DOCK8 is expressed in microglia, and it regulates microglial activity during neurodegeneration in murine disease models

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Dedicator of cytokinesis 8 (DOCK8) is a guanine nucleotide exchange factor whose loss of function results in immunodeficiency, but its role in the central nervous system (CNS) has been unclear. Microglia are the resident immune cells of the CNS and are implicated in the pathogenesis of various neurodegenerative diseases, including multiple sclerosis (MS) and glaucoma, which affects the visual system. However, the exact roles of microglia in these diseases remain unknown. Herein, we report that DOCK8 is expressed in microglia but not in neurons or astrocytes and that its expression is increased during neuroinflammation. To define the role of DOCK8 in microglial activity, we focused on the retina, a tissue devoid of infiltrating T cells. The retina is divided into distinct layers, and in a disease model of MS/optic neuritis, DOCK8-deficient mice exhibited a clear reduction in microglial migration through these layers. Moreover, neuroinflammation severity, indicated by clinical scores, visual function, and retinal ganglion cell (RGC) death, was reduced in the DOCK8-deficient mice. Furthermore, using a glaucoma disease model, we observed impaired microglial phagocytosis of RGCs in DOCK8-deficient mice. Our data demonstrate that DOCK8 is expressed in microglia and regulates microglial activity in disease states. These findings contribute to a better understanding of the molecular pathways involved in microglial activation and implicate a role of DOCK8 in several neurological diseases.

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found in the brain of MS patients (35–37). Interestingly, minocycline, a tetracycline antibiotic that prevents microglial activation, is in clinical trials for treatment of MS and producing promising results (38), further supporting the role of microglia in MS. As mentioned above, a role of DOCK8 in myeloid and lymphoid cells has been reported; however, a role of microglial DOCK8 in neuroinflammation is unknown.

Microglial activation is observed in various retinal degenerative diseases, including glaucoma (39). Glaucoma is a neurodegenerative disease of the eye, and it is one of the major causes of blindness worldwide. The pathology of glaucoma is characterized by progressive degeneration of the optic nerve and retinal ganglion cells (RGCs), which are found in the retina. The optic nerve injury (ONI) model is an experimentally induced model of glaucoma and is a good model for investigating microglial responses as their activation is clearly demonstrated (40–43). Also, this model is particularly useful for studying a role of microglial DOCK8 in disease as T cells do not infiltrate into the retina, allowing the examination of the role of DOCK8 in microglia independently of DOCK8 in T cells, unlike in the EAE model. Currently, information on microglial dynamics in the retina is limited due to a lack of tools for visualizing and capturing morphological and functional changes of these cells in diseased states. Therefore, a new method to visualize changes in microglial morphology would be desired to further understand the role of microglia in disease states.

In this study, we examined whether DOCK8 is expressed in neural cells and determined whether microglial DOCK8 plays a role in neurodegenerative diseases using mouse MS and glaucoma models. We focused on the retina, a tissue that is structured in distinct layers, allowing monitoring of migration of microglia easily and where the pathology is not associated with infiltrating T cells. We visualized morphology of microglia in reconstructed 3D images to demonstrate microglial activation during healthy and disease states.

Results

DOCK8 expression is increased in microglia during neuroinflammation

First, we examined the interaction of DOCK8 with Cdc42, Rac1, and RhoA. Immunoblot analysis revealed that DOCK8 binds to Cdc42 but not to Rac1 or RhoA (Fig. 1A). Consistently, DOCK8 possessed GEF activity for Cdc42 but not for Rac1 or RhoA (Fig. 1B). These data suggest that DOCK8 selectively activates Cdc42.

