exclusively produced by neurons in CNS and induces proliferation of microglia [6]. We also showed that IL-34 attenuated the neurotoxic effect of oligomeric amyloid beta (Aβ) *in vitro* and intracerebroventricular administration of IL-34 ameliorates the impairment of associative learning in an AD mouse model [6]. Another study also demonstrated that IL-34 rescued neuronal damage in mouse models of AD and kinate-induced neurotoxicity [7]. These findings suggest distinct functions of IL-34 in the development of various CNS disorders. However, the precise functions of IL-34 in the CNS still remain to be elucidated.

The blood-brain barrier (BBB) is a tight seal composed of capillary endothelial cells, pericytes, and astrocytes [8]. The BBB contributes to maintenance of CNS homeostasis by limiting the entry of plasma components, erythrocytes, and immune cells from the circulating blood [9–11]. Tight junction (TJ) plays an important role in the barrier function of the BBB, which is composed by TJ proteins including claudins, occludin, and zonula occludens-1 (ZO-1) [12]. BBB disruption is frequently associated with synaptic and neuronal dysfunction in various neurological disorders such as multiple sclerosis (MS), AD, Parkinson's disease, and amyotrophic lateral sclerosis [13, 14]. Pro-inflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α), interferon-γ, and IL-17, are thought to downregulate the expression of tight junction proteins and contribute to the transmigration of inflammatory immune cells into the CNS, which exacerbates neuroinflammation in these diseases [14–19].

In this study, we found that the CNS capillary endothelial cells as well as microglia express CSF1R. We also showed that IL-34 restored pro-inflammatory cytokine–induced BBB disruption by upregulating the expression levels of tight junction proteins such as claudin-5 and occludin. These findings suggest the presence of neuronal regulation of BBB functions via IL-34, and upregulation of IL-34 in the CNS may be a novel therapeutic strategy against neuroinflammatory and neurodegenerative disorders.

Materials and Methods

Reagents

Recombinant mouse IL-1β, TNF-α, and IL-34 were purchased from R&D Systems (Minneapolis, MN, USA). The c-fms/CSF1R tyrosine kinase inhibitor GW2580 was used as a blocker of CSF1R signaling (Millipore, Bedford, MA, USA). Dylight 594–labeled tomato lectin was used as a capillary endothelial cell marker (Vector Laboratories, Burlingame, CA, USA).

Animals

All protocols were approved by the Animal Experiment Committee of Nagoya University (approved number: 14018). C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan).

Cells

Primary neuronal cultures were prepared from the cortices of C57BL/6 mouse embryos at embryonic Day 17 as described previously [20]. Briefly, cortical fragments were dissociated into single cells in dissociation solution (Sumitomo Bakelite, Akita, Japan), and resuspended in neuron culture medium (Sumitomo Bakelite). Neurons were seeded onto 12-mm polyethylenimine-coated glass coverslips (Asahi Techno Glass Corp., Chiba, Japan) at a density of 5.0×10^4 cells/well in 24-well culture plates and were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. The purity of the cultures was >95% as determined by NeuN-specific immunostaining. Mouse brain capillary endothelial cell line MBEC4 [21] was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Confluent monolayer of MBEC4 cells was used as an established BBB model as described previously [22].

BBB Permeability assay

The permeability of MBEC4 cell monolayers was measuring transendothelial electrical resistance (TER) as described previously [22]. Confluent monolayer of MBEC4 cells on the 24-well transwell inserts (3-μm pore size) were incubated with or without 20 ng/ml TNF-α, 20 ng/ml IL-1β, 0–100 ng/ml IL-34, or 1 μmol/L GW2580 for 24 h. TER was measured using a Millicell-ERS (Millipore). Resistances of blank filters were subtracted from those of filters with cells before final resistances ($\Omega \cdot cm^2$) were calculated. Assays were carried out in five independent trials.

Immunocytochemistry

Primary neurons and MBEC4 cells were fixed with 4% paraformaldehyde for 10 min, permeabilized using 0.1% Triton X-100 for 5 min, and blocked using 5% normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. Neurons were incubated with rabbit anti-mouse IL-34 polyclonal antibodies (ProSci, Poway, CA, USA), mouse anti-mouse microtubule-associated protein–2 (MAP-2) monoclonal antibody (Chemicon, Temecula, CA, USA) overnight at 4℃ followed by a 1-h incubation with Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). MBEC4 cells were stained using rabbit anti-mouse CSF1R polyclonal antibodies (Abcam, Cambridge, UK) overnight at 4℃ followed by a 1-h incubation with Alexa-conjugated secondary antibodies (Invitrogen). Nuclei were counterstained with Hoechst 33342 (Invitrogen). Images were analyzed using a deconvolution fluorescent microscope system (BZ-8000, Keyence, Osaka, Japan).

Immunohistochemistry

Brains and lumbar spinal cords from C57BL/6J mice were fixed with 4% paraformaldehyde overnight, equilibrated in 20% sucrose with PBS for 48 hours,

embedded in Tissue Tek O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and frozen at −80℃ overnight. Coronal brain sections and transverse spinal cord sections (20 μm-thick) were prepared using a cryostat. Sections were permeabilized using 0.3% Triton X-100 after blocking with 5% normal goat serum in PBS for 1 h. Sections were incubated with rabbit anti-mouse IL-34 polyclonal antibodies (ProSci), mouse anti-mouse MAP-2 monoclonal antibody (Chemicon), rabbit anti-mouse CSF1R polyclonal antibodies (Abcam), and Dylight 594–labeled tomato lectin (Vector Laboratories) overnight at 4℃ followed by a 1-h incubation with Alexa-conjugated secondary antibodies (Invitrogen). Images were analyzed using a deconvolution fluorescent microscope system (BZ-8000, Keyence).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

MBEC4 cells were cultured at a concentration of 4×10^5 cells/well in 24-well culture plates and stimulated with 100 ng/ml IL-34 for 24 h. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs encoding mouse IL-34 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were generated by RT-PCR using SuperScript II (Invitrogen), Blend Taq DNA polymerase (Toyobo, Osaka, Japan), and the following specific primer sets:

CSF1R forward primer: 5′-AAGCAGAAGCCGAAGTACCA-3′
CSF1R reverse primer: 5′-GTCCCTGCGCACATATTTCAT-3′
GAPDH forward primer: 5′-TGTGTCCGTCGTGGATCTGA-3′
GAPDH reverse primer: 5′-CCTGCTTCACCACCTTCTTGA-3′

Western Blotting

MBEC4 Cells were lysed in TNES buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, and 0.1% SDS) with protease inhibitor mixture (Complete Mini EDTA-free; Roche Diagnostics, Basel, Switzerland). Cell lysate proteins dissolved in Laemmli sample buffer (20 μg/well) were separated on 4–20% SDS-polyacrylamide gels (Mini-Protean TGX; Bio-Rad, Hercules, CA, USA) and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA) as described previously [23]. The membranes were blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20, and then incubated overnight at 4℃ with rabbit anti-mouse Zonula Occludens–1 (ZO-1) polyclonal antibodies, rabbit anti-mouse occludin polyclonal antibodies, rabbit anti-mouse claudin-5 polyclonal antibodies (Invitrogen), and mouse anti-β-actin monoclonal antibody (Sigma). After an overnight incubation with primary antibodies at 4℃, each blot was probed with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare). Blots were then visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and quantitated using

a CS Analyzer 3.0 system (Atto, Tokyo, Japan). Assays were carried out in five independent trials.

