Activated microglia-derived macrophage-like cells exacerbate brain edema after ischemic stroke correlate with astrocytic expression of aquaporin-4 and interleukin-1 alpha release

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1. Introduction

Brain edema following brain infarction affects mobility and mortality. The mechanisms underlying this process remain to be elucidated. Animal studies have shown that aquaporin-4 (AQP4) expression in astrocytes increases after stroke, and its deletion significantly reduces brain swelling. Recently, two kinds of cells, resident microglia-derived macrophage-like cells (MG-MΦ) and bone marrow-derived macrophages (BM-MΦ), have been reported to accumulate in the ischemic core and stimulate adjacent astrocytes. Therefore, we hypothesized that these cells play crucial roles in the expression of AQP4 and ultimately lead to exacerbated brain edema. To verify this hypothesis, we investigated the role of MG- or BM-MΦ in brain edema using a rat model of transient middle cerebral artery occlusion and rat astrocyte primary cultures. AQP4 expression significantly increased in the peri-infarct tissue at 3–7 days post-reperfusion (dpr) and in the core tissue at 5 and 7 dpr, which synchronized with the expression of Iba1, Il1α, Tnf, and C1qa mRNA. Interleukin (IL)-1α treatment or coculture with MG- and BM-MΦ increased AQP4 expression in astrocytes, while an IL-1 receptor type I antagonist reduced these effects. Furthermore, aggravated animals exhibited high expression of Aqp4 and Il1α mRNA in the ischemic core at 7 dpr, which led to the exacerbation of brain edema. MG-MΦ signature genes were highly expressed in the ischemic core in aggravated rats, while BM-MΦ signature genes were weakly expressed. These findings suggest that IL-1α produced by MG-MΦ induces astrocytic AQP4 expression in the peri-infarct and ischemic core tissues, thereby exacerbating brain edema. Therefore, the regulation of MG-MΦ may prevent the exacerbation of brain edema.

Keywords:
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Brain edema is a leading cause of early death following ischemic stroke (White et al., 1979; Wu et al., 2018) and occurs in 10%–20% of patients with ischemic stroke (Hofmeijer et al., 2008); therefore, the prevention of brain edema is important for the satisfactory progression of patients. Brain edema is defined as swelling that occurs due to the accumulation of excess water in the brain parenchyma (Simard et al., 2007) and is associated with stroke (Manley et al., 2000; Nishioka et al., 2016), head trauma (Başarslan et al., 2015), tumors (Papadopoulos et al., 2004b), and inflammation (Papadopoulos and Verkman, 2007). The two primary types of brain edema that are associated with ischemic stroke are cytotoxic and vasogenic edema (Klatzo, 1994). Cytotoxic edema refers to cellular swelling that primarily affects astrocytes, while vasogenic edema involves the accumulation of excess fluid in the extracellular space of the brain parenchyma because of a leaky blood-brain barrier (BBB). However, the mechanisms underlying brain edema remain to be elucidated.

Aquaporin-4 (AQP4) is a bidirectional water transporting protein

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that is highly expressed at astrocytes in the BBB and cerebrospinal fluid (CSF)-brain interfaces (Frigeri et al., 1995; Rash et al., 1998). The location of AQP4 suggests that it is involved in water movement between the fluid compartments (e.g., the blood and CSF) and brain parenchyma. Highly polarized AQP4 expression is also found in astrocyte foot processes near, or in direct contact with, blood vessels (Nielsen et al., 1997). AQP4 has been reported to have a dual role, contributing to the fluid compartments (e.g., the blood and CSF) and brain parenchyma. Therefore, we hypothesized that MG-MΦ can contribute to neuronal injury and disease. During this process, they undergo changes in the expression of many genes (Anderson et al., 2016; Zamanian et al., 2012) and form a glial scar following ischemic stroke (Choudhury and Ding, 2016; Sotoniiew, 2015). Previous studies have shown that reactive astrocytes can both hinder and enhance central nervous system (CNS) recovery following stroke (Choudhury and Ding, 2016; Sims and Yew, 2017). Following stroke, resident microglia-derived macrophage-like cells (MG-MΦ) and bone marrow-derived macrophages (BM-MΦ) accumulate in the ischemic core (Kronenberg et al., 2018; Rajan et al., 2019; Ritzel et al., 2015; Sugimoto et al., 2014). Further, results from discriminatory analyses of gene expression revealed that BM-MΦ were more skewd toward an M2 neuroprotective phenotype than MG-MΦ (Kronenberg et al., 2018; Rajan et al., 2019). These MΦ can contribute to neuronal injury (Hu et al., 2012; Lakhan et al., 2009; Mabuchi et al., 2000) or support neuronal recovery (Imai et al., 2007; Kanazawa et al., 2017; Smirkin et al., 2010). Liddelow et al. (2017) reported that proinflammatory microglia secreted interleukin (IL)-1β, tumor necrosis factor (TNF), and complement component 1, subcomponent q (C1q) and that the simultaneous activity of these factors was necessary and sufficient for astrocyte activation. Additionally, these factors induce the formation of a subtype of astrocytes that are strongly neurotoxic and rapidly kill neurons (Liddelow et al., 2017).