Cdc42 plays a role in the migration of inflammatory cells (44), and therefore, we investigated whether DOCK8 is involved in neuroinflammation. For this, we first assessed DOCK8 expression in various cell types in the CNS, including neurons, astroglia, retinal Müller glia, and microglia. Immunoblotting analyses revealed that DOCK8 expression was found in microglia but not in any other cell types we tested (Fig. 1C). Furthermore, immunocytochemical analysis revealed that DOCK8 was expressed diffusely in the cytoplasm of microglia (Fig. 1D). Because DOCK8 plays important roles in immune responses (10–12, 17), we investigated whether inflammatory mediators affect DOCK8 expression levels in microglia. We
found that DOCK8 expression levels in primary cultured microglia were drastically up-regulated by stimulation with lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) (Fig. 1E). To confirm whether this is also true in vivo, we investigated DOCK8 expression levels in the spinal cord and optic nerve in EAE mice. Western blotting analyses demonstrated that DOCK8 expression levels were significantly increased in both the spinal cord and optic nerve of EAE mice compared with those of normal mice (Fig. 2, A and B). Double-immunohistochemical staining revealed that DOCK8 expression was strongly increased in the EAE spinal cord and colocalized with Iba1 (Fig. 2, C and D) and TREM2 (Fig. S1), a transmembrane glycoprotein that has been shown to be linked to DOCK8 by gene network analysis in Alzheimer’s disease brain (45). Together, these results suggest that DOCK8 expression in microglia is increased during neuroinflammation.

We also examined whether DOCK8 expression is increased in human MS. Immunohistochemical analysis of the occipital lobe and medulla oblongata revealed that DOCK8-immunopositive cells were drastically increased in the lesion site compared with the nonlesion site of human MS tissues (Figs. 2E and S2). Moreover, some DOCK8-positive cells in the lesion site appeared with microglia-like morphology (Fig. 2E, arrows). These results demonstrated that DOCK8 expression is increased during neuroinflammation in both mice and humans.

**DOCK8 is critical for activation of microglia in EAE**

To investigate a role of microglial DOCK8 during neuroinflammation, we generated DOCK8-deficient (DOCK8−/−) mice. Primary cultured microglia from DOCK8−/− mice showed cell morphology similar to that from WT mice (Fig. 3A), and expression levels of actin-regulating proteins were not

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**Figure 2. DOCK8 expression is up-regulated in mouse and human neuroinflammation.** A and B, immunoblot analysis of DOCK8 in the spinal cord (A) and optic nerve (B) of WT EAE mice. The DOCK8 expression is increased in the EAE spinal cord and optic nerve. Error bars represent S.E. (n = 4 mice for normal and n = 5 mice for EAE in A; n = 4 mice for normal and EAE in B) and data were analyzed by a Mann–Whitney U test. C and D, double immunostaining of the white matter of the spinal cord (L1–L3) (C) and optic nerve (D) of WT EAE mice using anti-Iba1 (red) and anti-DOCK8 (green) antibodies. Arrowheads indicate increased expression of DOCK8 in microglia in the optic nerve of WT EAE mice. E, immunostaining of the paraffin sections of the occipital lobe and medulla oblongata from a patient with MS using an anti-DOCK8 antibody; “normal” indicates an area from the MS patient without a lesion. In the lesion site, DOCK8-positive cells were detected, and some of them seemed to show microglia-like morphology (arrows). Scale bars, 100 μm (C), and 50 μm (D and E).
altered by a DOCK8 deficiency (Fig. 3B). We induced EAE in WT and DOCK8−/− mice and found that DOCK8 deficiency ameliorated EAE symptoms (Fig. 3C). Histopathological analysis with luxol fast blue and hematoxylin and eosin staining demonstrated that demyelination and activation of astrocytes were reduced in the spinal cord of DOCK8−/− EAE mice (Fig. S3).
Moreover, an increased number of microglial cells are detected in WT EAE mice but not in DOCK8<sup>−/−</sup> EAE mice (Fig. 3D). To assess changes in microglial morphology in vivo in detail, we developed a simple method to create a 3D image reconstruction of tissues to allow visualization of a 3D image of microglia using simultaneous application of the tissue clearing technique and immunostaining. This method captured the differences in microglial morphology among various tissues (retina, optic nerve, and spinal cord) and among different layers (the inner plexiform layer (IPL) and the outer plexiform layer (OPL)) within the retina (Fig. S4). EAE-induced changes in the microglial number were clearly demonstrated by the 3D image reconstruction of the spinal cord (Fig. 3E). The number of microglia in the spinal cord and optic nerve of WT EAE mice was increased by more than 3–4 times compared with that of normal WT mice, whereas the number of microglia in DOCK8<sup>−/−</sup> EAE mice was similar to that of normal WT mice (Fig. 3F).