Statistical analysis

Statistical significance was analyzed with one-way analysis of variance followed by post-hoc Tukey's test, using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

IL-34 is exclusively expressed in CNS neurons

In the previous study, we have reported that IL-34 protein is primarily expressed in neurons whereas IL-34 mRNA expression was detected in neurons and astrocytes [6]. First, we confirmed the expression pattern of IL-34 in the CNS using immunostaining in mouse primary cortical neurons, brains, and spinal cords. As shown in Fig. 1, IL-34 protein was exclusively expressed in neurons in the CNS.

CNS capillary endothelial cells expressed IL-34 receptor CSF1R

Next, we examined the expression pattern of IL-34 receptor CSF1R protein in the CNS using immunostaining. In addition to microglia, CNS microvessels were also immunopositive for CSF1R (Fig. 2A, green). Its staining pattern in the microvessels was identical to that of tomato lectin (Fig. 2A, red and arrows in the overlap images) which selectively binds to the surface of capillary endothelial cells [24], suggesting that CNS capillary endothelial cells express CSF1R. Meninges and large vessel adventitia were also stained with CSF1R and tomato lectin. Although CSF1R has been detected on fibroblasts and smooth muscle cells which are the main components of meninges and adventitia [1, 7], the meninges and adventitia showed strong non-specific binding of antibodies and lectin. Therefore, the positive staining in meninges and adventitia may be artifact.

Furthermore, mouse brain capillary endothelial cell line MBEC4 cells strongly express CSF1R protein (Fig. 2B, green). MBEC4 cells constitutively express CSF1R mRNA, and stimulation with IL-34 did not alter CSF1R expression level (Fig. 2C). These data indicate that CNS capillary endothelial cells constitutively express CSF1R and are potential target of IL-34 in the CNS, as well as microglia.

IL-34 restored BBB disruption via CSF1R signaling in endothelial cells

BBB disruption is a common pathological feature of various neurological diseases, and inflammatory cytokines such as IL-1β and TNF-α have been considered as causative factors that damage BBB integrity by downregulating TJ proteins in BBB endothelial cells [14–16, 25, 26]. To investigate whether IL-34 affects the BBB

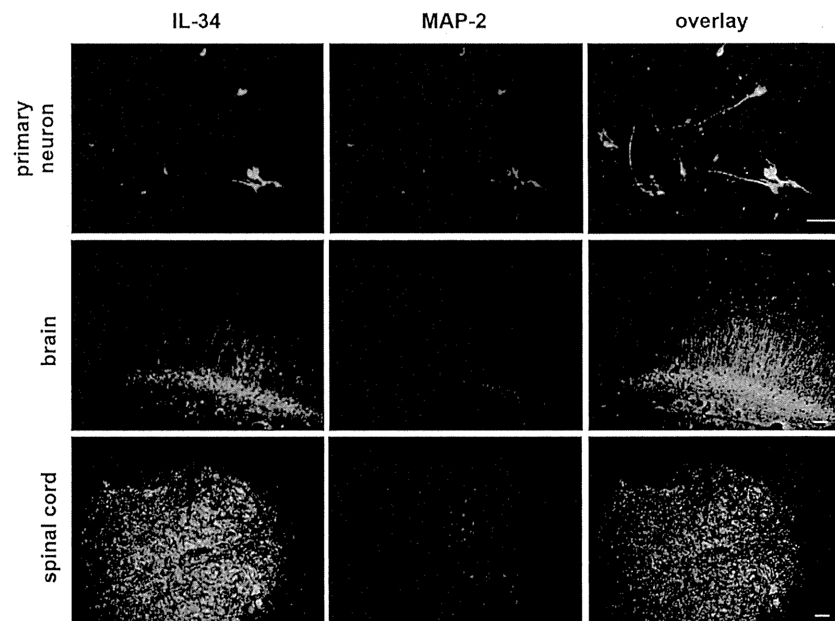

Fig. 1. IL-34 is produced by neurons in the CNS. Immunofluorescence images of primary cortical neurons, brain sections, and lumbar spinal cord sections. Green, IL-34; red, MAP-2; blue, Hoechst nuclear counterstain. Scale bar, 50 μm.

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integrity, we evaluated BBB permeability by measuring TER in MBEC4 cell monolayer as an *in vitro* BBB model [22]. IL-34 significantly ameliorated a decrease in TER induced by IL-1β and TNF-α in a dose dependent manner (Fig. 3), whereas treatment with IL-34 alone did not alter untreated BBB integrity (data not shown). Moreover, addition of CSF1R signal inhibitor GW2580 ablated the effect of IL-34 on BBB (Fig. 3). These results indicate that IL-34 restored pro-inflammatory cytokine–mediated BBB disintegrity via CSF1R signaling in endothelial cells.

IL-34 upregulated TJ proteins in BBB endothelial cells

Next, we assessed whether IL-34 alters the expression levels of TJ proteins that are sensitive to pro-inflammatory cytokines [14–16]. Western blotting analysis detected that major TJ proteins such as claudin-5 and occludin were significantly downregulated by treatment with IL-1β and TNF-α (Fig. 4). Addition of IL-34 reversed the expression levels of these TJ proteins (Fig. 4), whereas treatment with IL-34 alone did not alter the expression levels of TJ proteins in untreated MBEC4 cells (data not shown). Addition of GW2580 canceled the effect of IL-34 on the expression of claudin-5 and occludin (Fig. 4). These data suggest that IL-34 rescues pro-inflammatory cytokine–induced BBB disruption via upregulating TJ proteins such as claudin-5 and occludin in BBB endothelial cells.

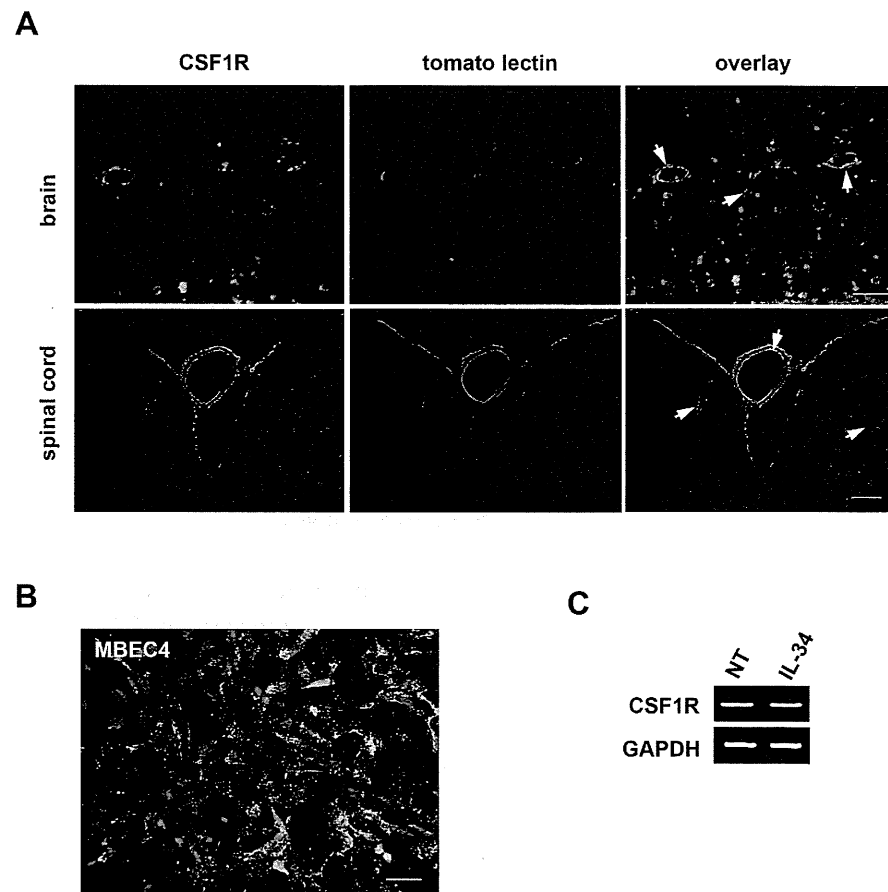

Fig. 2. CNS endothelial cells express CSF1R. (A) Immunofluorescence images of brain sections, and lumbar spinal cord sections. Green, CSF1R; red, tomato lectin; blue, Hoechst nuclear counterstain. Arrows indicate CSF1R-immunopositivity in the capillary endothelial cells. Scale bar, 50 μm. (B) Immunofluorescence image of mouse brain capillary endothelial cell line MBEC4. Green, CSF1R; blue, Hoechst nuclear counterstain. Scale bar, 50 μm. (C) RT-PCR data for CSF1R. Stimulation with IL-34 did not alter the expression of CSF1R in MBEC4 cells.