Therefore, we hypothesized that MG-MΦ and/or BM-MΦ play crucial roles in the expression of AQP4 in astrocytes and ultimately lead to exacerbated brain edema. To verify this hypothesis, we investigated the effects of MG- or BM-MΦ in the ischemic lesion on the expression of AQP4 and development of brain edema using a rat model of transient middle cerebral artery occlusion (tMCAO) and primary cultures of rat astrocytes.

2. Material and methods

2.1. Animals and surgical procedures

All animal experiments were approved by the Animal Ethics Committee of Osaka University Graduate School of Medicine and were performed according to the guidelines outlined in the Guide for the Institute of Experimental Animal Sciences Faculty of Medicine, Osaka University. Seven-week-old male Wistar rats (n = 190; body weight 180–216 g; Kiwa Laboratory Animals, Wakayama, Japan) were subjected to tMCAO for 90 min as described previously (Matsumoto et al., 2007a). Rats that were subjected to tMCAO were weighed before the experiments, and rats with more than 0.9-fold weight loss at 2 days post-reperfusion (dpr) were excluded from the analysis. Neonatal rats (n = 50; 0 to 2-day-old Wistar rats; Kiwa Laboratory Animals) were used for primary astrocyte cultures.

2.2. Primary cell cultures

Rat primary astrocyte cultures were prepared as described previously (Tanaka et al., 1998). Briefly, whole forebrains of neonatal rats were dissected and dissociated into individual cells using a nylon bag with 160-μm pores. The dissociated cells were a mixture of glial cells, including microglial cells, astrocytes, and oligodendrocyte progenitors, that were plated on poly-L-lysine (PLL)-coated 75 cm² flasks and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin-amphotericin B suspension (PSA; Wako Pure Chemical Industries). After 11 days, the astrocytes were purified using the anti-GLAST (ACSA-1) MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Next, the purified cells were seeded on PLL-coated 6- or 24-well Falcon Companion tissue culture plates (Corning, NY, USA) or PLL-coated coverslips (Matsunami Glass, Osaka, Japan) and maintained in E2 medium (serum-free and PSA-supplemented DMEM-based medium containing 10 mM HEPES [pH7.4]; Thermo Fisher Scientific, Waltham, MA, USA), 4.5 mg/mL glucose, 10 μg/mL insulin, 38.7 nM sodium selenite, 5.5 μg/mL transferrin (Thermo Fisher Scientific), and 1 mg/mL BSA (Sigma-Aldrich, St.

### Abbreviations

- AQP4: aquaporin-4
- BBB: blood-brain barrier
- BM-MΦ: bone marrow-derived macrophages
- C1q: complement component 1, subcomponent q
- CNS: central nervous system
- CSF: cerebrospinal fluid
- DMEM: Dulbecco’s modified Eagle’s medium
- dpr: days post-reperfusion
- EGFP: enhanced green fluorescent protein
- FBS: fetal bovine serum
- GR: number and rate of granulocytes
- HCT: hematocrit rate
- Hgb: hemoglobin concentration
- IL: interleukin
- IL-1RA: IL-1 receptor antagonist
- IL-1RI: IL-1 receptor type I
- LYM: number and rate of lymphocytes
- MCH: mean corpuscular hemoglobin
- MCHC: mean corpuscular hemoglobin concentration
- MCV: mean corpuscular volume
- MG-MΦ: microglia-derived macrophage-like cells
- MO: number and rate of monocytes
- MPV: mean platelet volume
- PBS: phosphate-buffered saline
- PCI: principal component 1
- PC2: principal component 2
- PCA: principal component analysis
- PCT: plateletcrit value
- PDW: platelet distribution width
- PLT: number of platelets
- PSA: penicillin-streptomycin-amphotericin B suspension
- qPCR: quantitative real-time RT-PCR
- RBC: number of red blood cells
- RDW: red blood cell distribution width
- TBST: tris-buffered saline containing 0.1% Tween-20
- tMCAO: transient middle cerebral artery occlusion
- TNF: tumor necrosis factor
- UV: unit variance
- WBC: number of white blood cells

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Louis, MO, USA)). For some experiments, E2 was supplemented with either recombinant rat IL-1α (3 ng/mL; Sigma-Aldrich), recombinant rat TNF-α (30 ng/mL; Sigma-Aldrich), complete component human C1q (400 ng/mL; Sigma-Aldrich), IL-1 receptor type I (IL-1RI) antagonist (100 μM; Calbiochem, La Jolla, CA, USA), and/or TNF-α antagonist (100 μM; Calbiochem).

2.3. MG- and BM-MΦ isolation

MG- and BM-MΦ cultures were prepared as described previously (Matsumoto et al., 2007b). MG- and BM-MΦ were isolated from the ischemic hemispheres at 7 dpr following tMCAO. The ischemic hemispheres were minced using scissors and triturated using a Pasteur pipette in 10 mL of phosphate-buffered saline (PBS) that contained 0.02% ethylenediaminetetraacetic acid (Sigma-Aldrich) on ice. The minced tissues were filtered through a nylon mesh bag with 160-μm pores and centrifuged at 1000 rpm for 5 min at 4 °C. Following centrifugation, the pellet was suspended in 10% FBS-DMEM, seeded on polystyrene dishes for suspension culture (Sumitomo, Tokyo, Japan), and incubated for 30 min in a CO2 incubator. Next, the dishes were rinsed with 3% FBS-DMEM to remove the unattached cells. Purified MG- and BM-MΦ were scraped off using a rubber scraper (Sumitomo) and seeded onto 0.4-μm pore size (high pore density) inserts (Corning) in 6- or 24-well plates or PLL-coated 24-well plates in E2 medium.