Immunostaining with a Ki67 antibody revealed that microglial cell proliferation was increased in the spinal cord of WT EAE mice but not in DOCK8<sup>−/−</sup> EAE mice (Fig. S5), whereas in the retina microglial cell proliferation was not detectable in WT EAE mice (Fig. S6). In addition, many microglia with de-ramified morphology were found in both the spinal cord and optic nerve from WT EAE mice but not from DOCK8<sup>−/−</sup> EAE mice (Fig. 3, E and F, and Movies S1 and S2). Taken together, these results demonstrate that DOCK8 deficiency suppresses microglial activation and decreases the number of microglia in the spinal cord and optic nerve during EAE.

**Role of microglial DOCK8 in retinal degeneration in EAE**

Our observations so far suggest that DOCK8 deficiency reduces microglial activation and ameliorates EAE severity. However, considering that in mice DOCK8 deficiency and DOCK8 mutation with abolished GEF activity for Cdc42 activation reduce T-cell infiltration into the CNS and affect T-cell function (Fig. S7) (46), microglial activity might be suppressed by transient treatment with CyD induced relocation of DOCK8 from the cytoplasm to the cell periphery where filopodia formation was detected (Fig. 5A, arrows). In addition, the number of cells with filopodia, after transient CyD treatment, was reduced in DOCK8<sup>−/−</sup> microglia compared with WT microglia (Fig. 5B), suggesting that DOCK8 is required for actin assembly in microglia. We next investigated the effects of DOCK8 on microglial cell migration. Using a Boyden chamber assay, we measured the chemotactic response of microglia migration from the control medium (top chamber) toward 10 μM adenosine triphosphate (ATP; bottom chamber). The motility of WT microglia was increased by ATP attraction, but this increase was significantly lower in DOCK8<sup>−/−</sup> microglia (Fig. 5C). Altogether, these results indicate that DOCK8 promotes microglial migration via the Cdc42-mediated actin assembly.

**Role of DOCK8 in phagocytosis of microglia**

To further confirm the role of microglial DOCK8, we used another model, ONI, an acute model of glaucoma. This model simulates the degeneration of RGCs that occurs in glaucoma, and T cells are not found in the retina (Fig. S8), which gives us an advantage to investigate a role of microglial DOCK8 without the effect of DOCK8 in T cells. Furthermore, microglial activity does not appear to play a role in RGC death in this model (Fig. 6A) (49, 50); this provides a good opportunity to study a role of DOCK8 in phagocytic activity because the level of any signals from dying or dead RGCs is considered to be similar in WT and DOCK8<sup>−/−</sup> mice. A 3D image reconstruction of the retina revealed that the number of microglia after ONI was strongly decreased in the GCL (red), IPL (green), and OPL (blue) in both WT and DOCK8<sup>−/−</sup> mice (Fig. 6, B and C). In WT mice, some dying RGCs were fully wrapped by microglia in the GCL (Fig. 7A). This observation was confirmed by the transverse sequential section images (Fig. 7B) and a 3D image reconstruction (Movie S3) of such cells. The number of phagocytic microglia was reduced in DOCK8<sup>−/−</sup> mice compared with that in WT mice (Fig. 7C). However, these data are not enough to demonstrate whether there is a difference in phagocytic activity between WT and DOCK8<sup>−/−</sup> microglia. To examine this point in more detail, we used a dye that fluoresces at an acidic pH such as in phagosomes in vitro. We found that phagocytic activity is significantly reduced in DOCK8<sup>−/−</sup> microglia compared with WT microglia (Fig. 7D).

**Discussion**

In this study, we report that DOCK8 is expressed in microglia and is involved in microglial migration and phagocytosis, at least in vitro. To the best of our knowledge, this is the first study to show that DOCK8 is expressed in microglia and affects their...
activity and to reconstruct 3D images of retinal microglia. The clearing technique we used to reconstruct 3D images of microglia is particularly useful for laminar tissues such as the retina to facilitate the visualization of immunostained cells on different focal plans of the tissue. We demonstrated clear morphological differences in ramified resting microglia in the retina, optic nerve, and spinal cord and in different layers of the retina (Fig. S4, C and D). These differences may simply reflect the available space within the layers, and future studies will include how these shapes affect activities of microglia in disease states.