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Discussion

IL-34 is widely expressed in a variety of tissues including brain. Because IL-34 shares the same receptor with CSF-1/M-CSF, it has similar functions on monocyte lineage cells such as induction of proliferation and differentiation of macrophages, Langerhans cells, osteoclasts, and microglia [3–5, 27, 28]. In the CNS, IL-34 is mainly released by neurons, especially when they are damaged [6]. Like CSF-1/M-CSF, IL-34 induces microglial proliferation. In addition, IL-34 enhances microglial neuroprotective functions by inducing anti-oxidant enzyme heme oxigenase-1 (HO-1), and amyloid degrading enzyme insulin degrading enzyme (IDE). Moreover, we also found that IL-34 induces microglial production of TGF-β which negatively regulates microglial activation [29]. TGF-β dose-dependently suppressed microglial proliferation by IL-34 but attenuated oligomeric amyloid

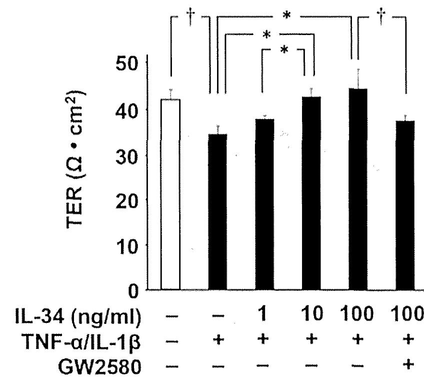

Fig. 3. IL-34 restores damaged BBB integrity. MBEC4 cells were treated with TNF-α (20 ng/ml) and IL-1β (20 ng/ml) in the presence of IL-34 (0–100 ng/ml) and GW2580 (1 μmol/l). TER of MBEC4 cell monolayer was measured after a 24-h incubation. Values are means ± SEM (n=5). *, $p<0.05$; †, $p<0.01$.

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β–mediated neurotoxicity [29]. The neuroprotective functions of IL-34 was partially suppressed by blockade of TGF-β receptor signaling, suggesting that neuroprotective effect of IL-34 was in part mediated by microglial TGF-β production in response to IL-34. Thus, IL-34 released from damaged neurons acts as a "Help-me" signal which induces microglial neuroprotective effects with

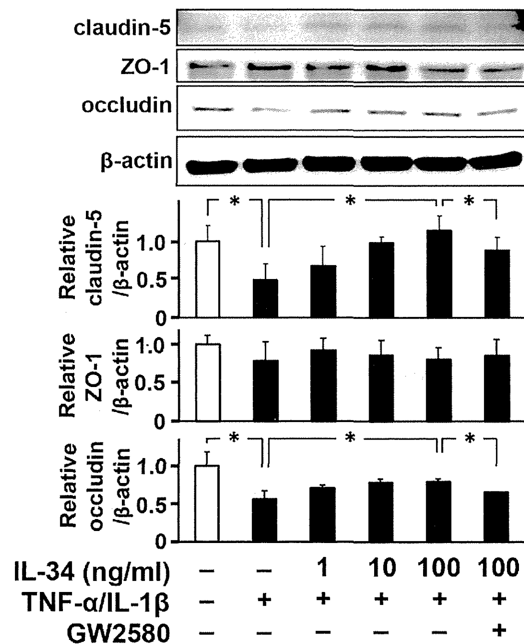

Fig. 4. IL-34 upregulates tight junction proteins in MBEC4 cells. MBEC4 cells were incubated with TNF-α (20 ng/ml) and IL-1β (20 ng/ml) in the presence of IL-34 (0–100 ng/ml) and GW2580 (1 μmol/L) for 24 h. Upper, representative images of Western blots for tight junction proteins. Bottom, quantified expression levels of tight junction proteins relative to those in untreated cells. Values are means ± SEM (n=5). *, $p<0.05$.

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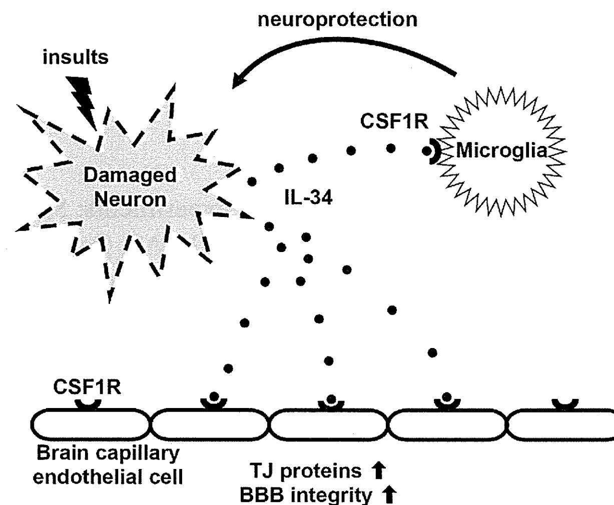

Fig. 5. Model of the roles of IL-34 in the CNS. Damaged neurons secrete IL-34 as a "Help-me" signal. IL-34 binds its receptor CSF1R which is mainly expressed in microglia and BBB endothelial cells. CSF1R signaling enhances neuroprotection in microglia and restores BBB disruption by upregulating TJ proteins in capillary endothelial cells.

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subsiding microglial activation (Fig. 5). The expression of CSF1R is reportedly high during early postnatal development, and is very low in adult brain [30]. IL-34 exhibited a broader regional expression than CSF-1/M-CSF, mostly without overlap, suggesting important role of IL-34–CSF1R signaling in regional neurogenesis. A previous study reported that CSF1R expression is increased in microglia of AD brains and microglia overexpressing CSF1R are neuroprotective [31]. Therefore, IL-34 produced by neurons [6] as well as CSF-1/M-CSF produced by astrocytes [32] may be involved in the development of neurodegenerative diseases such as AD via microglial CSF1R signaling.

In this study, we have shown that BBB endothelial cell expresses CSF1R and is a novel target of IL-34. BBB disruption has been implicated as a pathogenesis of various neurological disorders including MS and AD. A recent study showed that amyloid β suppressed expression of TJ protein ZO-1 in BBB endothelial cells via receptor for advanced glycation end products (RAGE) and claimed that amyloid β-RAGE interaction may be a potential molecular pathway in breakage of BBB integrity [33]. In addition, pro-inflammatory cytokines such as IL-1β, TNF-α, IFN-γ, and IL-17 have been considered as the candidates to increase BBB leakage [14–19]. Our findings revealed a novel function of IL-34–CSF1R signaling on the maintenance of BBB integrity via upregulating major TJ proteins claudin-5 and occludin in capillary endothelial cells (Fig. 5). A major downstream target of CSF1R signaling is cAMP responsive element-binding protein (CREB), which modulates the transcription of TJ proteins [34, 35]. Taken together, IL-34 released from damaged neurons may functions as a "Help-me" signal toward restoration of CNS homeostasis via microglia and BBB endothelial cells (Fig. 5). Our study clarified the presence of neuronal regulation of BBB functions via IL-34–CSF1R

signaling. IL-34–CSF1R pathway may be novel therapeutic target for neuroinflammatory and neurodegenerative disorders such as MS and AD.

