METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.M. performed all experiments, with the exception of the portions indicated below. C.T. supported immunohistochemical analyses. C.T. and S.M. helped with *in vitro* experiments. H.M. and H.F. provided the autopsy samples from individuals with multiple sclerosis. R.M. and T.Y. designed the experiments. T.Y. coordinated and directed the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. SJL/J mice were obtained from Charles River Japan. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Osaka University.

Induction of a targeted EAE lesion. Adult female SJL/J mice (6–8 weeks old) were subcutaneously immunized with an emulsion of 50 μg bovine MBP (Wako) in complete Freund's adjuvant (Difco) containing 500 μg *Mycobacterium tuberculosis* H37Ra (Difco) on day 0. This immunization protocol alone rarely induces disseminated disease; the few mice in which dissemination occurred after this protocol were excluded from experiment. After 21–23 d, we performed a dorsal laminectomy at Th3–Th4 and injected 1 μl of a cytokine mixture composed of 250 ng recombinant mouse tumor necrosis factor α (TNF-α) (R&D) and 150 U recombinant mouse interferon γ (IFN-γ) (R&D) dissolved in PBS over a 3-min period (CST coordinate: 0.5 mm depth at the midline) into the dorsal column of the thoracic spinal cord. Mice received an intravenous injection of 200 ng pertussis toxin (List Biological Laboratories) in 100 μl PBS on the same day and at 48 h after the injection of TNF-α and IFN-γ.

EAE scores. Individual mice were scored for disease severity on the basis of the following scale: 0, no clinical disease; 0.5, piloerection; 1, tail weakness; 1.5, tail paralysis; 2, hindlimb weakness; 3, hindlimb paralysis; 3.5, forelimb weakness; 4, forelimb paralysis; or 5, moribund or death.

Hindlimb placing test. Independent testing of each hindlimb for placement on a tabletop in response to gentle, purely tactile or proprioceptive (with joint movement) stimulation applied to the front or top of the hindfoot was conducted. Each test was repeated for each hindlimb up to six times or until three correct responses were obtained. The number of completed placing responses (maximum, three) for each hindlimb was noted, and the results for both hindlimbs were added to obtain a hindlimb placing score (minimum, zero; maximum, six)[8].

Histology and immunohistochemistry. At 0, 7, 14, and 28 d after induction of targeted EAE, mice were transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Spinal cord and brain tissues were post-fixed with 4% PFA in PBS at 4 °C overnight, immersed in 30% sucrose in PBS and then embedded in optimal cutting temperature compound (Tissue-Tek) for frozen sectioning. Cross-sections were cut at 20-μm thickness on a cryostat and mounted on Matsunami adhesive silane-coated slides (Matsunami Glass). For histology, sections were stained with H&E (Wako).

For immunohistochemistry, sections were permeabilized in PBS containing 0.1% Triton X-100 and 0.5% BSA for 1 h at room temperature. The sections were then incubated with primary antibodies overnight at 4 °C and then incubated with fluorescently labeled secondary antibody for 3 h at room temperature. The primary antibodies used were as follows: rabbit prostaglandin I synthase (1:100, 100023, Cayman), rabbit IP receptor (1:100, 160070, Cayman), rabbit NeuN (1:100, MAB377, Covance), rabbit amyloid β (A4) precursor protein (APP) (1:100, A8718, Sigma), mouse GFAP (1:100, G3893, Sigma), rat PDGF receptor-α (1:100, 558774, BD Pharmingen), rabbit CD31 (1:100, ab28364, Abcam), rat CD105 (1:50, endoglin; 550546, BD Bioscience), rabbit cox2 (1:100, 160106, Cayman), mouse Ki67 (1:100, 556003, BD Bioscience), rabbit PKC-γ (1:100, sc-211, Santa Cruz), rat CD4 (1:100, 550278, BD Bioscience) and rat CD11b (1:100, 550282, BD Bioscience) antibodies. Alexa Fluor 488- or 568-conjugated goat antibody to rabbit IgG, goat antibody to rat IgG and goat antibody to mouse IgG (Invitrogen) were used as secondary antibodies. The mouse on mouse (MOM) kit (Vector) was used for Ki67 immunohistochemistry.