Microglia play important roles in neuroinflammation, and its migration to the lesion site is necessary for the propagation of neuroinflammation. One of the key events for microglial migration is rearrangement of the actin cytoskeleton by Rho GTPases. Rho GTPases have diverse functions to regulate a variety of cellular processes, including morphogenesis, migration, neuronal development, and cell division (3, 44, 51–53). In particular, Cdc42 plays a crucial role in directing cell migration and chemotaxis (54–56). Our findings indicate that DOCK8 specifically activates Cdc42, and the lack of DOCK8 impairs cell

Figure 4. DOCK8 deficiency preserves visual function and reduces microglial migration in the retinas of EAE mice. A, representative images of FG-labeled RGCs in WT and DOCK8−/− EAE mice. FG-labeled RGCs were counted. RGC survival is greatly increased in DOCK8−/− EAE mice. Error bars represent S.E. (n = 4 eyes). B, visual responses in WT and DOCK8−/− EAE mice by measuring multifocal electoretinogram. The visual responses are better preserved in DOCK8−/− EAE mice compared with WT EAE mice. Error bars represent S.E. (n = 6 eyes for WT normal; n = 8 eyes for DOCK8−/− normal; n = 7 eyes for WT EAE; n = 11 eyes for DOCK8−/− EAE). C, layer-specific microglia distribution in the EAE mouse retina. Microglia were detected by immunostaining of Iba1 in the transparent whole retina. The GCL (red), IPL (green), OPL (blue), and stack images (three layers) of retinal microglia are shown. The Iba1-positive cells are detected in the GCL of WT EAE mice but not in DOCK8−/− EAE mice. Scale bar, 100 μm. D, quantitative analysis of Iba1-positive cells in the GCL, IPL, and OPL. The data suggest that migration from the IPL to the GCL is inhibited in DOCK8−/− EAE mice. Error bars represent S.E. (n = 6 eyes for WT normal; n = 8 eyes for DOCK8−/− normal; n = 5 eyes for WT and DOCK8−/− EAE) and data were analyzed by a Mann–Whitney U test. Scale bars, 100 μm.
In our study, we used potential in some CNS disorders, including MS and glaucoma. Findings from this study alone cannot determine how microglial DOCK8 affects survival or death of retinal neurons (69–71). Future studies will include generation and use of microglia-specific DOCK8−/− mice in disease models to explore the therapeutic potential of microglial DOCK8 in CNS disorders.

**Experimental procedures**

**Experimental animals**

DOCK8−/− mice were generated by homologous recombination in C57BL/6 mouse embryonic stem cells. The animals were treated in accordance with the Tokyo Metropolitan Institute of Medical Science guidelines for the care and use of animals, and all animal experiments were approved by the Institutional Animal Care and Use Committee of the Tokyo Metropolitan Institute of Medical Science.

**Preparation of primary cultured cells**

Primary cultured cortical neurons (75, 76), astrocytes (48), microglia, and Müller glia (69, 72) were prepared as described previously. For induction of DOCK8 expression, cultured microglia were stimulated with LPS (10 ng/ml; EMD Millipore, Billerica, MA) or TNFα (50 ng/ml; PeproTech, Rocky Hill, NJ) for 24 h. These cells were directly lysed with a sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 15% glycerol) and subjected to immunoblotting. For analysis of filopodia formation, cultured microglia were stimulated with 10 μM CyD (EMD Millipore) for 15 min and then washed and cultured in normal culture medium for an additional 15 min. After fixation with 3.7% formaldehyde for 20 min, cells were stained with rhodamine-labeled phalloidin (1:1000; Cytoskeleton, Denver, CO) for visualizing actin filaments.

**Immunostaining of cultured microglia**

Primary cultured microglia were fixed with 3.7% formaldehyde for 20 min and stained with 0.03% crystal violet (Fujifilm, Tokyo, Japan) or anti-DOCK8 antibody (1:1000; EMD Millipore). Alexa Fluor–conjugated donkey anti-rabbit IgG (1:100; Thermo Fisher Scientific, Waltham, MA) was used as a secondary antibody. For visualizing F-actins, cells were stained with rhodamine-labeled phalloidin (1:1000). Cells with or without filopodia were counted manually in two randomly chosen areas of 0.05 mm² per well for a total of 4 wells. Quantitative analysis of the signal bands was carried out using NIH ImageJ software 1.46r.