Author Contributions

Conceived and designed the experiments: SJ HT AS. Performed the experiments: SJ. Analyzed the data: SJ HT TM AS. Contributed reagents/materials/analysis tools: YS JK TM HH YC YW. Wrote the paper: SJ HT AS.

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Interleukin-1β Induces Blood–Brain Barrier Disruption by Downregulating Sonic Hedgehog in Astrocytes

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Abstract

The blood–brain barrier (BBB) is composed of capillary endothelial cells, pericytes, and perivascular astrocytes, which regulate central nervous system homeostasis. Sonic hedgehog (SHH) released from astrocytes plays an important role in the maintenance of BBB integrity. BBB disruption and microglial activation are common pathological features of various neurologic diseases such as multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease. Interleukin-1β (IL-1β), a major pro-inflammatory cytokine released from activated microglia, increases BBB permeability. Here we show that IL-1β abolishes the protective effect of astrocytes on BBB integrity by suppressing astrocytic SHH production. Astrocyte conditioned media, SHH, or SHH signal agonist strengthened BBB integrity by upregulating tight junction proteins, whereas SHH signal inhibitor abrogated these effects. Moreover, IL-1β increased astrocytic production of pro-inflammatory chemokines such as CCL2, CCL20, and CXCL2, which induce immune cell migration and exacerbate BBB disruption and neuroinflammation. Our findings suggest that astrocytic SHH is a potential therapeutic target that could be used to restore disrupted BBB in patients with neurologic diseases.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

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These authors contributed equally to this work.

Introduction

The blood–brain barrier (BBB) is a tight seal composed of capillary endothelial cells, pericytes, and perivascular astrocytes [1]. The BBB contributes to homeostasis in the central nervous system (CNS) by limiting the entry of plasma components, erythrocytes, and immune cells from the circulating blood [2]. Astrocytes play a pivotal role in maintenance of BBB integrity via contact-dependent mechanisms and release of trophic factors [3–5]. In addition, a recent study revealed that Sonic hedgehog (SHH) released from astrocytes promotes BBB formation and integrity by upregulating tight junction (TJ) proteins in capillary endothelial cells [6]. Without SHH, its receptor Patched-1 (Ptch-1) suppresses a G-coupled–protein receptor Smoothened (Smo) which is critical for the activation of a transcription factor Gli-1 [7]. Gli-1 is an important regulator of TJ protein expression and BBB formation. SHH binds and inactivates Ptch-1, which allows Smo to activate Gli-1, which upregulates TJ proteins and enhances BBB integrity. Disruption of BBB integrity is frequently observed in neurologic diseases such as multiple sclerosis (MS), Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease, suggesting that infiltrating molecules and immune cells from the blood perturb CNS homeostasis and exacerbate these disorders [8–13]. Microglial activation is another characteristic pathologic feature in these diseases [14]. Activated microglia release various cytotoxic factors such as nucleic acids, glutamate, reactive oxygen species (ROS), proteases, and pro-inflammatory cytokines/chemokines [15]. Interleukin-1β (IL-1β) is a major microglial pro-inflammatory cytokine that acts on both endothelial cells and astrocytes to increase BBB permeability [16–18]. However, the mechanisms of BBB disruption by IL-1β have not been fully elucidated. In this study, we demonstrated that IL-1β suppressed SHH expression in astrocytes and increased BBB permeability by downregulating TJ proteins in endothelial cells. Moreover, IL-1β stimulated astrocytes to secrete pro-inflammatory chemokines such as CCL2, CCL20, and CXCL2, which induce the migration of immune cells such as neutrophils, monocytes, macrophage, dendritic cells, and pathogenic T cells. Our findings reveal novel mechanisms of BBB disruption by IL-1β, and suggest that SHH could be used therapeutically against various neurologic diseases.

Methods

Cell cultures

Protocols for animal experiments were approved by the Animal Experiment Committee of Nagoya University (The approval number: 13122).

Mouse primary astrocyte–rich cultures were prepared from primary mixed glial-cell cultures of newborn C57BL/6J mice (SLC, Hamamatsu, Japan), as described previously [19,20]. The purity of astrocytes was >95%, as determined by immunostaining with antibody against glial fibrillary acidic protein. Cells were cultured in maintenance medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 5 μg/ml bovine insulin, and 0.6% glucose). Astrocytes were plated at a density of 1×10^4 cells/well in 96-well multidishes, 1×10^5 cells/well in 24-well multidishes, or 5×10^5 cells/well in 6-cm culture dishes. For IL-1β treatment, the cells were incubated with or without 2 ng/ml mouse recombinant IL-1β (R&D Systems, Minneapolis, MN, USA) for 24 h, and then astrocyte conditioned media (ACM) were collected and used for subsequent experiments.

The mouse brain capillary endothelial cell line, MBEC4 (a kind gift from Dr. T. Tsuruo) [21], was maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and used as an established BBB model.

BBB permeability assay

We used MBEC4 monolayers as an *in vitro* BBB model, as described previously [22]. The permeability of MBEC4 monolayers was evaluated using fluorescein isothiocyanate–labeled bovine serum albumin (FITC-BSA) as a marker. Confluent monolayers of MBEC4 cells on Transwell inserts (3 μm pore size; BD Falcon, Franklin Lakes, NJ, USA) were incubated for 24 h with 2 ng/ml IL-1β, ACM, IL-1β-treated ACM, 1–100 ng/ml recombinant mouse SHH (R&D systems), 0.01–1 μM purmorphamine (a Smo agonist) (Merck Millipore, Billerica, MA, USA), or 0.3–30 μM cyclopamine (a Smo inhibitor) (Merck Millipore). Next, the monolayers were washed with assay buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4). This buffer (1 ml) was added to the outside of the insert (the abluminal side). Assay buffer containing 4% FITC-BSA (Sigma-Aldrich, St. Louis, MO, USA) was loaded on the luminal side of the insert and incubated for 1 h. The concentration of FITC-BSA in the abluminal chamber was determined by measuring the fluorescence (excitation, 480 nm; emission, 530 nm) using a Wallac 1420 $ARVO_{MX}$ (PerkinElmer Japan, Yokohama, Japan). Assays were carried out in five independent trials.