2.4. Expression of recombinant rat AQP4

HEK-293T cells were kindly provided by Dr. Hidekazu Tanaka, College of Life Sciences, Ritsumeikan University, Shiga, Japan, and maintained in 10% FBS-DMEM. HEK-293T cells (2.5 × 10^4 cells/cm^2) were seeded in 4- or 6-well plates, or PLL-coated coverslips, one day before transfection. The next day, the expression plasmids containing gene encoding rat Aqp4 (VB190626-1034msf; VectorBuilder Inc., Chicago, IL, USA) were introduced into HEK-293T cells using a Lipofectamine® 3000 (Invitrogen, Carlsbad, CA, USA) cell transfection kit. These cells were then used to select AQP4 antibodies for western blotting and immunohistochemistry (Fig. S1).

2.5. Brain water content determination

The ratio of wet-weight to dry-weight (wt/dry ratio) was obtained to evaluate brain water content and brain edema on 7 dpr. Dry-weight was measured after drying the brain tissue at 100 °C for 24 h. The wet-weight and dry-weight of the ischemic and contralateral hemispheres were recorded separately, and brain edema was calculated using the wet-weight/dry-weight formula (Nishioka et al., 2016).

2.6. Triphenyltetrazolium chloride stain

Rats that were subjected to tMCAO were euthanized using CO2 gas at 1, 2, 3, 5, and 7 dpr. Brains were dissected, and 2-mm-thick coronal sections were prepared at the caudoputamen level. The sections were incubated with 0.1% 2,3,5-triphenyltetrazolium hydrochloride (Wako Pure Chemical Industries) for 5 min at room temperature to identify ischemic lesions. Tissues from the cerebral cortex that represented the ischemic core, peri-infarct tissue, and contralateral cortex were dissected (Fig. S2). These tissues were then processed for mRNA isolation.

2.7. Quantitative real-time RT-PCR (qPCR)

Total brain tissue RNA was isolated using the mirVana™ miRNA isolation kit (Thermo Fisher Scientific). Brain tissues were homogenized using a bead homogenizer (TAITEC Co., Saitama, Japan), while cultured cells were isolated using ISOGEN® reagent (Nippon Gene, Tokyo, Japan). RNA was reverse-transcribed using ReverTra Ace® qPCR RT master mix with gDNA remover (Toyobo, Osaka, Japan). qPCR analysis was performed in duplicate using a CFX Connect™ real-time system (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR® premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio Inc., Shiga, Japan). All the gene-specific mRNA expression values were normalized to the expression value of Gapdh mRNA. The primer sequences for each gene are listed in Table S1.

2.8. Immunohistochemistry

Immunofluorescence staining of ischemic brain sections was performed as described previously (Matsumoto et al., 2007a,b). Briefly, cultured cells were plated onto PLL-coated coverslips and fixed using 4% paraformaldehyde. Next, the sections or cells were incubated with primary antibodies against AQP4 (1:100; rabbit polyclonal, AB3594; Chemicon, Temecula, California, USA), CD1 (1:250; mouse monoclonal, ab64543; Abcam, Cambridge, UK), GFAP (1:250; mouse monoclonal, MAB360; Chemicon), Iba1 (1:250; rabbit polyclonal, 019–19741; Wako Pure Chemical Industries, 1:100; goat polyclonal, ab10719; Abcam), IL-1α (1:50; goat polyclonal, ab9875; Abcam), Neun (1:250; rabbit monoclonal, ab177487; Abcam), PDGFβRI (1:250; mouse monoclonal, ab69506; Abcam), or TNF (1:50; mouse monoclonal, 60,291-1-lg; Proteintech, Rosemont, IL, USA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 0.1% BSA. After washing with TBST, the sections or cells were further incubated with DyLight® 488-, 594-, and 647-labelled secondary antibodies (1:1000; Invitrogen), and Hoechst 33342 (1:1000; Invitrogen) was used for nuclear staining. Finally, the immune-stained specimens were observed under a fluorescence microscope (BX-ZX10; Keyence, Osaka, Japan).

2.9. Western blotting

The brain tissues and cultured cells were lysed using Laemmli’s sample solution. Brain tissues were homogenized using a bead homogenizer (TAITEC Co.), and the lysates were electrophoresed using a 15% acrylamide gel, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies against β-actin (1:500; mouse monoclonal, A5441; Sigma-Aldrich) and AQP4 (1:100; rabbit polyclonal, AQP-004; Alomone, Jerusalem, Israel). Following incubation with a horseradish peroxidase-linked secondary antibody (1:5000; GE Healthcare, Marlborough, MA, USA), the immunoreactions were visualized using Western BLOT Chemiluminescence HRP Substrate (Takara Bio Inc.). Immunoreactive bands were analyzed by densitometry using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). The densitometry data were standardized against the immunoreactivity of β-actin.