To estimate the length of vessels, the sections were immunostained with either CD105-specific or CD31-specific antibodies. The length of the vessels in the gray matter was measured in the sections around the targeted EAE lesion (segments Th3–Th4, just rostral to the lesion center). The means were calculated from 5–7 sections spaced 100 μm apart[28].

To assess demyelination in the spinal cord, we performed myelin staining by using a green fluorescent lipophilic dye (FluoroMyelin, Invitrogen) according to the manufacturer's instructions.

Axonal tract tracing. The hindlimb CST was labeled with a 10% solution of BDA (10,000 MW; Invitrogen). BDA was slowly injected into the hindlimb area of the motor cortex (coordinates from bregma: 2.0 mm posterior/2.0 mm lateral, 2.0 mm posterior/2.5 mm lateral, 2.5 mm posterior/2.0 mm lateral and 2.0 mm posterior/2.5 mm lateral, 0.4 μl per site, all at a depth of 0.8 mm into cortex) using a glass capillary attached to a microsyringe. Two weeks after the BDA injections, mice were transcardially perfused with 4% PFA in PBS. The sections of the spinal cord were cut horizontally at 20-μm thickness on a cryostat and mounted on Matsunami adhesive silane-coated slides. For visualizing the BDA, the sections were incubated in PBS containing 0.3% Triton X-100 for 1 h, followed by incubation with Alexa Fluor 488–conjugated streptavidin (1:500, Invitrogen) for 2 h at room temperature.

To quantify the number of sprouting axons, the number of CST collaterals crossing the line positioned in the gray matter was measured as previously reported[29]. We drew a horizontal line through the central canal and across the lateral rim of the gray matter. Two vertical lines were drawn to divide the horizontal line into two equal parts, starting from the central canal to the lateral rim. Only fibers crossing the two lines were counted in each section. To correct for variations in tracing efficiency, the number of CST collaterals per section were normalized to the total number of labeled CST axons on each side; 4–6 sections spaced 100 μm apart were examined. The average number of CST collaterals per section was divided by the number of labeled CST fibers and multiplied by the number of CST fibers at the level of the medulla[30].

Propriospinal neurons were labeled with Alexa Fluor 555–conjugated CTB (Invitrogen). Under anesthesia, half of the L1–L2 vertebrates were laminectomized. The tracer was injected into the exposed spinal cord 200 μm lateral to the midline, 0.4 μl per site, at a depth of 300 μm. At each location, the glass capillary was left in position for an additional 1 min before withdrawal. All the sections were analyzed using a fluorescence (Olympus BX51, DP71) or confocal laser-scanning microscope (Olympus FluoView FV1000). The number of contacts of BDA-labeled axons with Alexa Fluor 555–labeled propriospinal neurons was determined as described[31] in the section around the EAE lesion (segments Th2–Th3, one segment above the lesion center). The average numbers of contacts per section were divided by the number of labeled CST fibers and multiplied by the number of counted CST fibers at the level of medulla. The means were calculated from each of five sections spaced 100 μm apart.

Surgical procedure. For pharmacological treatments in mice, anesthetized female SJL/J mice underwent laminectomy at thoracic level Th3–Th4, and a cannula from the Alzet osmotic minipump (model numbers 1002 and 1004, Alzet Corp) was placed under the dura at the thoracic cord immediately after injection of the cytokine mixture. The osmotic minipump was filled with vehicle solution (saline), CAY10441 (0.31 μg per kg of body weight per d over 2 or 4 weeks) or iloprost (0.36 μg per kg of body weight per d over 2 or 4 weeks), and the pump was placed subcutaneously in the back. To selectively kill spinal cord neurons, the excitotoxic glutamate agonist NMDA (Sigma) was stereotaxically injected into the spinal cord gray matter at Th2–Th3 at a 0.3 μl volume of 1 mM NMDA per site and a depth of 800 μm.