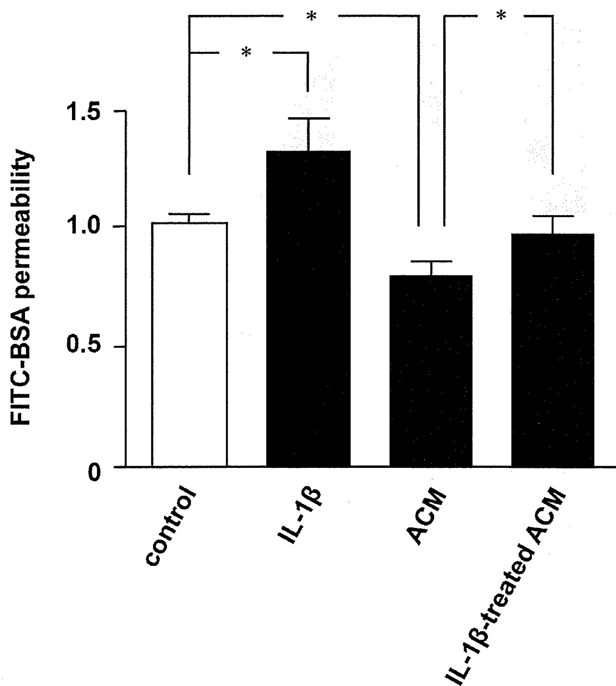

Figure 1. IL-1β abolishes the protective effect of astrocytes on BBB integrity. MEBC4 cells were treated for 24 h with 2 ng/ml IL-1β, ACM, or IL-1β–treated ACM for 24 h. FITC-BSA was loaded onto the luminal side of the insert for 1 h, and then the FITC-BSA levels on the abluminal side were analyzed. All quantitative data are expressed as means ± SEM (n = 5), normalized to the corresponding values from untreated cells. *, $p<0.001$.
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Quantitative reverse transcription-PCR

Astrocytes were collected after a 6-h incubation with 0.02–2 ng/ml IL-1β. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and reverse transcribed with SuperScript III (Life Technologies, Carlsbad, CA, USA) as described previously [23]. Expression levels of mRNAs encoding SHH, CXCL2, CCL2, and CCL20 were evaluated by quantitative PCR (qPCR) using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) on a Rotor-Gene Q real-time PCR cycler (Qiagen). The following mouse gene–specific primers and probes were obtained from Applied Biosystems: *Shh*, Mm00436528_m1; *Cxcl2*, Mm00436450_m1; *Ccl2*, Mm00411241_m1; *Ccl20*, Mm01268754_m1; *Actb*, Mm00607939_s1; and *Gapdh*, Mm99999915_g1. Gene expression values were determined by the $\Delta\Delta C_T$ method. Levels of the mRNAs of interest were normalized to the geometric mean of *Actb* and *Gapdh* levels. Assays were carried out in five independent trials.

Enzyme-linked immunosorbent assay (ELISA)

Astrocyte conditioned media were collected after a 24-h incubation with 0.02–2 ng/ml IL-1β, and then assessed for protein levels using ELISA kits specific for mouse SHH, CXCL2, CCL2, and CCL20 (R&D systems). Assays were carried out in five independent trials.

Western blotting

MBEC4 cells were incubated with ACM, 2 ng/ml IL-1β, 100 ng/ml SHH, 1 μM purmorphamine, or 30 μM cyclopamine for 24 h. To assess the protein expression levels of occludin and zonula occludens-1 (ZO-1), cells were lysed in TNES buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, and 0.1% SDS) with protease inhibitor mixture (Complete Mini EDTA-free; Roche Diagnostics, Basel, Switzerland) and a phosphatase inhibitor mixture (Sigma-Aldrich) as described previously [24,25]. Cell lysate proteins dissolved in Laemmli sample buffer (30 μg/well) were separated on 4–20% SDS-polyacrylamide gels (Mini-Protean TGX; Bio-Rad, Hercules, CA, USA) and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20, and then incubated overnight at 4°C with rabbit anti–claudin-5 monoclonal antibody (Zymed Laboratories, South San Francisco, CA, USA), rabbit anti-occludin polyclonal antibody (Zymed Laboratories), rabbit anti–ZO-1 polyclonal antibody (Zymed Laboratories), or mouse anti–β-actin monoclonal antibody (clone AC-15; Sigma-Aldrich), followed by incubation with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) for 1 h at room temperature. The signals were visualized using SuperSignal West Dura chemiluminescent substrate (Thermo Fisher Scientific,

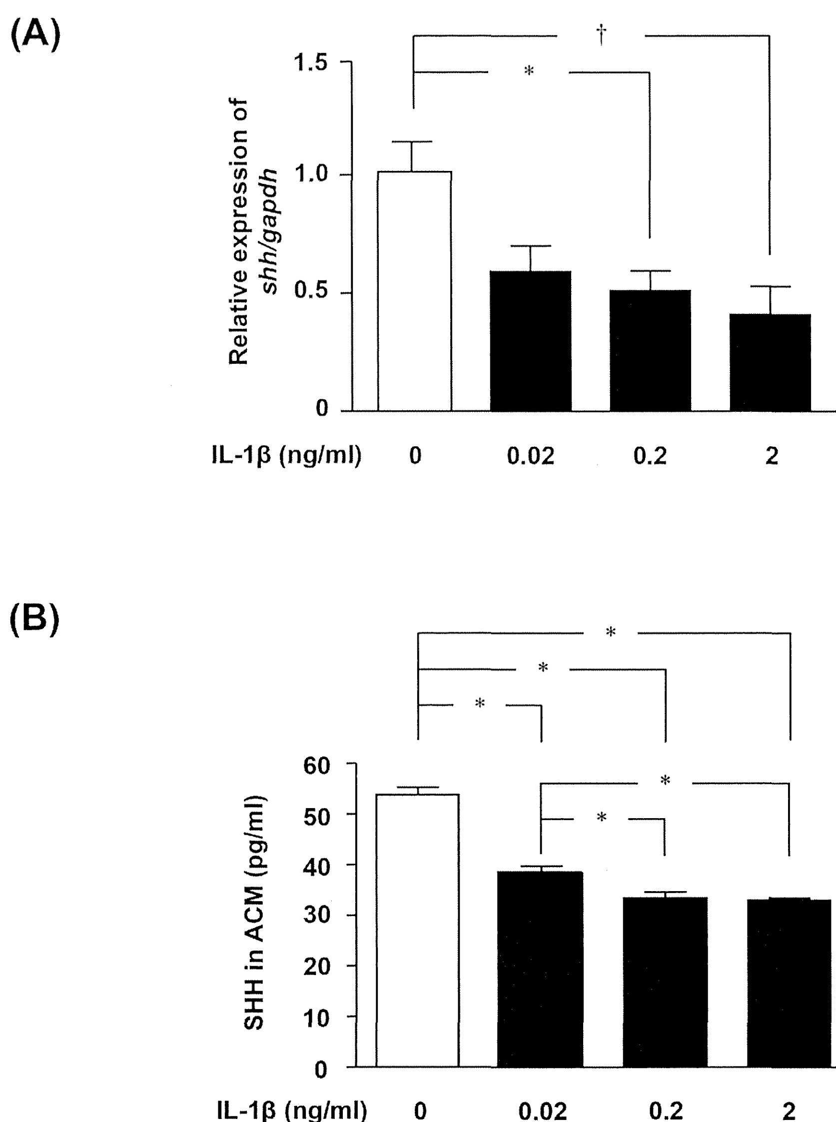

Figure 2. IL-1β downregulates SHH production in astrocytes. (A) *Shh* mRNA levels in astrocytes, determined by qPCR. Astrocytes were treated with IL-1β for 6 h. Values are means ± SEM (n = 5). *, $p<0.05$; †, $p<0.01$. (B) Protein levels of SHH in ACM, determined using ELISA. Astrocytes were treated with IL-1β for 24 h. Values are means ± SEM (n = 5). *, $p<0.001$.
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Waltham, MA, USA), and quantitated using a CS Analyzer 3.0 system (Atto, Tokyo, Japan). Assays were carried out in five independent trials.

Statistical analysis

Statistical significance was analyzed with one-way analysis of variance followed by post-hoc Tukey's test, using GraphPad Prism6 (GraphPad Software, La Jolla, CA, USA).

