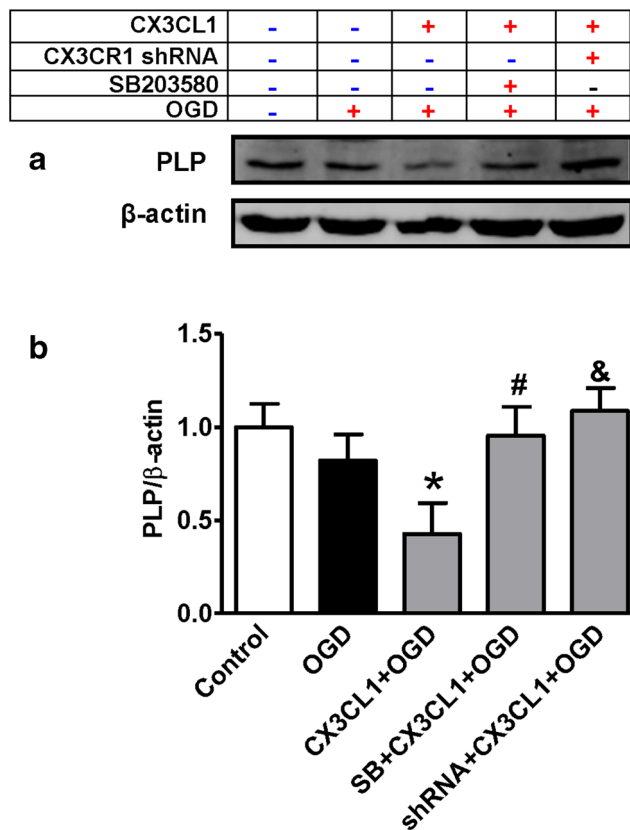
We then investigated the effects of CX3CL1/CX3CR1 axis and p38MAPK pathway on expression of myelin PLP in oligodendrocytes in the co-cultures with BV2 microglia under ischemia. BV2 microglia were pre-infected with CX3CR1 shRNA (MOI=10) for 72 h or pre-treated with 20  $\mu$ M of SB203580 for 1 h, and then the co-cultures of oligodendrocytes with BV2 microglia were subjected to OGD for 4 h followed by reperfusion for 20 h in the presence of 0 or 50 ng/ml of CX3CL1. After the treatments, the PLP expression was detected by Western blot analysis.

It was found that OGD has a role to slightly reduce PLP expression in oligodendrocytes (Fig. 2), and in the presence of 50 ng/ml of CX3CL1, OGD treatment led to a further reduction in PLP expression. PLP expression in CX3CL1+OGD group was significantly lower than that in OGD or control groups. However, pre-treatment of BV2 microglia with CX3CR1 shRNA or SB203580 completely prevented the reduction PLP expression in oligodendrocytes induced by CX3CL1 and/or OGD. PLP expression in both of SB+CX3CL1+OGD and shRNA+CX3CL1+OGD group was significantly higher than that in CX3CL1+OGD group and no significant difference from the control (Fig. 2).

### Effects of CX3CL1/CX3CR1 Axis and p38MAPK on Proliferation of Oligodendrocytes Co-Cultured with BV2 Microglia Under Ischemia

We also examined whether CX3CL1/CX3CR1 axis and p38MAPK pathway are involved in the changes in oligodendrocytes proliferation in the co-cultures with BV2 microglia under ischemia. BV2 microglia were pre-infected with CX3CR1 shRNA or negative shRNA (MOI=10) for 72 h or pre-treated with 20  $\mu$ M of SB203580 for 1 h, and then the co-cultures of oligodendrocytes with BV2 microglia were subjected to OGD for 4 h followed by reperfusion for 20 h in the presence of 0 or 50 ng/ml of CX3CL1. After the treatments, the proliferation of oligodendrocytes was detected by BrdU assay.