To determine the size of the lesion, we perfused mice transcardially with 4% PFA in PBS 3 d after NMDA injection. Horizontal sections of the spinal cord (30-μm thick) were taken at 150-μm intervals, including the site of NMDA injection. The sections were stained with NeuroTrace red fluorescent Nissl stain (Invitrogen). We assessed the length of the lesion in the longitudinal direction by analyzing the area of cell loss in each section.

Preparation and transfection of siRNA. Mouse IP receptor siRNA and PGIS siRNA were synthesized by Invitrogen (Stealth siRNA). The sense and antisense strands of siRNA were as follows: IP receptor siRNA1, 5′-GAAAGGCUGUC UUCCAACGCCUCAA-3′ (sense) and 5′-UUGAGGCGUUGGAAGACAGC CUUUC-3′ (antisense); IP receptor siRNA2, 5′-UCUCGGGCACGAGAGGA UGAAGUUU-3′ (sense) and 5′-AAACUUCAUCCUCUCGIGCCCGAGA-3′ (antisense); PGIS siRNA1, 5′-GGGAAGAGUUAUGCCAUCAACAGCA-3′ (sense) and 5′-UGCUGCCGAUGGCAUAACUCUUCCC-3′ (antisense); PGIS siRNA2, 5′-GGGAGAGAUGCUGCCAGCUUCCUUA-3′ (sense) and 5′-UAAG GAAGCUGGCAGCAUCUCUCCC-3′ (antisense). The 5′ ends of these siRNAs were labeled with Alexa Fluor 555. Transfection of the IP receptor siRNA with

cultured cortical neurons was performed using the Nucleofector transfection device (Amaxa) according to the manufacturer's protocols.

Transfection of PGIS siRNA with cultured endothelial cells was performed using Lipofectamine 2000 (Invitrogen). The cells were lysed 72 h after transfection and then subjected to western blot analysis.

For *in vivo* experiments, aliquots of the stock solution were mixed with i-Fect transfection reagent (Neuromics). The mice received intracortical injections (2 μg siRNA μl^{-1}; coordinates from bregma: 2.0 mm posterior/2.0 mm lateral, 2.0 mm posterior/2.5 mm lateral, 2.5 mm posterior/2.0 mm lateral and 2.0 mm posterior/2.5 mm lateral, all at a depth of 0.8 mm into cortex) of either a lipid-encapsulated IP receptor–selective siRNA or i-Fect–encapsulated nontargeting double-stranded RNA (dsRNA) (control mismatch siRNA) on days 0 and 14 after induction of targeted EAE. BDA was simultaneously injected in the mixture with the siRNAs. Injection of siRNA or the transfection reagents alone into motor cortex did not cause any signs of toxicity at the behavioral level assessed in open-field test (data not shown).

For knockdown of PGIS in vascular endothelial cells *in vivo*, PGIS siRNA or nontargeting dsRNA in saline (50 μg siRNA ml^{-1}) was rapidly injected within 5–10 s into the mouse tail vein on days 0, 7 and 14 after induction of targeted EAE. This procedure was previously shown to achieve selective gene transfer to vascular endothelial cells[32]. Injection of either the siRNA or saline alone into the vein did not result in any signs of toxicity, as assessed by behavior (data not shown).

Western blot analysis. The tissues or cells were homogenized in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA containing protease inhibitor (Roche). The lysates were clarified by centrifugation at 13,000*g* at 4 °C for 20 min, and the supernatants were collected and normalized for protein concentration. Proteins were separated by 8% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). After blocking with PBS containing 5% skim milk and 0.05% Tween 20, the membranes were incubated with primary antibodies.