Results

IL-1β suppressed the protective effect of astrocytes on BBB integrity

First, we confirmed the effects of IL-1β and astrocytes on BBB integrity using MBEC4 monolayers as an *in vitro* BBB model. Astrocyte conditioned media (ACM) significantly decreased the permeability of BBB (Fig. 1). Treatment with IL-1β alone significantly increased the permeability of BBB, and conditioned media from IL-1β–stimulated astrocytes lost the ability to increase BBB integrity (Fig. 1). These findings suggested that IL-1β disrupts BBB integrity not only directly, but also indirectly via astrocyte dysfunction.

IL-1β decreased astrocytic production of SHH

Next, we focused on SHH, a soluble factor released from astrocytes that plays an important role in BBB maintenance. Specifically, we investigated whether IL-1β affects astrocytic SHH expression. Treatment with IL-1β significantly decreased *Shh* mRNA levels in astrocytes in a dose-dependent manner (Fig. 2A). Similar results were obtained for SHH protein levels in ACM using specific ELISA (Fig. 2B).

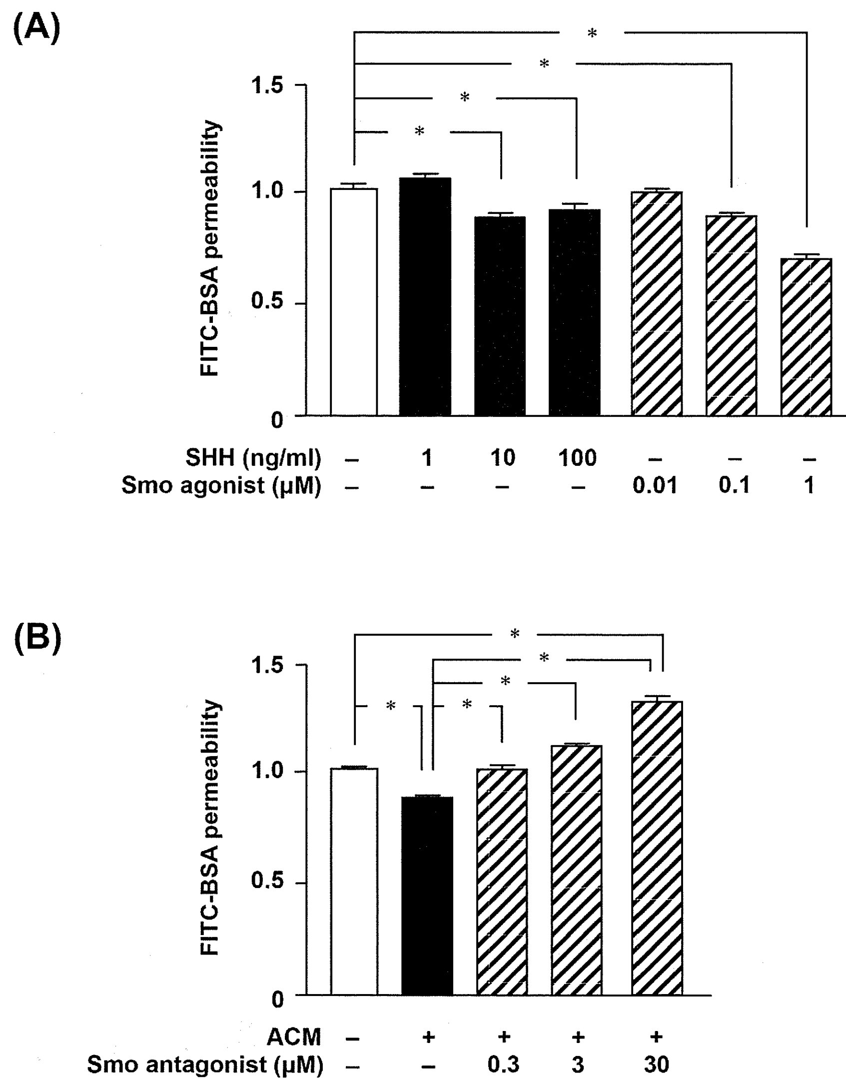

Figure 3. SHH signaling is critical for maintenance of BBB integrity. (A) MBEC4 cells were treated with SHH or the Smo agonist purmorphamine for 24 h. (B) MBEC4 cells were treated with ACM or the Smo antagonist cyclopamine for 24 h. FITC-BSA was loaded onto the luminal side of the insert for 1 h, and then the FITC-BSA levels on the abluminal side were analyzed. All quantitative data are expressed as means ± SEM (n = 5), normalized to the corresponding values from untreated cells. *, $p<0.001$.
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SHH produced by astrocytes is critical for maintenance of BBB integrity by upregulating tight junction proteins

Next, we examined the effect of astrocytic SHH signaling on BBB function. SHH or the Smo agonist (i.e. a SHH signaling enhancer) purmorphamine significantly decreased BBB permeability (Fig. 3A). By contrast, the Smo antagonist (i.e. a SHH signaling inhibitor) cyclopamine abolished the astrocytic effect on the maintenance of BBB function (Fig. 3B). The expression levels of such TJ proteins as claudin-5, occludin, and ZO-1 were closely correlated with BBB integrity (Fig. 4A–C): levels of these proteins were highest when permeability was lowest. Activation of SHH signaling by ACM, SHH, or purmorphamine resulted in significant upregulation of these proteins, whereas the Smo antagonist cyclopamine ablated the astrocytic effect on their expression (Fig. 4A–C). These observations suggested that SHH produced by astrocytes plays a critical role in BBB integrity by upregulating expression of TJ proteins.

IL-1β stimulated pro-inflammatory chemokine production in astrocytes

Finally, we assessed the effects of IL-1β on the production of pro-inflammatory chemokines in astrocytes. Treatment with IL-1β significantly increased the mRNA and protein expression levels of CXCL2, CCL2, and CCL20 in astrocytes (Fig. 5A and 5B). These data imply that IL-1β also activates astrocytes to release these pro-inflammatory chemokines; induces migration of immune cells such as neutrophils, monocytes, macrophage, dendritic cells, and pathogenic T cells; and leads to further BBB disruption and neuroinflammation.

Discussion

IL-1β is considered to be a critical factor for astrocyte activation in various neurologic disorders [26]. IL-1β binds to its receptor, IL-1R, whose downstream signaling activates nuclear factor-κB (NF-κB), a key player in the immune and inflammatory response