We demonstrated that OGD treatment induced a significant increase in the number of BrdU-positive oligodendrocytes as compared with the control group. In the presence of 50 ng/ml of CX3CL1, OGD treatment led to a remarkable decrease in the number of BrdU-positive cells (Fig. 3). BrdU-positive cells in CX3CL1+OGD group were significantly lower than those in OGD group. However, the positive cells in SB+CX3CL1+OGD group were found to be significantly higher than those in CX3CL1+OGD group, suggesting the existence of a promoting role of SB203580 on oligodendrocyte proliferation. In addition, we did not find any statistical differences in the number of BrdU-positive oligodendrocytes between OGD and NC+OGD groups or between CX3CL1+OGD and NC+CX3CL1+OGD groups. However, the number of BrdU-positive cells in shRNA+OGD or shRNA+CX3CL1+OGD group was significantly higher than that in OGD or



**Fig. 2** CX3CL1 reduced the expression of myelin proteolipid protein (PLP) in oligodendrocytes via CX3CR1/p38MAPK signaling pathway in the co-cultures with BV2 microglia under ischemia. BV2 microglia were pre-infected with CX3CR1 shRNA (shRNA) (MOI=10) for 72 h or pre-treated with 20  $\mu$ M of SB203580 (SB) for 1 h, and then the co-cultures of oligodendrocytes with BV2 microglia were subjected to OGD for 4 h followed by reperfusion for 20 h in the presence of 0 or 50 ng/ml of CX3CL1. After the treatments, the PLP expression was detected by Western blot analysis. **a** A representative experiment of Western blot of PLP. **b** Quantification of the level of PLP in oligodendrocytes. Data were presented as mean  $\pm$  SEM ( $n=5$ ). \* $P<0.05$  vs. OGD (and control), # $P<0.05$  vs. CX3CL1+OGD, & $P<0.05$  vs. CX3CL1+OGD

CX3CL1+OGD group, respectively (Fig. 3). The findings showed that silencing CX3CR1 expression in BV2 microglia by pre-treatment with CX3CR1 shRNA could improve oligodendrocyte proliferation. The data also implied that CX3CL1 inhibits oligodendrocyte proliferation probably via activation of CX3CR1 and p38MAPK signaling in the co-cultures with BV2 microglia under ischemia.

#### CX3CR1 shRNA or SB203580 Attenuated the Enhanced Expression in TNF- $\alpha$ and IL-1 $\beta$ Induced by CX3CL1 in OGD-Treated BV2 Microglia

To further investigate the mechanisms involved in the detrimental effect of CX3CL1/CX3CR1 axis on oligodendrocytes under ischemia, we investigated the effects of CX3CL1, CX3CR1

shRNA, and SB203580 on the expression of microglia cytokines TNF- $\alpha$  and IL-1 $\beta$  in OGD-treated BV2 microglial cells in vitro. The treatment with CX3CL1 (50 ng/ml) plus OGD induced a significant increase in expression of TNF- $\alpha$  and IL-1 $\beta$  in BV2 microglia as compared with the cells treated with OGD only (Fig. 4). The increased expression in TNF- $\alpha$  (Fig. 4a) and IL-1 $\beta$  (Fig. 4b) induced by OGD and CX3CL1 could be dramatically suppressed by pre-treatment of the cells with CX3CR1 shRNA or the p38MAPK inhibitor. These findings demonstrated that the increased expression of TNF- $\alpha$  and IL-1 $\beta$  induced by the addition of CX3CL1 in OGD-treated BV2 microglia is CX3CR1- and also p38MAPK-dependent.

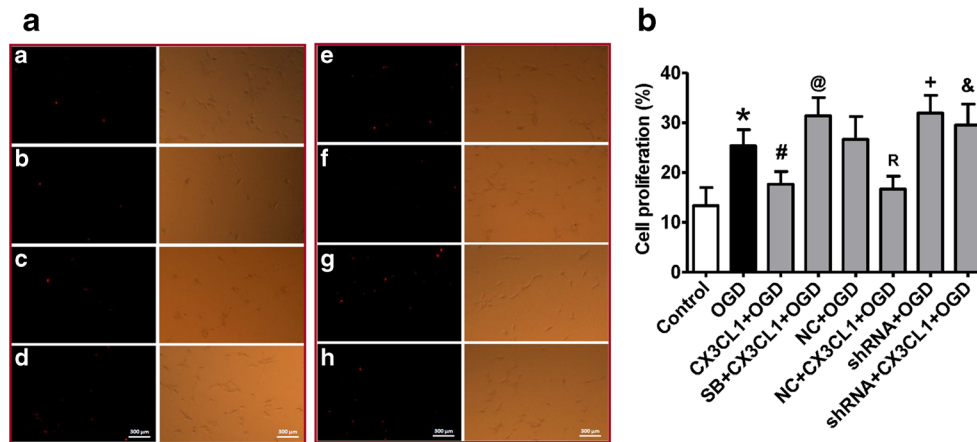
#### Effects of CX3CR1 shRNA on CX3CR1 Protein Expression and p38 Phosphorylation in Microglia

