Supporting Information

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SI Text

Animals. Male Wistar rats (200–230 g) were obtained from Japan SLC (Hamamatsu). Male IFN-γR–deficient mice (*ifngr*$^{-/-}$) that were backcrossed to C57BL/6J for 10 generations (B6.129S7-*Ifngr1*^{tm1Agt}/J, Generations: N10F15 [20-DEC-06]) were purchased from The Jackson Laboratory, and their background wild-type control C57BL/6J mice (male) were purchased from Japan Charles River. The C57BL/6J mice were offspring of stock originally obtained from The Jackson Laboratory. Male Lyn-deficient mice (*lyn*$^{-/-}$) that were backcrossed to C57BL/6J Slc (Japan SLC) for more than 10 generations were kindly provided by Prof. Tadashi Yamamoto (Division of Oncology, Department of Cancer Biology, The Institute of Medical Science, The University of Tokyo) (1), and their background wild-type control C57BL/6J Slc (male) were used as the corresponding controls of *lyn*$^{-/-}$. Male mice lacking $P2X_4R$ (*p2rx4*$^{-/-}$) that were backcrossed to C57BL/6J Jcl (Clea Japan) for more than 10 generations were kindly provided by Prof. Joji Ando (Department of Biomedical Engineering, Graduate School of Medicine, The University of Tokyo) (2); we used C57BL/6J Jcl (Clea Japan) as the corresponding control mice. All mice used were age ≈9–11 weeks at the start of each experiment. Rats and mice were housed individually and in groups of ≈2 or 3 per cage, respectively, at a temperature of 22 ± 1 °C with a 12-h light-dark cycle (light on 8:30 to 20:30), and were fed food and water ad libitum.

Behavioral Studies. All experimental procedures were performed under the guidelines of Kyushu University. The PWT was measured using calibrated von Frey filaments (rats: 0.4–15.1 g; mice: 0.02–2.0 g; Stoelting) before and after intrathecal administration of recombinant IFN-γ (10–1,000 U; Calbiochem) or injury of a unilateral L5 spinal nerve (an animal model of neuropathic pain) in Wistar rats (3, 4) or mice (*ifngr*$^{-/-}$, *lyn*$^{-/-}$ (1), *p2rx4*$^{-/-}$ (2), and each background wild-type control C57BL/6J) (5, 6). For intrathecal administration to rats, under isoflurane anesthesia, a 32-gauge intrathecal catheter (ReCathCo) was inserted through the atlanto-occipital membrane into the lumbar enlargement and externalized through the skin (6). Intrathecal administration to mice was performed according to a procedure described previously (7) using a 25-μl Hamilton syringe with 30-gauge needle. Minocycline (40 mg/kg; Sigma) was administered i.p. once a day from day 0 (30 min before IFN-γ administration) to day 7.

In Situ Hybridization. The lumbar spinal cords of normal rats fixed with Tissue Fixative (Genostaff) were embedded in paraffin. For in situ hybridization as a first staining, tissue sections (6 μm) were de-waxed, rehydrated, fixed with 4% paraformaldehyde, and then treated with proteinase K. Hybridization was performed with digoxigenin-labeled antisense or sense probe for the rat IFN-γR (NM_ 053783, sequence position 997-1713) at a concentration of 100 ng/ml in the Probe Diluent (Genostaff) at 60 °C for 16 h. After hybridization, sections were washed, treated with 50% formamide, and then incubated with RNase. Then, sections were washed and incubated with anti-DIG alkaline phosphatase-conjugated antibody (1:1,000; Roche Diagnostics GmbH). Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich). For immunohistochemistry as a second staining after in situ hybridization, the sections were treated 3% hydrogen peroxide in PBS for 15 min and with Protein Block (Dako). Sections were incubated with the anti-ionized calcium binding adapter molecule 1 (Iba1) rabbit polyclonal antibody (1:1,000; Wako), treated with Histofine Simplestain rat MAX-PO (MULTI) (Nichirei) for 30 min, and then incubated with diaminobenzidine.