2.10. Determination of IL-1α and TNF levels

Purified MG- and BM-MΦ were cultured in E2 medium for 24 h. The levels of IL-1α and TNF in the supernatants were determined using a rat IL-1α ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) and a rat TNF-α ELISA kit (R&D Systems, Inc.), respectively, according to each manufacturer’s instructions.

2.11. Neurologic scores

Neurologic impairment was scored using the modified neurologic severity scores (mNSS) (Chen et al., 2001). The mNSS is composite of motor, sensory, reflex, and balance tests. Possible scores range from 0 to 18. One point is awarded for the inability to perform a test, or for lack of a tested reflex; thus, the higher the score, the more severe the injury.

2.12. Blood count

Bloods were collected from the inferior vena cava using a heparin-containing syringe. Whole bloods were measured for 18 items: number...
of white blood cells, red blood cells, and platelets; number and rate of lymphocytes, monocytes, and granulocytes; hemoglobin concentration; hematocrit rate; mean corpuscular volume; mean corpuscular hemoglobin; mean corpuscular hemoglobin concentration; red blood cell distribution width; mean platelet volume; platelet distribution width; and plateletcrit value. This was performed using a fully automatic blood cell counter (PCE-210N; Erma Inc., Tokyo, Japan).

2.13. Statistical analysis

For data analyses, we first verified the normality of the distributions to identify the appropriate statistical tools to use for this study. Values are expressed as the mean ± standard error of the mean. Data were analyzed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). A one-way ANOVA with Dunnett’s or Tukey’s post-hoc test was used to identify significant differences between the groups. Additionally, correlations between two variables were analyzed using the Pearson’s correlation test. The threshold for significance was set at p < 0.05. Further, the principal component analysis (PCA) of relative expression levels of signature genes for MG- and BM-MΦ was conducted using SIMCA 15 (Sartorius Stedium data analytics, Östra Strandgatan, Umeå, Sweden), and the data was normalized by unit variance (UV).

3. Results

3.1. Correlation of brain edema with AQP4 expression after tMCAO

The progression of brain edema was first detected dynamically (at 6 h and 1, 3, 5, 7, and 14 dpr following tMCAO) based on the wet/dry ratio (Fig. 1A). We found that brain edema began to increase at 1 dpr following tMCAO and remained elevated until 7 dpr, and then tended to decrease at 14 dpr (Fig. 1A). Next, to investigate the relation between brain edema and AQP4 expression, we evaluated the spatial-temporal profile of Aqp4 mRNA expression following tMCAO using qPCR.
Figure 1B. Result from qPCR revealed that Aqp4 mRNA began to increase at 3 dpr in the peri-infarct tissue and 5 dpr in the core, and it remained elevated until 7 dpr in the peri-infarct and core tissues (Fig. 1B). These findings demonstrate that there may be a strong relationship between brain edema and AQP4 expression. To further identify AQP4 expressing cells in the ischemic brain at 7 dpr, we immunohistochemically stained brain sections with AQP4 (Fig. 1C), or co-stained with AQP4, GFAP, and Iba1 (Fig. 1D). Reactive astrocytes that strongly expressed GFAP, a marker for astrocytes, formed gliosis in the peri-infarct tissue. We then drew the border between the peri-infarct tissue and ischemic core with reference to the gliosis (Fig. 1D). Results from our immunohistochemistry analysis revealed that the expression of AQP4 was stronger in the peri-infarct tissue and the striatum and callosum of the ischemic core than that in the contralateral cortex (Fig. 1C and D). These AQP4+ cells also co-expressed GFAP (yellow arrowheads in Fig. 1D). However, Iba1-positive (Iba1+) microglia and macrophages

Fig. 2. Expression of Iba1, IL-1α, TNF, and C1q in the ischemic brain following transient middle cerebral artery occlusion (tMCAO). (A–E) Real-time qRT-PCR analysis of Aif1 (Iba1) (A), Il1a (B), Tnf (C), C1qa (D), and Iib1 (E) mRNA expression in the ischemic core, peri-infarct tissue (peri), and contralateral cortex (cont) of tMCAO rats (n = 3). (F) Representative images of Iba1 (green), TNF (red), and IL-1α (pink) immunofluorescence staining in the peri-infarct tissue and ischemic core of the ischemic brain at 7 days post-reperfusion. Hoechst 33258 fluorescence (blue) indicates the nuclei. Blue and yellow arrowheads indicate TNF+/IL-1α+/Iba1+ and TNF+/IL-1α+/Iba1+ cells, respectively (n = 6).

(G) The cells number of TNF+/IL-1α+/Iba1+, TNF+/IL-1α+/Iba1+, TNF+/IL-1α+/Iba1+, or TNF+/IL-1α+/Iba1+ in the ischemic core, peri, and cont of tMCAO rats (n = 6). Data are expressed as the mean ± SEM. * , ** , and +++ indicate p < 0.05, 0.01, and 0.001, respectively, as compared to the sham (S) group or cont. +++ indicates p < 0.001, as compared to peri.
scarcely co-expressed GFAP and accumulated in the peri-infarct tissue and ischemic core (blue arrowheads in Fig. 1D). These results demonstrate that AQP4 is expressed in astrocytes in the peri-infarct tissue and ischemic core.