Finally, we investigated the effect of CX3CR1 shRNA on CX3CR1 protein expression by incubating BV2 microglia with or without CX3CR1 shRNA (MOI=10) for 72 h. The Western blot analysis showed that CX3CR1 shRNA could significantly inhibit CX3CR1 protein expression (Fig. 5a). We also examined the effect of CX3CL1 and/or CX3CR1 shRNA on p38 phosphorylation and demonstrated that CX3CL1 could stimulate p38 phosphorylation in BV2 cells, which could be significantly blocked by treatment with CX3CR1 shRNA (Fig. 5b).

#### Discussion

In the present study, we demonstrated that addition of CX3CL1 induced a further increase in the number of apoptotic oligodendrocytes in the co-cultures with BV2 microglia under conditions of OGD treatment. We also showed that silencing CX3CR1 expression in BV2 microglia by pre-infected the cells with CX3CR1 shRNA led to a significant reduction in the number of apoptotic oligodendrocytes in the OGD-treated co-cultures. The numbers of apoptotic cells in the co-cultures treated with OGD plus shRNA or CX3CL1 and shRNA were significantly higher than those in the co-cultures treated with OGD only. These demonstrated for the first time that the CX3CL1/CX3CR1 axis plays a destructive rather than beneficial effect on oligodendrocyte under ischemic conditions. In addition, we found that inhibition of p38MAPK significantly reduced the increased numbers of apoptotic oligodendrocytes induced by treatment with CX3CL1 and ischemia. This implied that the destructive effect of CX3CL1 and CX3CR1 on oligodendrocyte is probably p38MAPK pathway-mediated.

Proteolipid protein (PLP) is an integral membrane protein expressed predominantly in oligodendrocytes in the brain [34–36]. PLP gene expression has been considered as a sensitive indicator of oligodendrocyte function in studies on ischemic stroke [37]. It has also been reported that the



**Fig. 3** The effects of CX3CL1/CX3CR1 axis and p38MAPK on proliferation of oligodendrocytes co-cultured with BV2 microglia under ischemia. BV2 microglia were pre-infected with CX3CR1 shRNA (shRNA) or negative shRNA (NC) (MOI=10) for 72 h or pre-treated with 20  $\mu$ M of SB203580 (SB) for 1 h, and then the co-cultures of oligodendrocytes with BV2 microglia were subjected to OGD for 4 h followed by reperfusion for 20 h in the presence of 0 or 50 ng/ml of CX3CL1. After the treatments, the proliferation of oligodendrocytes

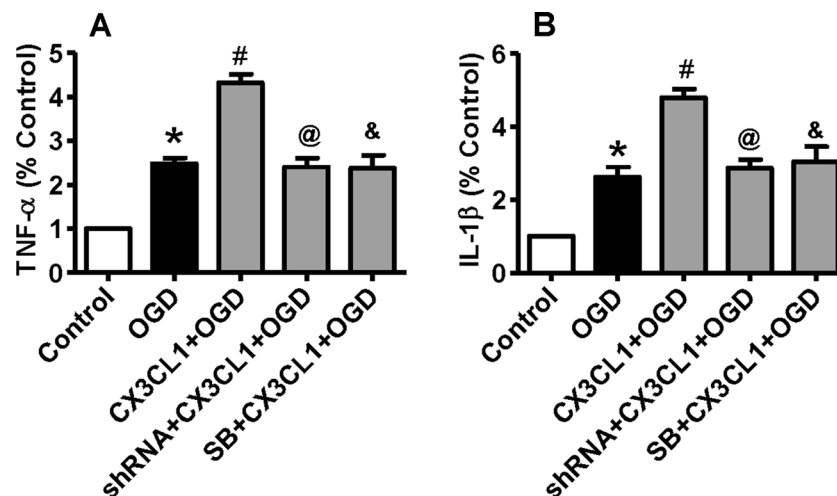
was detected by BrdU assay as described in the “Materials and Methods” section. **a** Representative photographs (a-h) of proliferation of oligodendrocytes by BrdU assay (the left panel shows BrdU assay and the right shows the same bright field). **b** The percentage of cellular proliferation in total cells. Data were presented as mean $\pm$ SEM ( $n=5$ ). \* $P<0.05$  vs. control; # $P<0.05$  vs. OGD; @ $P<0.05$  vs. CX3CL1+OGD; <sup>R</sup> $P<0.05$  vs. NC+OGD; <sup>+</sup> $P<0.05$  vs. NC+OGD; & $P<0.05$  vs. NC+CX3CL1+OGD