Immunohistochemistry. Anesthetized animals were perfused transcardially with PBS, followed by 4% paraformaldehyde. The L5 segments of the spinal cord were removed, postfixed in the same fixative for 3 h at 4 °C, and placed in 30% sucrose solution for 24 h at 4 °C. Transverse L5 spinal cord sections (30 μm) were cut and processed for immunohistochemistry with antibodies for phospho-Tyr-701-STAT1 (1:100), $P2Y_{12}$ receptor (1:1,000; kindly provided by David Julius, University of California, San Francisco) (6), phospho-Tyr-416-SFK (1:400, Cell Signaling), Lyn (1:200; Wako), and $P2X_4R$ (1:1,000; Alomone). The cell types in the dorsal horn were identified using the following markers: microglia, OX-42 (1:1,000; Serotec) and Iba1 (1:2,000; Wako); astrocytes, GFAP (1:500; Chemicon); spinal cord neurons, NeuN (1:200; Chemicon), MAP2 (1:500; Chemicon), and ED-1 (1:500; Serotec). The numbers of Iba1-positive cells with clearly visible cell bodies in the spinal dorsal horn were counted and normalized by the constant area (0.3 mm^2 in rats and 0.15 mm^2 in mice). To visualize proliferating cells, BrdU (75 mg/kg; Sigma) was injected i.p. into rats 22 h after intrathecal administration of IFN-γ; 2 h later, tissue from BrdU-treated rats was fixed by 4% paraformaldehyde. Spinal sections (30 μm) were incubated with 2N HCl to denature DNA and then were incubated with 0.1 M boric acid. After a rinse with PBS, sections were incubated with the primary antibody for BrdU (anti-BrdU, 1:2,000; Chemicon) and the marker of microglia Iba1 (1:2,000′ Wako). Another marker of proliferation, Ki-67 (anti-Ki-67, 1:100′ Dako), was used also. Following incubation, the spinal sections were incubated with secondary antibodies conjugated to Alexa Fluor™ 488 or 546 (1:1,000′ Molecular Probes). The spinal cord sections were analyzed using an LSM510 Imaging System (Zeiss).

PKH26 Labeling of Circulating Macrophages. For fluorescence labeling of peripheral macrophage, rats were anesthetized by isoflurane, and the red fluorescent dye PKH26-PCL (100 μM; Sigma) or the corresponding vehicle was slowly injected intravenously in a total volume of 500 μl. Forty-eight hours after PKH26 infusion, blood was collected into a 1-ml insulin syringe filled with 200 μl of NaCl-EDTA as an anticoagulant. The lysis of red blood cells was performed in a total volume of 15 ml of lysis buffer for 5 min at room temperature. After a wash by centrifuge (300 × *g* for 5 min), the cell pellet was resuspended in 0.5 ml of staining buffer with OX-42 antibody (1:100) and incubated for 30 min on ice. Following a wash, the cells were incubated with secondary antibody conjugated to Alexa Fluor™ 488 (1:500; Molecular Probes) for another 10 min on ice. The stained cells were resuspended in 1% paraformaldehyde/PBS buffer and stored at 4 °C. The sample cells were analyzed using a FACS Calibur HG flow cytometer (Becton Dickinson) with CellQuest Pro software (Becton Dickinson). Non-stained cells with the same preparation were included as the negative control. To detect PKH26-labeled macrophages within the dorsal horn, rat tissue was fixed 3 d after intrathecal administration of IFN-γ or PBS, and L5 spinal sections were used for immunohistochemical analyses. Recombinant IFN-γ or PBS was administrated intrathecally to rats through the catheter 48 h after the i.v. infusion of PKH26.

Microglial Culture. Rat primary cultured microglia were prepared in accordance with a method described previously (4, 8). In brief, a mixed glial culture was prepared from neonatal Wistar rats and maintained for 10–16 d in DMEM with 10% FBS. Immediately before experiments microglia were collected as the floating cells over the mixed glial culture by a gentle shake of the culture flasks. The microglia were transferred to coverslips for subsequent experiments. The cultures were $>$ 99% pure, as determined by immunostaining for OX-42 and Iba1. For the experiments of microglia infusion, primary cultured microglia (2×10^4 cells/5 μl) from neonatal wild-type C57BL/6J (Japan Charles River) or from *ifngr*$^{-/-}$ mice were intrathecally infused to *ifngr*$^{-/-}$ mice using a 25-μl Hamilton syringe with 30-gauge needle. Six h after the infusion of microglia, recombinant IFN-γ (10 U) was administered intrathecally. In light of the survivability of primary cultured microglial cells that had been taken out of cell culture environments, the PWT was measured 1 d after intrathecal administration of IFN-γ.