**Immunoblot analysis**

Immunoblotting was carried out as reported previously (75). The optic nerve and spinal cord were freshly isolated from WT and DOCK8−/− mice and then homogenized in cold PBS. Total
homogenates of the optic nerve and spinal cord or total cell lysates of primary cultured cells were heated at 100 °C for 5 min. Proteins were separated by SDS-PAGE and transferred to an Immobilon-P filter (EMD Millipore). Membranes were incubated with an antibody against DOCK8 (1:1000), Iba1 (1:1000; Abcam, Cambridge, UK), Cdc42 (1:1000; Cell Signaling Technology, Danvers, MA), Rac1 (1:1000; BD Biosciences), RhoA (1:1000; BD Biosciences), Wiskott–Aldrich syndrome protein (WASP) (1:1000; Epitomics, Burlingame, CA), WASP-family verprolin-homologous protein (WAVE) (1:1000; BD Biosciences), and actin (1:1000; BD Biosciences) in PBS containing 0.05% Tween 20 (PBS-T) and 2.5% skimmed milk. Membranes were washed in PBS-T and incubated with horseradish peroxidase–conjugated secondary antibodies against mouse IgG (1:1000; Cell Signaling Technology), rabbit IgG (1:1000; Cell Signaling Technology), or goat IgG (1:1000; Santa Cruz Biotechnology). Labeled proteins were detected using ChemiLumi One Ultra (Nakalai Tesque, Kyoto, Japan). Quantitative analysis of the signal bands was carried out using NIH ImageJ software 1.46r.

**Binding assay for small G proteins**

GST-Cdc42, GST-Rac1, and GST-RhoA (84) were purified from bacterial lysates using GSH-agarose (GSH-Sepharose 4B, Thermo Fisher Scientific). After 24 h of transfection with pCMV-full-length DOCK8, COS-7 cells were washed twice with PBS and lysed with an EDTA buffer (1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA containing a protease inhibitor mixture (Roche Applied Science)) and centrifuged. The supernatants were incubated with agaroase bead–associated GST fusion proteins for 1 h at 4 °C and washed four times. DOCK8 bound to small GTPase proteins was resolved by

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**Figure 6.** DOCK8 deficiency reduces microglial migration in the retina following ONI. A, representative images of FG-labeled RGCs in WT and DOCK8−/− mice after ONI. FG-labeled RGCs were counted. There was no difference in RGC survival between WT and DOCK8−/− mice following ONI. Error bars represent S.E. (n = 3 eyes). B, layer-specific distribution of microglia in mouse retinas 5 days after ONI. Microglia were detected by immunostaining of Iba1 in the transparent whole retina. GCL (red), IPL (green), OPL (blue), and stack images (three layers) of retinal microglia are shown. C, quantitative analysis of Iba1-positive cells in the GCL, IPL, and OPL. ONI up-regulates the number of Iba1-positive cells in all retinal layers, but the extent of increase in the GCL is smaller in DOCK8−/− mice compared with WT mice. Error bars represent S.E. (n = 6 eyes) and data were analyzed by a Mann–Whitney U test. Scale bars, 100 μm (A) and 50 μm (B).
SDS-PAGE and assessed by immunoblotting with an anti-DOCK8 antibody (1:1000).

**Measurement of the activity of Rho-family proteins**

The activities of Cdc42, Rac1, and RhoA were measured as described previously (75). GST-CRIB and GST-Rhotekin (84) were purified from bacterial lysates using GSH-agarose (Thermo Fisher Scientific). After 24 h of transfection, COS-7 cells were washed twice with PBS and lysed with a lysis buffer (1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 10 mM MgCl₂ containing protease inhibitor mixture) and then centrifuged. The resulting supernatants were incubated with agarose bead–associated GST fusion proteins for 45 min at 4 °C. The beads were washed four times with lysis buffer, the bound small GTPase proteins were subjected to SDS-PAGE, and immunoblot analysis was performed with anti-Cdc42, -Rac1, and -RhoA antibodies.