3.2. Expression of IL-1α, TNF, and C1q in the ischemic brain of tMCAO rats

As shown in Fig. 1D, numerous AQP4+ astrocytes and Iba1+ cells were observed in the peri-infarct tissue and ischemic core. Activated microglia have previously been shown to induce reactive astrocytes by secreting IL-1α, TNF, and C1q (Liddelow et al., 2017). Therefore, we hypothesized that AQP4 expression in astrocytes would be modulated by these factors that are secreted from activated microglia and/or macrophages. First, we assessed the expression of AQP4 by qPCR (Fig. 2A). The AQP4 mRNA began to increase at 3 dpr in the peri-infarct tissue and the core and remained elevated until 7 dpr in the core (Fig. 2A). Expression levels of Il1a, Tnf, C1qa, and Iba1 mRNA displayed concomitant changes in the core and were highest at 7 dpr (Fig. 2A-D). The expression of Il1b mRNA did not correspond to AQP4 mRNA level and was the highest at 1 dpr in the core and declined thereafter (Fig. 2E). Fig. 2F shows the localization of TNF+/IL-1α+ cells in the ischemic brain at 7 dpr. Two types of Iba1+ cells—TNF+/IL-1α+/Iba1+ (blue arrowheads) and TNF-/IL-1α-/Iba1+ (yellow arrowheads)—appeared in the peri-infarct tissue and ischemic core (Fig. 2F). These cells had short, thick processes, enlarged somata, and were abundant in the peri-infarct tissue and ischemic core. In the ischemic core, TNF+/IL-1α+/Iba1+ cells accounted for approximately one-third of Iba1-positive cells (Fig. 2G). These findings demonstrate that the changes in the expression of IL-1α, TNF, and C1q are similar to those of AQP4.

3.3. IL-1α increased AQP4 expression in cultured neonatal rat astrocytes

To verify the effects of IL-1α, TNF, and C1q on AQP4 expression in astrocytes, we cultured primary rat astrocytes in vitro. Astrocytes were cultured with or without recombinant IL-1α, TNF, and C1q for 24 h. Results from the qPCR and western blotting analyses revealed that IL-1α and TNF increased Aqp4 mRNA (Fig. 3A) and IL-1α increased AQP4 protein levels (Fig. 3B), respectively. Additionally, when the astrocytes were preincubated with a specific antagonist of IL1-R1 and/or TNF for 3 h, the factor-induced AQP4 expression was inhibited (Fig. 3A and B). Immunofluorescence staining showed that astrocytes that were cultured with IL-1α and TNF were activated with short, thick processes and enlarged somata (Fig. 3C and Cc), and these morphological changes were suppressed by each antagonist (Fig. 3C and Ce). Therefore, these data suggest that IL-1a and TNF induce AQP4 expression in cultured astrocytes; however, C1q does not.

3.4. Isolated MG- and BM-MΦ from the ischemic core increased AQP4 expression in cultured neonatal rat astrocytes

To investigate whether MG- and BM-MΦ in the ischemic core expressed Il1a and Tnf mRNA and proteins, MG- and BM-MΦ were isolated from the ischemic core at 7 dpr. The isolated MG- and BM-MΦ expressed Iba1, but not GFAP, CD31, NeuN or PDGFRβ, a marker for astrocyte, endothelial cell, neuron, or pericyte, respectively (Fig. S3). In addition, Iba1+ cells contained TMEM119-positive and TMEM119-negative cells. TMEM119 is a microglial marker that discriminates resident microglia from blood-derived macrophages (Fig. S3A) (Bennett et al., 2016; Satoh et al., 2016). Il1a and Tnf mRNA were highly expressed in MG- and BM-MΦ, but scarcely expressed in cultured astrocytes (Fig. 4A). Furthermore, IL-1α and TNF proteins were detected in the conditioned medium of MG- and BM-MΦ that were cultured for 24 h (Fig. 4B). Next, isolated MG- and BM-MΦ were seeded on the cell culture inserts that incorporated polyethylene terephthalate track-etched membranes (0.4-μm high pore density), and the cultured astrocytes were seeded on the companion plates. After the inserts containing MG- and BM-MΦ and astrocytes were cocultured for 24 h, we evaluated the AQP4 expression in the astrocytes. MG- and BM-MΦ cocultures increased the

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**Fig. 3.** Effects of IL-1α, TNF, and C1q on AQP4 expression in cultured neonatal rat astrocytes. (A) Real-time qRT-PCR analysis of Aqp4 mRNA expression in astrocytes treated with IL-1α (3 ng/mL), TNF (30 ng/mL), or C1q (400 ng/mL) after treatment with antagonists (IL-1RI; 100 μM or TNF; 100 μM; n = 8). (B) Western blot analysis showing the protein levels of AQP4 in astrocytes treated with IL-1α, TNF, or C1q after antagonist treatment (n = 6). (C) Representative images of AQP4 (green) and GFAP (red) immunofluorescence staining in astrocytes. Hoechst 33258 fluorescence (blue) indicates the nuclei (n = 6). Data are expressed as the mean ± SEM. * and ** indicate p < 0.05 and 0.001, respectively, as compared to the control (Con). +, ++, and +++ indicate p < 0.05, 0.01, and 0.001, respectively, as compared to IL-1α or TNF alone.
expression of Aqp4 mRNA (Fig. 4C) and its protein (Fig. 4D). When the astrocytes were preincubated with IL-1RI and TNF antagonists for 3 h, MG- and BM-MΦ-induced AQP4 expression was inhibited (Fig. 4C and D). Furthermore, astrocytes that were cocultured with MG- and BM-MΦ, as well as IL-1α and TNF-treated cells, were observed as activated cells (Fig. 4Eb). Therefore, these data suggest that MG- and BM-MΦ-co-cultures induce AQP4 expression in cultured astrocytes.