oligodendrocyte-specific PLP message declined at 12 h in the ischemic core and was almost absent at 24 h in an adult mouse stroke model (middle cerebral artery occlusion) [38]. This acute reduction in a myelin message demonstrated ischemia-induced oligodendroglial injury [37]. To understand whether CX3CL1/CX3CR1 axis is also involved in the changes in oligodendrocyte function under ischemia, we investigated the effects of CX3CL1/CX3CR1 axis on myelin PLP expression in oligodendrocytes.

In contrast to the effect on the number of apoptotic oligodendrocytes, the addition of CX3CL1 was found to lead to a further decrease rather than increase in PLP expression in

oligodendrocytes in the co-cultures with BV2 microglia under conditions of OGD treatment. However, the detrimental effect of CX3CL1 on PLP expression in oligodendrocytes completely disappeared when BV2 microglia was pre-infected with CX3CR1 shRNA or pre-treatment with SB203580. The findings indicated that the reduction in oligodendrocyte function or PLP expression by CX3CL1 treatment is CX3CR1- and p38MAPK pathway-dependent and also associated with the decrease in oligodendrocyte survival in the co-cultures with BV2 microglia under ischemia.

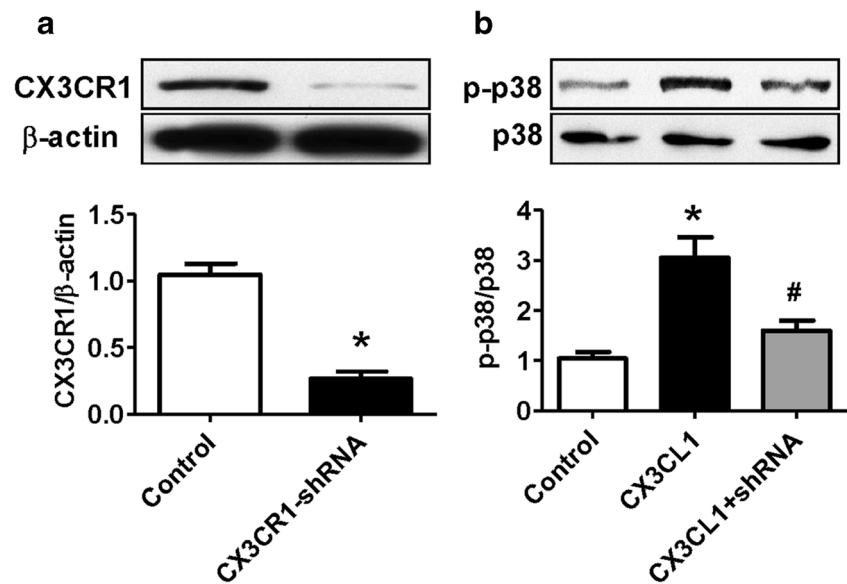
CX3CL1 markedly reduced the numbers of oligodendrocytes by increasing cell apoptosis as well as decreased PLP



**Fig. 4** CX3CR1 shRNA or SB203580 significantly reduced the increased expression of TNF- $\alpha$  and IL-1 $\beta$  induced by OGD and CX3CL1 in BV2 microglia in vitro. BV2 microglial cells were infected with CX3CR1 shRNA (shRNA) for 72 h or pre-treated with 20  $\mu$ M of SB203580 only for 1 h, then subjected to OGD for 4 h in the presence of