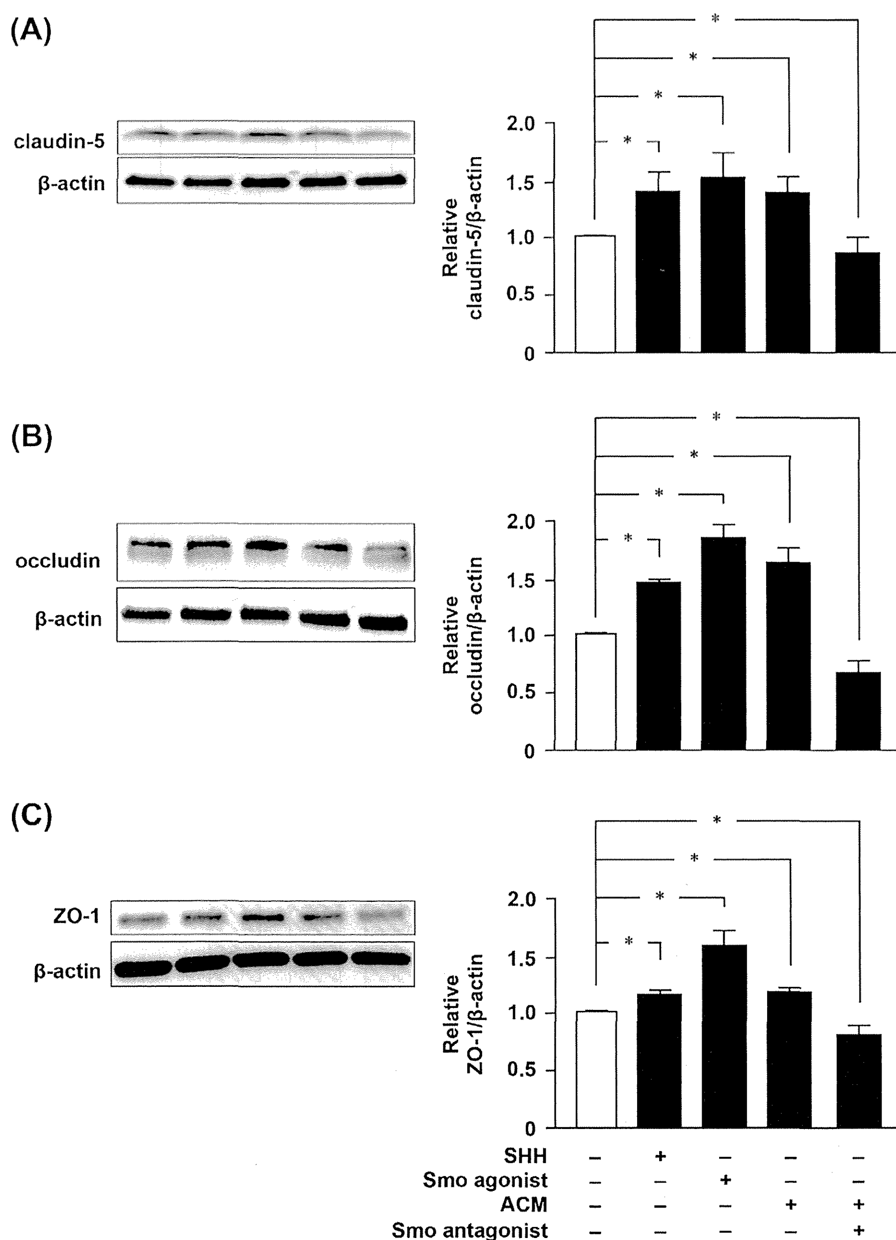

Figure 4. Astrocytic SHH signaling regulates expression of tight junction proteins in BBB. Western blotting of claudin-5 (A), occludin (B) and ZO-1 (C) in MBEC4 cells. Cells were treated for 24 h with ACM, SHH (100 ng/ml), the Smo agonist purmorphamine (1 μM), or the Smo antagonist cyclopamine (30 μM). All quantitative data are expressed as means ± SEM (n = 5), normalized to the corresponding values from untreated cells. *, $p < 0.05$.
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in astrocytes [27,28]. NF-κB promotes transcription of mediators of inflammation, such as pro-inflammatory cytokines/chemokines [29]. In addition, NF-κB also increases neurotrophic factor production in astrocytes [30,31]. Therefore, IL-1β plays two opposing roles in astrocytes. Microglia are the main source of IL-1β in the CNS. A variety of stimuli, such as damage-associated molecular pattern molecules (DAMPs), amyloid β, and pro-inflammatory cytokines, trigger microglial IL-1β production via an inflammasome-dependent mechanism [32,33]. Upregulation of IL-1β is observed in a broad spectrum of neurological diseases, including infections, trauma, stroke, and epilepsy, as well as chronic neurologic diseases such as MS, Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease [32]; BBB disruption is associated with progression of these diseases [34]. IL-1β increases BBB permeability by downregulating TJ proteins [17,18]. In addition, IL-1β also induces astrocytes to release vascular endothelial growth factor, which increases BBB permeability [16]. Thus, IL-1β induces BBB breakdown via both direct and indirect pathways.

Here, we propose another novel mechanism for IL-1β–mediated BBB disruption. SHH is a critical activator of Smo–Gli-1 signaling which upregulates TJ proteins and enhances BBB integrity (Fig. 6A). A decrease in SHH allows Ptch-1 to suppress Smo–Gli-1 signaling. In the healthy state, astrocytes release SHH,

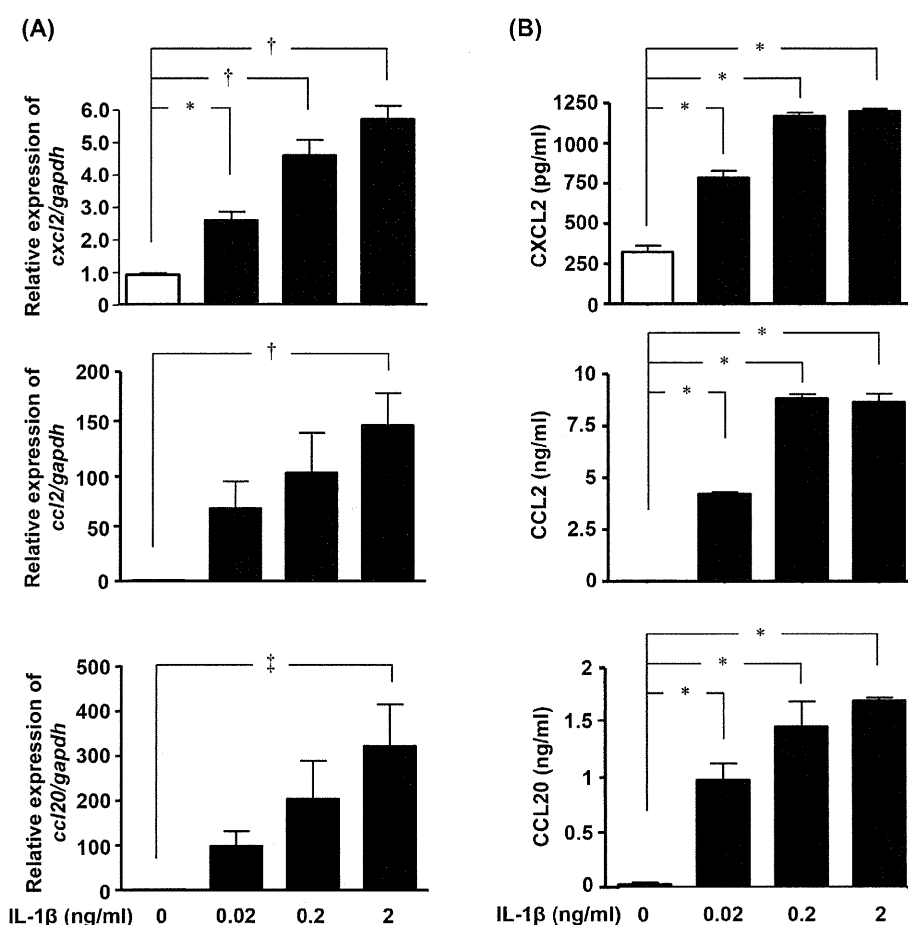

Figure 5. IL-1β upregulates production of pro-inflammatory chemokines in astrocytes. (A) *Cxcl2*, *Ccl2*, and *Ccl20* mRNA levels in astrocytes, determined by qPCR. Astrocytes were treated with IL-1β for 6 h. Values are means ± SEM (n = 5). *, $p<0.05$; †, $p<0.01$. (B) Protein levels of CXCL2, CCL2, and CCL20 in astrocytes, determined by ELISA. Astrocytes were treated with IL-1β for 24 h. Values are means ± SEM (n = 5). *, $p<0.001$.
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which upregulates TJ proteins in endothelial cells and maintains BBB integrity (Fig. 6B, left). Once pathogenic stimuli activate microglia to release IL-1β (Fig. 6B, right), it suppresses SHH production in astrocytes, downregulates TJ proteins in endothelial cells, and disrupts BBB integrity. Moreover, IL-1β–stimulated astrocytes secrete the pro-inflammatory chemokines CXCL2, CCL2, and CCL20, which induce migration of immune cells such as neutrophils, monocytes, macrophage, dendritic cells, and pathogenic T cells. Infiltration of these cells exacerbates BBB disintegrity and subsequent neuroinflammation.