Fig. 4. Effects of microglia-derived macrophage-like cells (MG-MΦ) and bone marrow-derived macrophages (BM-MΦ) isolated from the ischemic core on AQP4 expression in cultured neonatal rat astrocytes. (A) Real-time qRT-PCR analysis of Il1a, Tnf, and C1q mRNA expression in MG- and BM-MΦ isolated from the ischemic core at 7 days post-reperfusion (n = 3). (B) Enzyme-linked immunosorbent assay of IL-1α and TNF protein in MG- and BM-MΦ-conditioned medium after culture for 24 h (n = 8). (C) Real-time qRT-PCR analysis of Aqp4 mRNA expression in astrocytes cocultured with MG- and BM-MΦ after treatment with antagonists (IL-1RI; 100 μM or TNF; 100 μM; n = 8). (D) Western blot analysis showing the protein levels of AQP4 in astrocytes cocultured with MG- and BM-MΦ after antagonist treatment (n = 9). (E) Representative images of AQP4 (green) and GFAP (red) immunofluorescence staining in astrocytes. Hoechst 33258 fluorescence (blue) indicates the nuclei (n = 6).

Data are expressed as the mean ± SEM. * and *** indicate p < 0.05 and 0.001, respectively, as compared to astrocytes cultured without MG-MΦ, BM-MΦ, and antagonists. +, ++, and +++ indicate p < 0.05, 0.01, and 0.001, respectively, as compared to astrocytes cocultured with MG- and BM-MΦ.

Fig. 5. The relationship between change of body weight, brain edema, and neurological score. (A) Correlation between body weight and brain water content (brain edema) at 7 days post-reperfusion (dpr) following transient middle cerebral artery occlusion (tMCAO; n = 15). (B) Correlation between body weight and neurological score at 7 dpr following tMCAO (n = 15).
3.5. IL-1α and IL-1R antagonists may regulate AQP4 expression in the ischemic brain

To assess the role of weight loss in stroke severity, we examined the correlation between weight loss and brain edema at 7 dpr and found that weight loss was strongly related to brain edema (Fig. 5A, p < 0.05). More importantly, the neurological score assessment also showed a correlation with weight loss at 7 dpr (Fig. 5B, p < 0.01). Next, we...
provided a severe level from weight loss after tMCAO as following. As shown in Fig. 6A, rats at 7 dpr were provided with a number (e.g., 1, 2 ... 12) that corresponded to the order of weight gain (i.e., a low-number indicated a more severe condition). Next, we compared the expression of Aqp4, Il1a, Tnf, and C1qa mRNA in each rat and discovered that the expression levels of Aqp4 and Il1a mRNA concomitantly changed with weight loss (Fig. 6B and C); however, we did not observe any changes in Tnf and C1qa (Fig. 6D and E). Interestingly, the expression changes of Il1b and Il1m mRNA followed the opposite pattern (Fig. 6F and G). Further, we examined the correlation between mRNA expression and weight gain and observed that there was a negative linear correlation between weight gain and Aqp4 (Fig. 6H, \( p < 0.05 \)) and Il1a (Fig. 6I, \( p < 0.05 \)) mRNA levels in the ischemic core. Furthermore, the correlation analysis showed that Il1b (Fig. 6L, \( p < 0.01 \)) and Il1m mRNA (Fig. 6M, \( p < 0.05 \)) in the ischemic core were positively correlated with weight gain. Tnf and C1qa mRNA levels were not related to weight gain (Fig. 6J and K). These data suggest that IL-1\( \alpha \) increase and endogenous IL-1R antagonist (IL-1RA) decrease may participate in AQP4 expression in vivo, and exacerbate brain edema and prognosis.