Western Blotting. The membrane fractions from spinal cord segments L4–L6 and whole-cell lysates of cultured microglial cells were prepared in accordance with methods described previously (5). Aliquots (0.5–2 μg) were subjected to a 10% polyacrylamide gel electrophoresis (BioRad), and proteins were transferred electrophoretically to nitrocellulose membranes. After blocking, the membranes were incubated with anti-P2X$_4$R rabbit polyclonal antibody (1:1,000) or anti-Lyn mouse monoclonal antibody (1:200) and then were incubated with HRP-conjugated secondary antibody (1:1,000). The blots were detected using a chemiluminescence method (ECL system; Amersham) and exposed to autoradiography films (Hyperfilm-ECL; Amersham). Bands were quantified using the software NIH Image J 1.36 (National Institutes of Health).

Statistics. Statistical analyses of the results were made with Student's *t* test, Student's paired *t* test, or 1-way ANOVA with a post hoc test (Dunnett's multiple comparison test).

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3. Kim SH, Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50:355–363.
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8. Nakajima K, et al. (1992) Identification of elastase as a secretory protease from cultured rat microglia. *J Neurochem* 58:1401–1408.

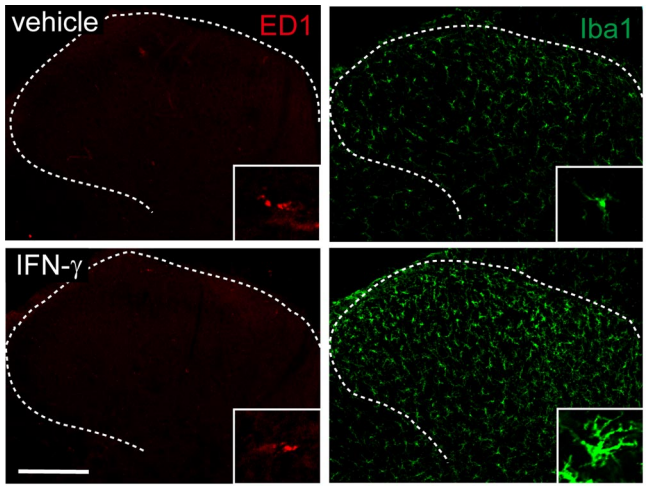

Fig. S1. IFN-γ administered intrathecally does not change immunofluorescence, morphology, or number of ED-1$^+$ macrophages in the dorsal horn. Photographs show immunofluorescence of ED-1 (red, *Left*) and Iba1 (green, *Right*) in the dorsal horn 3 d after IFN-γ (1,000 U) administration in rats. (Scale bar, 200 μm.) Insets are images at high magnification (50 μm wide).