50 ng/ml of CX3CL1. After the treatments, the measurements of TNF- $\alpha$  and IL-1 $\beta$  were conducted using ELISA as described in the “Materials and Methods” section. Data were presented as mean $\pm$ SEM ( $n=3$ ). \* $P<0.05$  vs. control; # $P<0.05$  vs. OGD; @ $P<0.05$  vs. CX3CL1+OGD; & $P<0.05$  vs. CX3CL1+OGD

**Fig. 5** The effects of CX3CR1 shRNA and/or CX3CL1 on CX3CR1 protein expression and p38 phosphorylation in microglia. **a** BV2 microglial cells were infected with or without CX3CR1 shRNA (MOI=10) for 72 h; CX3CR1 expression was then determined by Western blot analysis. **b** BV2 microglial cells were treated with 50 ng/ml of CX3CL1 for 4 h and then infected with or without CX3CR1 shRNA for 72 h. After the treatments, p38 phosphorylation was detected by Western blot analysis. Data were presented as mean±SEM ( $n=3$ ). \* $P<0.05$  vs. control, # $P<0.05$  vs. CX3CL1



expression in the cells after ischemia. However, it is unclear whether this is due to increased cell loss or reduced proliferation. We therefore examined the effects of CX3CL1/CX3CR1 axis on oligodendrocyte proliferation in the co-cultures with BV2 microglia under ischemia. The addition of CX3CL1 was found to inhibit significantly the increased proliferation of oligodendrocytes induced by ischemia which is reflecting by the reduced number of BrdU-positive cells. The findings also showed that the inhibiting effect of CX3CL1 on oligodendrocyte proliferation could be suppressed by pre-treatment of BV2 microglia with CX3CR1 shRNA or SB. The data indicated that inhibition of CX3CL1 on oligodendrocyte proliferation is a CX3CR1 and p38MAPK signaling-mediated process. The findings also implied that reduction in the numbers of oligodendrocyte induced by CX3CL1 is associated with the increased cell loss as well as the reduced secondary proliferation.

It has been suggested that oligodendrocyte injury induced by ischemia is associated with the molecules released from the activated microglia [16, 39, 40]. Once activated, microglia are thought to release a variety of inflammatory and cytotoxic mediators such as TNF- $\alpha$ , IL-1 $\beta$ , and reactive oxygen species contributing to cell damage and death [41–45]. In the present study, we demonstrated that treatment with OGD or OGD plus CX3CL1 could induce a significant increase in expression of TNF- $\alpha$  and IL-1 $\beta$  in BV2 microglia in vitro. We also found that the reduced expression of CX3CR1 by CX3CR1 shRNA or treatment with p38MAPK inhibitor could lead to a remarkable reduction in expression of these microglia cytokines in BV2 microglia after OGD. These results suggested that the detrimental effect on oligodendrocyte induced by ischemia might be due to the significantly increased release of these

harmful cytokines by activated microglia via CX3CL1/CX3CR1 axis and p38MAPK pathway.

In summary, we demonstrated for the first time that CX3CL1/CX3CR1 axis has a destructive effect on oligodendrocytes by promoting apoptosis and inhibiting myelin PLP expression and proliferation under ischemia via p38MAPK signaling pathway. It is highly likely that under in vivo conditions, ischemia could promote neurons to produce and release CX3CL1 and microglia to express CX3CR1. The increased CX3CL1 and CX3CR1 can lead to an increase in the numbers of activated microglia by the increased interactions of CX3CL1 with CX3CR1 and then activating microglia p38MAPK/PKC signaling pathway. This, in turn, increases production of harmful cytokines in microglial cells. The increased harmful cytokines, in the end, led to oligodendrocyte injury. However, many aspects on the above mechanisms we proposed need to be confirmed by further studies. The relevant studies are important for fully understanding the mechanisms involved in the development of oligodendrocyte injury, which may be able to suggest new therapeutic strategies to preserve or restore oligodendrocyte and white matter function and structure after ischemia.

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**Conflict of Interest** The authors declare that they have no competing interests.

**Author Contributions** Y.L., Y. K., and Z.M.Q. conceived and supervised the study; X.M.W. and Q.Q.L. performed the experiments; Y.L. and X.M.W. contributed to the analysis of data. Y. K. and Z.M.Q. wrote the manuscript.

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