### 3.6. Aggravated rats expressed the signature genes for microglia at high levels

The expression levels of the signature genes in the ischemic core relative to the expression levels of signature genes in the contralateral cortex were calculated using qPCR (Table S2) and applied to PCA. PCA demonstrates the similarity and differences in the relative expression patterns of samples in a score plot and explains the contribution of each gene to the constructing score plot in a loading plot. The scores of rats with lower weights were plotted in the principal component 1 (PC1)-positive/principal component 2 (PC2)-negative area in the score plot (Fig. S4A). While, the scores of rats with higher weights were plotted in the PC1-negative/PC2-positive area in the score plot (Fig. S4A). The signature genes for MG-M\( \Phi \), such as P2ry12, Olfm3, Gpr34, and Pros1, were plotted in the PC1-negative/PC2-positive area in the loading plot, while the signature genes for BM-M\( \Phi \), such as Ccr2 and Mybl2, were plotted in the PC1-positive/PC2-negative area (Fig. S4B). However, the score of Rat No. 8 was plotted far away from the others. Additionally, based on the results from the Smirnov-Grubbs test (\( p < 0.01 \)), Rat No. 8 was considered an outlier with respect to Tmem119 and Cx3cr1 expressions (Table S2). Thus, the relative expression levels of the signature genes, except for Rat No. 8, were applied to PCA. The scores of rats with weights that were lower and higher were plotted in PC1-positive and PC1-negative areas, respectively, in the score plot (Fig. 7A). All the signature genes for MG-M\( \Phi \), including P2ry12, Tmem119, Olfm3, Gpr34, Pros1, and Cx3cr1, were plotted in the PC1-positive area in the loading plot, while signature genes for BM-M\( \Phi \), such as Ccr2 and Mybl2, were plotted in the PC1-negative area (Fig. 7B). When considering the PCA...
score and loading plots together, the signature genes for MG-MΦ were expressed at high levels in aggravated (i.e., low-number) rats, while BM-MΦ were expressed at high levels in recovered (i.e., high-number) rats. However, Iba1 mRNA levels were not related to weight gain (Fig. 7C and D). These data suggest that the presence of MG-MΦ in the ischemic core may lead to brain edema and poor outcomes.

4. Discussion

AQP4 mediates water homeostasis in both normal and pathophysiological conditions and is the most important type of aquaporin in the central nervous system. In the present study, we found that the progression of brain edema paralleled Aqp4 mRNA expression following ischemic stroke (Fig. 1A and B). Several studies have shown that changes in AQP4 expression correlate with brain edema after ischemic stroke (De Castro Ribeiro et al., 2006; Taniguchi et al., 2000). Brain edema after stroke, that generates mainly cytotoxic and ionic edema in the early stages (<24 h), is also known to be associated with a second peak of vasogenic edema after 2–4 days; this is termed the biphasic edema response (Throne et al., 2014). In the present study, brain edema also showed a biphasic edema response at 1 and 7 dpr. Furthermore, brain edema and Aqp4 mRNA expression were elevated in aggravated rats, which were determined by weight loss (Figs. 5A, 6B and 6H). This shows that brain edema and AQP4 expression are positively correlated. Results from several studies also indicate that the reduction of AQP4 reduces brain edema and infarct volume in the ischemic brain (Hirt et al., 2009; Nishioka et al., 2016; Taniguchi et al., 2000; Yano et al., 2017). Furthermore, Manley et al. (2000) described that the absence of AQP4 prevented the formation of edema and improved neurological outcomes in a permanent ischemia model in AQP4-deficient mice. On the other hand, AQP4-dependent water efflux at the astrocyte end-foot appears to be responsible for clearance of vasogenic edema, as AQP4 knockout impairs the ability to efficiently clear the edema (Papadopoulos et al., 2004a). However, with regards to long-term outcome after stroke, the lack of AQP4 has been reported to have an overall beneficial role, associated with improved neuronal survival and reduced neuroinflammation (Hirt et al., 2017). Wang et al. (2015) reported that inhibiting AQP4 expression protected BBB integrity through alleviating astrocyte swelling after ischemic brain injury. These reports suggest that AQP4 plays a crucial and complex role in the pathophysiology of edema. Therefore, it is important to carefully characterize the type of brain edema and consider the time-point at which it occurs.

Food intake and energy-balance are regulated centrally by the hypothalamus, including the lateral hypothalamic area, arcuate nucleus, paraventricular nucleus, ventromedial and dorsomedial hypothalami (Schwartz et al., 2000). Among them, the lateral hypothalamic area plays a central role in the increase of food intake, the destruction of the areas leads to diminution or failure of eating and drinking behavior (Anand and Brobeck, 1951). The lateral hypothalamic area located in the peri-infarct tissue and/or ischemic core in tMCAO rats in this study. Therefore, the injured size of lateral hypothalamic area is caused by ischemic stroke may determine the degree of failure the food intake. Therefore, weight loss may correlate with brain edema and neurological score (Fig. 5). Furthermore, we also analyzed the blood cell components at 7 dpr after tMCAO. As a result, there was a negative linear correlation between weight gain and red blood cells (p < 0.05), hemoglobin concentration (p < 0.01), and hematocrit rate (p < 0.05; Table S3). These data indicate that severely rats were dehydrated and supports our thought.

The change in Aqp4 mRNA expression following tMCAO was well correlated with the changes in the expression of Iba1, Il1a, Tnf, and C1qa mRNA, but not Il1b (Figs. 1B and 2A-E), Liddelow et al. (2017) reported that activated microglia induced neurotoxic reactive astrocytes by secreting IL-1α, TNF, and C1q. Indeed, we revealed that IL-1α significantly enhanced AQP4 expression in vitro, and these effects were inhibited by specific antagonists (Fig. 3). Furthermore, cocultures of MG- and BM-MΦ revealed the same results (Fig. 4). As shown in Fig. 2F, Tnf'/IL-1α+/Iba1+ cells accumulated in the peri-infarct tissue and ischemic core. Therefore, IL-1α and TNF produced by Iba1+ cells may be at least one of the factors inducing AQP4 expression in astrocytes in the peri-infarct tissue and ischemic core. However, the expression levels of Aqp4 and Il1a mRNA, but not Tnf mRNA, and brain edema appeared to concomitantly change in vivo (Figs. 5A, 6B-D, and 6H-J). These results suggest that IL-1α may play a major role in the exacerbation of brain edema following ischemic stroke.