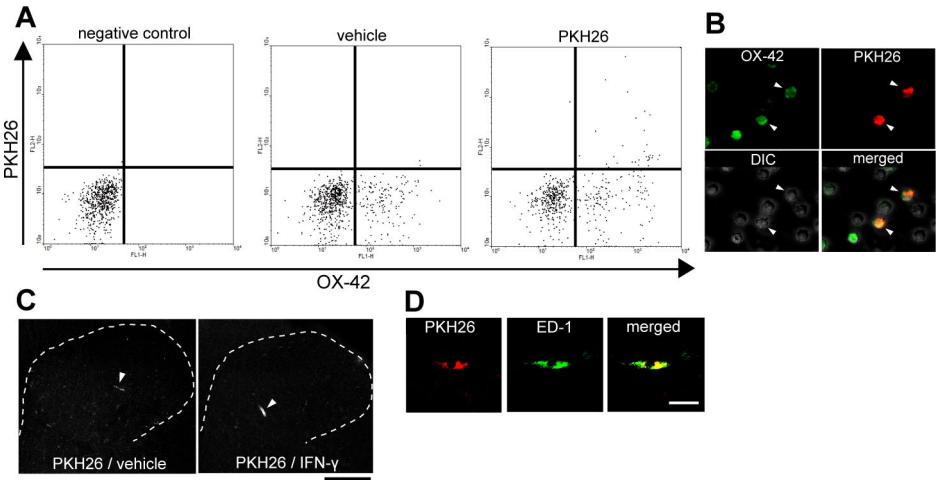

Fig. S2. Spinal IFN-γ injection does not cause infiltration of circulating macrophage in the spinal dorsal horn. (*A*) Representative dot plots for flow cytometry of PKH26- and OX-42-stained blood cells taken from rats 48 h after i.v. injection of PKH26 or vehicle. (*B*) Photographs show that a subpopulation of PKH26-labeled blood cells is immunostained with OX-42 (arrowheads). (DIC: differential interference contrast.) (*C*) Photographs show fluorescence of PKH26 (arrowheads) in the spinal dorsal horn 3 d after intrathecal administration of vehicle or IFN-γ (1,000 U). (Scale bar, 200 μm.) (*D*) $PKH26^{+}$ cells in the dorsal horn are immunolabeled with ED-1. (Scale bar, 20 μm.)

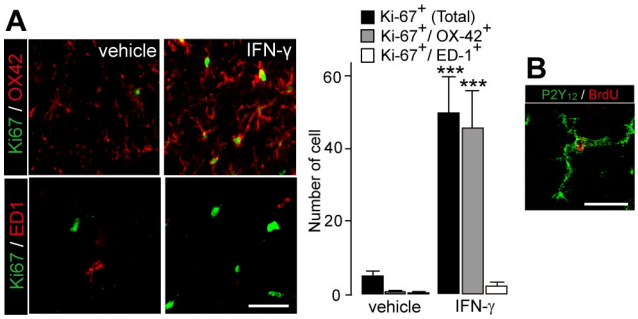

Fig. S3. Spinal IFN-γ injection causes resident microglia, but not macrophages, to proliferate in the dorsal horn. (*A*) Double immunofluorescence labeling of Ki-67 (green) with OX-42 (red, *Upper*) or ED-1 (red, *Lower*) in the dorsal horn 1 d after IFN-γ (1,000 U) administration. (Scale bar, 50 μm.) The number of Ki-67$^+$ED-1$^+$ cells, Ki-67$^+$OX-42$^+$ cells, and Ki-67$^+$ cells in the dorsal horn of rats (vehicle: $n = 3$; IFN-γ: $n = 5$, ***, $P < 0.001$ vs. vehicle). Data are mean ± SEM. (*B*) Double immunofluorescence labeling of P2Y$_{12}$R (green) with BrdU (red) in the dorsal horn 1 d after IFN-γ (1,000 U) administration. (Scale bar, 20 μm.)

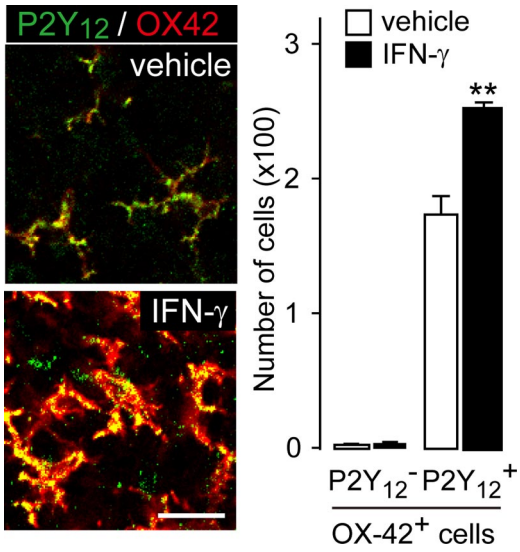

Fig. S4. Resident microglia, but not macrophages, undergo proliferation by intrathecal IFN-γ administration. (*Left*) Double immunofluorescence labeling of $P2Y_{12}R$ (green) with OX-42 (red) in the dorsal horn 3 d after administration of IFN-γ (1,000 U) in rats. (Scale bar, 20 μm.) (*Right*) The number of $P2Y_{12}R^+$ OX-42^+ cells and $P2Y_{12}R^-$ OX-42^+ cells in the dorsal horn of rats (vehicle: $n = 3$; IFN-γ–treated: $n = 4$, **, $P < 0.01$ vs. vehicle). Data are mean ± SEM.