For detection, a fluorescence-conjugated secondary antibody and an electrogenerated chemiluminescence system (GE Healthcare) were used. The membrane was exposed to an imaging system (LAS-3000, Fujifilm) according to the manufacturer's specifications. The protein bands were quantified using ImageJ software. The following antibodies were used: mouse α-tubulin (1:1,000, sc-5286, Santa Cruz), rabbit antibodies to prostaglandin I synthase (1:1,000, 100023, Cayman) and rabbit antibodies to IP receptor (1:1,000, 160070, Cayman). Horseradish peroxidase–conjugated mouse IgG–specific and rabbit IgG–specific antibodies were used as secondary antibodies (1:5,000, Cell Signaling Technology).

Primary culture of cortical neurons. Cortical neurons used for primary cultures were prepared from cerebral cortices obtained from mice at postnatal day 1. The cerebral cortices were dissociated by trypsinization (treatment with 0.25% trypsin in PBS for 15 min at 37 °C) followed by resuspension in DMEM containing 10% (v/v) heat-inactivated FBS (Gibco) and trituration. For the neurite outgrowth assay, the isolated cells were plated on culture dishes coated with 0.1 mg ml^{-1} collagen type IV (Sigma) and 0.1 mg ml^{-1} fibronectin (Sigma) at a density of 1–2 × 10^4 cells per cm^2 in DMEM supplemented with 2% (v/v) B27 (Invitrogen). After culturing for 24 h, cells were immunostained with mouse β-tubulin–specific (Tuj1; 1:100, MMS-425P, Covance) and rat Ctip2-specific (1:1,000, ab18465, Abcam) antibodies to measure the neurite length of subcortical projection neurons. The following pharmacological reagents were used in the cultures: 100 μM Sp-cAMPS (Sigma) and 100 μM Rp-cAMPS (Sigma). To analyze the effect of prostacyclin, 1 μM CAY10441 (Cayman) and 1 μM iloprost (Cayman) were used. All the reagents were added to the cortical neuron cultures 30 min before the start of the coculture assay.

Primary culture of endothelial cells. Primary cultures of mouse brain capillary endothelial cells were prepared from 3-week-old female SJL/J mice. Mouse cerebral cortices and spinal cords, free of meninges, were digested in a mixture of 1 mg ml^{-1} collagenase type 2 (Worthington) and 6.7 μg ml^{-1} DNase (Sigma) in DMEM for 1.5 h at 37 °C. The cell pellet was separated by centrifugation in 20% BSA-DMEM (1,000*g*, 10 min) and then incubated for another 45 min at 37 °C with a mixture of 1 mg ml^{-1} collagenase-dispase (Roche) and 6.7 μg ml^{-1} DNase in DMEM at 37 °C. Microvessel endothelial cell clusters were separated on a 33% continuous Percoll gradient (GE Healthcare), collected and plated on culture dishes coated with 0.1 mg ml^{-1} collagen type IV and 0.1 mg ml^{-1} fibronectin. Endothelial cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM glutamine (Gibco), 2 ng ml^{-1} basic fibroblast growth factor (bFGF) (R&D) and 4 μM hydrocortisone (Sigma); in addition, the medium was supplemented with 1 μg ml^{-1} puromycin (Sigma). The absence of contamination in the endothelial cell culture was confirmed by immunostaining with the endothelial cell marker CD31. For the coculture experiments, cortical neurons and endothelial cells were grown separately on the lower wells and upper chambers of a transwell coculture system, respectively.

Cortical evoked CDPs. The sensory motor cortex and lumber spinal cord were exposed in mice anesthetized with ketamine hydrochloride (100 mg per kg of body weight, intraperitoneally (i.p.)) and xylazine hydrochloride (5 mg per kg of body weight, i.p.). Cortical evoked potentials were elicited by electrical stimulation (10 square-wave pulses at 330 Hz, 50 μA, 200 ms; STG4002, Multichannel Systems) of the left sensory motor cortex using 50-μm–diameter Teflon-coated tungsten wires (A-M systems) lowered 0.8 mm into the cortex. Postsynaptic potentials evoked by the cortical stimuli were recorded with a silver ball electrode placed medially on the contralateral cord surface using a PowerLab (AD Instruments). At each recording site (L1–L2), 20 responses were averaged and stored for off-line analysis of amplitude and latency.