In this study, the Smo antagonist cyclopamine decreased TJ protein expression levels and BBB integrity exceeding the physiological levels (Figs. 3 and 4). Previous reports suggested that unidentified endogenous ligands of Smo seem to activate this signaling although SHH is the main regulator of Smo–Gli-1signaling [35,36]. Our data also imply the presence of endogenous ligand(s) of Smo.

BBB disruption is a common pathologic feature of neurologic disorders such as stroke, MS, Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease. Therefore, restoration of BBB integrity has been recognized as a therapeutic target for treatment of these diseases [8,37]. In fact, both glucocorticoids and interferon β, both of which have been widely used for MS treatment, decrease BBB permeability [38,39]. Moreover, the efficacy of the α4-integrin antagonist natalizumab has also demonstrated the utility of BBB-targeting drugs in treating MS [40]. By contrast, excessive immunosuppression resulting from conventional therapies for MS sometimes causes progressive multifocal leukoencephalopathy [41]. Thus, from the perspective of adverse effects, restoration of TJ proteins represents a superior therapeutic approach. Inhibition of IL-1β is a promising potential method for restoring BBB integrity [42]; however, a previous study indicated that simple blockade of IL-1β runs the risk of increasing BBB disruption, because this cytokine also enhances the protective effects of astrocytes on the BBB [26]. Treatment with SHH may circumvent this dilemma, allowing reinforcement of BBB integrity without loss of the beneficial effects of IL-1β.

During development, SHH signaling is primarily involved in CNS morphogenic events [43], whereas in adulthood, SHH participates in vascular proliferation, neurogenesis, and tissue repair in the CNS [44]. Dysregulation of SHH occurs in a variety of neurologic disorders; therefore, activation of the SHH signaling pathway, which would enhance neurogenesis and gliogenesis, has been proposed as a potential therapeutic approach for treatment of these diseases [45]. Downregulation of SHH has been observed in MS brains [46], and interferon-β treatment improves symptoms in a MS rodent model, concomitant with reduced BBB breakdown and elevated SHH expression [47]. Taken together, these

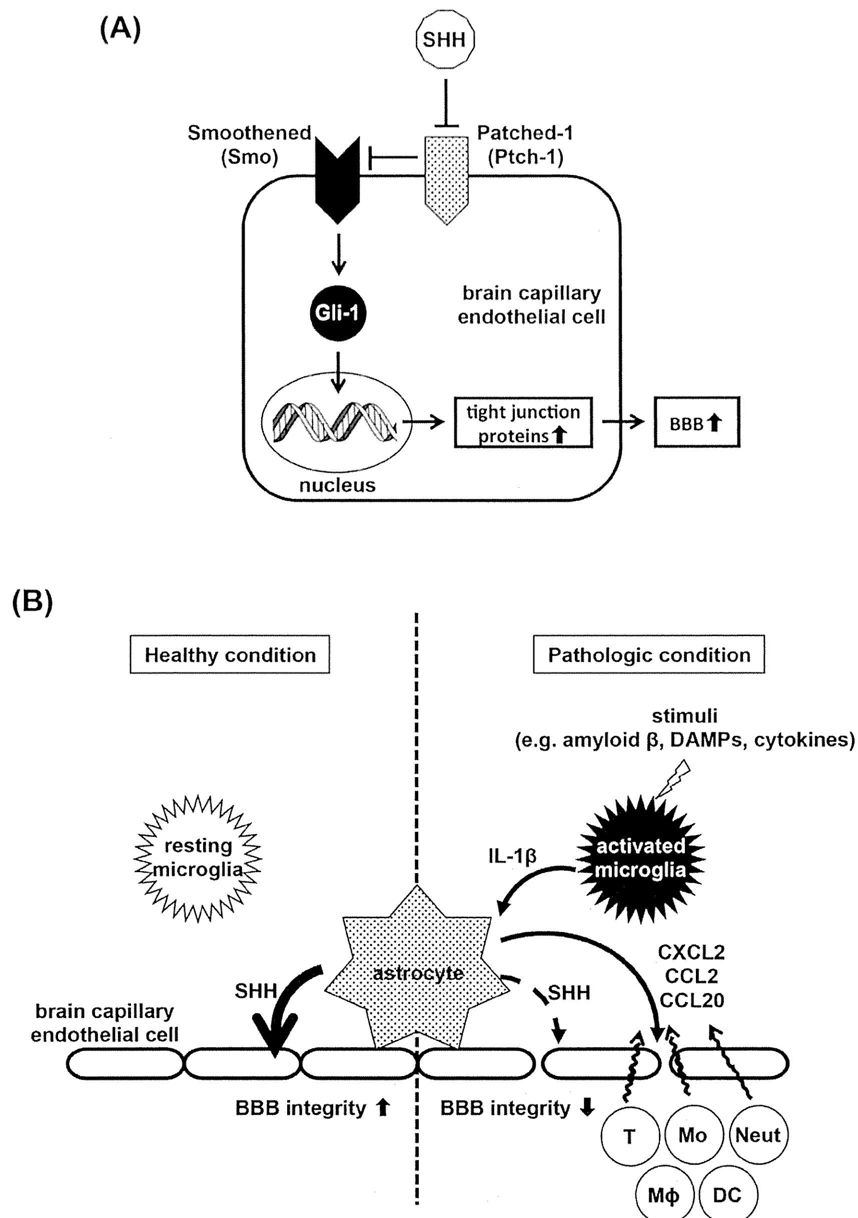

Figure 6. Model of the roles of the SHH and IL-1β pathways in the BBB. (A) Model of SHH signaling pathway in brain capillary endothelial cells. Secreted SHH binds and inactivates its receptor Patched-1, which allowed Smoothened to activate the transcription factor Gli-1. Gli-1 upregulates tight junction proteins and enhances BBB integrity. (B) Model of BBB breakdown by IL-1β. Under healthy conditions (left), astrocytes secrete SHH to upregulate tight junction proteins in endothelial cells and maintain BBB integrity. Under pathologic conditions (right), pathogenic stimuli such as amyloid β, DAMPs, or cytokines induce microglia to release IL-1β. IL-1β suppresses astrocytic SHH production, leading to downregulation of tight junction proteins in endothelial cells and disintegrity of the BBB. IL-1β also activates astrocytes to release pro-inflammatory chemokines such as CXCL2, CCL2, and CCL20. These chemokines induce migration of immune cells, thereby worsening BBB disruption and neuroinflammation. Neut, neutrophils; Mo, monocytes; MΦ, macrophage; DC, dendritic cells; T, T cells.
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observations suggest that SHH exerts a synergistic therapeutic effect by promoting CNS tissue repair while reinforcing the BBB.

This study reveals a novel mechanism for IL-1β–mediated BBB disruption: downregulation of SHH expression in astrocytes. Our findings suggest that stimulation of astrocytic SHH production could promote restoration of BBB integrity, and may therefore be useful in treating a variety of neurologic disorders.

Author Contributions

Conceived and designed the experiments: HT YS AS. Performed the experiments: YW SJ YS. Analyzed the data: YW HT SY TM. Contributed reagents/materials/analysis tools: YC HH BP JK. Contributed to the writing of the manuscript: YW HT AS.

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