The IL-1 family includes IL-1α, IL-1β, and IL-1RA. Although IL-1α and IL-1β are derived from different genes, they both bind to the same receptors, IL-1RI and IL-1 receptor type II, and exhibit similar biological activities (Colotta et al., 1993). Iio et al. (2006) reported that IL-1β induced AQP4 expression through the NF-κB pathway in cultured rat astrocytes. Therefore, IL-1α-induced AQP4 expression may also be mediated by the NF-κB pathway. IL-1RA is a naturally occurring anti-inflammatory molecule and physiologically active inhibitor of IL-1 (Apte and Voronov, 2002). As shown in Fig. 6C and I, the expression of Ile6 mRNA was elevated in aggravated rats as compared with that in mild rats, and Il1r expression was low (Fig. 6G and M). Numerous studies have demonstrated that the administration of IL-1RA reduced brain edema and infarct volume in experimental models of ischemic stroke (Oprića et al., 2004; Selton et al., 1996; Stroemer and Rothwell, 1997; Yang et al., 1997; Zhang et al., 2017). Furthermore, some studies in humans demonstrated that IL-1RA was an effective therapeutic candidate for patients with stroke (Emsley et al., 2005; Maysami et al., 2016) and severe traumatic brain injury (Helmy et al., 2014). Therefore, IL-1RA may provide therapeutic effects for patients with ischemic stroke by inhibiting IL-1α-induced AQP4 expression in astrocytes and brain edema.

Two types of Iba1+ cells—TNF'/IL-1α+/Iba1+ and Tnf'/IL-1α+/Iba1+—were observed in the peri-infarct tissue and ischemic core (Fig. 2F). Additionally, evidence from several studies suggests that MG- and BM-MΦ accumulate in ischemic lesions (Kronenberg et al., 2018; Rajan et al., 2019; Ritzel et al., 2015). Further, Rajan et al. (2019) found that the ratio of CD11b+CD45low cells (MG-MΦ) to CD11b+CD45high cells (BM-MΦ) was 1:1 at 3 dpr and 5:1 at 7 dpr using flow cytometry. Furthermore, using gene expression profiling, studies have demonstrated that the expression of Il1a and Tnf in MG-MΦ was significantly higher than that in BM-MΦ (Kronenberg et al., 2018; Rajan et al., 2019; Ritzel et al., 2015). Therefore, Tnf'/IL-1α+/Iba1+ and Tnf'/IL-1α+/Iba1+ cells may be considered MG-MΦ and BM-MΦ, respectively. Kronenberg et al. (2018) identified genes that were differentially expressed in the two cell types after stroke using discriminatory analysis; therefore, results from this analysis revealed that the category MG-MΦ > BM-MΦ contained P2ry12, Tmem119, Olfm13, Gpr34, Pros1, and Cx3cr1, while BM-MΦ > MG-MΦ contained Ccr2 and Myb2. We also evaluated the expression of these genes in the brain at 7 dpr following tMCAO and revealed that the signature genes for MG-MΦ were expressed at high levels in aggravated (i.e., low-number) rats, while BM-MΦ were expressed at high levels in recovered (i.e., high-number) rats (Fig. 7B). Therefore, IL-1α produced by MG-MΦ may induce astrocytic AQP4 expression and exacerbate brain edema. Several studies have also described the beneficial effects of BM-MΦ on stroke outcome (Cha et al., 2015; Gleim et al., 2012; Wattanamit et al., 2016). Blocking monocyte recruitment after stroke led to increased infarct size (Wattanamit et al., 2016), worsened post-stroke sensory-motor performance (Kronenberg et al., 2018), and abolished recovery (Cha et al., 2015). Furthermore, Kronenberg et al. (2018) reported that BM-MΦ were more skewed toward an M2 neuroprotective phenotype than MG-MΦ. Therefore, poor outcomes may be dependent on the rate of MG-MΦ in the ischemic core of the brain.
5. Conclusions

The release of IL-1α from the ischemic core may induce astrocytic AQM4 expression in the peri-infarct tissue and core and consequently exacerbate brain edema. Therefore, inhibiting IL-1α-signaling by MG-ΜΦ in the ischemic core may be a novel therapeutic strategy for the treatment of ischemic stroke.

Author contributions

Yukie Murata: Data curation, Investigation, Writing - review & editing. Kana Sugimoto: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Writing - original draft, Writing - review & editing. Chihpin Yang: Investigation. Kazuo Harada: Formal analysis, Software, Writing - review & editing. Rina Gono: Investigation. Teiji Harada: Investigation. Yohei Miyashita: Formal analysis. Kazuma Higashisaka: Formal analysis. Ryuchi Katada: Funding acquisition, Formal analysis. Junya Tanaka: Supervision, Writing - review & editing. Hiroshi Matsumoto: Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2020.104848.

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