T cell proliferation and cytokine analysis. Spleens were harvested from control-, iloprost- and CAY10441-treated EAE mice on day 7. Freshly isolated spleen was prepared and treated with ammonium-chloride-potassium (ACK) lysing buffer (Lonza) to remove erythrocytes. The cell suspension was filtered through a fine mesh screen. Single cells were plated at a density of 5 × 10^6 cells ml^{-1} in RPMI 1640 medium supplemented with glutamine (Gibco), sodium pyruvate, penicillin, streptomycin, 2-mercaptoethanol (Wako) and 10% FBS. To re-stimulate the T cells, 50 μg ml^{-1} MBP was initially added to the culture. After stimulation for 24 h, cell proliferation was estimated by the measurement of BrdU incorporation into newly synthesized cellular DNA using the Cell Proliferation ELISA and BrdU (colorimetric) kit (Roche) according to the manufacturer's instructions. To measure the production of cytokines, the splenocytes obtained from mice with EAE were cultured with 50 μg ml^{-1} MBP peptide. After stimulation for 72 h, supernatants were collected, and the expression of a panel of cytokines was measured using the Q-Plex Mouse Cytokine Array (Quansys Biosciences) according to the manufacturer's instructions. To measure cytokine expression in the spinal cord, tissues were homogenized in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA containing protease inhibitor. Samples were centrifuged at 15,000*g* for 20 min at 4 °C. The supernatant was aliquoted and stored at −80 °C. The expression of a panel of cytokines was measured using the Q-Plex Mouse Cytokine Array (Quansys Biosciences) according to the manufacturer's instructions.

Enzyme immunoassay. PGI_2 synthesis in cell culture and spinal cord tissue was assessed by quantifying its stable metabolite (6-keto–PGF1-α; Cayman) according to the manufacturer's instructions. To measure 6-keto–PGF1-α expression in the spinal cord, tissues were homogenized in 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% NP-40, 10% glycerol and protease inhibitor. The lysates were clarified by centrifugation at 13,000*g* at 4 °C for 20 min, and the supernatants were collected and normalized for protein concentration. To measure 6-keto–PGF1-α expression in conditioned media, vascular endothelial cells were plated at a density of 5 × 10^5 cells ml^{-1} in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1 mM HEPES, 2 mM glutamine, 2 ng/ml bFGF and 4 μM hydrocortisone. After 1 d in culture, the culture supernatant was collected and stored at −20 °C.

Immunohistochemical staining of human tissues. We obtained autopsied spinal cord tissues from three individuals with relapsing-remitting multiple sclerosis. We formalin fixed the spinal cord samples, embedded them in paraffin and cut them into 4-μm–thick sections for immunohistochemistry.

We then deparaffinized, washed and subjected the sections to an antigen-retrieval procedure by heating the sections in citrate buffer. We incubated tissue samples with primary antibodies specific for human CD105 (1:10, M352701, Dako) and PGIS (1:100, 160640, Cayman) and Alexa Fluor 488– or 568–conjugated goat mouse IgG and goat rabbit IgG (Invitrogen) secondary antibodies. The research protocol was approved by the Human Use Review Committees of the Graduate School of Medicine, Osaka University, and Toneyama National Hospital, for the Protection of Human Subjects. Informed consent was obtained from all subjects.

Statistical analyses. Data are presented as mean ± s.e.m. For clinical scores, significance between groups was examined using Bonferroni's test. Other statistics were analyzed using either an unpaired Student's *t* test, repeated measures ANOVA or one-way ANOVA followed by Scheffe's tests or Tukey's tests. $P < 0.05$ was considered to be significant.

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