**Chemotaxis assay**

Migration assays were performed using Boyden-type 96-well plates (ChemoTx system, Neuroprobe, Gaithersburg, MD). Microglia were suspended in Dulbecco’s modified Eagle’s minimal essential medium without serum. The same medium containing ATP (50 ng/ml; BioVision, Mountain View, CA) was used to fill each well. Polycarbonate filters with 8-µm pores were placed in contact with the liquid, and cells were dispensed over each well. Cells were seeded on polycarbonate filters and incubated at 37 °C (5% CO₂) for 3 h. The cells that remained on the top surface of the filter were wiped with a Kimwipe, and cells under the filter (migrated cells) were fixed with 3.7% formaldehyde for 10 min. The filter was then stained with 0.03% crystal violet, and individual fields were counted.

**Phagocytosis assay**

The phagocytic activity was measured using pHrodo™ Green Zymosan Bioparticles™ (Life Technologies) according to the manufacturer’s instructions. pHrodo Green conjugates are nonfluorescent outside the cell at a neutral pH but fluoresce brightly green at an acidic pH such as in phagosomes. In brief, microglia were cultured in Dulbecco’s modified Eagle’s minimal essential medium containing 10% fetal bovine serum and 0.5 mg/ml fluorescently labeled zymosan particles for 20, 60, and 120 min. After fixation, images were obtained with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Fluorescence intensity was determined using NIH ImageJ software 1.46r.

**Histological analyses**

Frozen (10-µm-thick) or paraffin (7-µm-thick) tissue sections of the retina, optic nerve, or spinal cord were examined with immunohistochemical analysis using anti-Iba1 (1:1000), anti-DOCK8 (1:1000), anti-CD3 (1:1000; Santa Cruz Biotechnology), anti-TREM2 (1:250; Abcam), anti-Ki67 (1:250; Abcam), or anti-glial fibrillary acidic protein (1:1000; Cell Signaling Technology) antibodies. Alexa Fluor–conjugated donkey anti-goat IgG, anti-rabbit IgG, and goat anti-rat IgG (1:1000) were used as secondary antibodies. For detection of myelin in spinal cord, tissues were embedded in paraffin wax, sectioned at a thickness of 7 µm, and stained with luxol fast blue followed by hematoxylin and eosin. Stained sections were examined using a microscope (BX51, Olympus, Tokyo, Japan) connected to a DP70 camera (Olympus). Images were processed and viewed using DP Manager software (v2.2.1.195; Olympus). Quantitative analysis of the
stained region was carried out using NIH ImageJ software version 1.46r.

**Preparation of transparent tissues and immunostaining**

Mice were anesthetized by isoflurane and perfused with Zamboni’s fixative (2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer). Tissues (retina, optic nerve, and spinal cord) were removed and postfixed in Zamboni’s fixative for 10 h at 4 °C. The retina and optic nerve as a whole tissue and spinal cord) were removed and postfixed in Zamboni’s fixative (2% paraformaldehyde and 15% picric acid) supplemented with 4% formaldehyde in 0.1 M phosphate buffer. Tissues were embedded in paraffin and sectioned at 8 μm. Sections were deparaffinized with xylene and rehydrated with graded alcohols. Immunostaining was performed using antibodies specific for various cellular and molecular markers. Primary antibodies were incubated overnight at 4 °C, followed by incubation with secondary antibodies conjugated to Fluorescein Isothiocyanate (FITC) or Alexa Fluor 647. Images were captured using a digital camera mounted on a fluorescence microscope.

**Induction and clinical scoring of EAE**

Female WT and DOCK8<sup>+/−</sup> mice were used for the experiments. EAE was induced with the myelin oligodendrocyte glycoprotein (MOG)<sub>35-55</sub> peptide (MEVGWYRSPFSRVVHLYRNGK) at 6–8 weeks of age as reported previously (48, 72, 81). To induce EAE, a total of 200 μg of MOG<sub>35-55</sub> (GenScript, Piscataway, NJ) emulsified in 200 μl of complete Freund’s adjuvant (Thermo Fisher Scientific) supplemented with 4 μg/ml Mycobacterium tuberculosis was injected subcutaneously into the lower flanks followed by an intraperitoneal injection of 106 viable Mycobacterium tuberculosis. Clinical signs of EAE were scored daily as follows: 0, no clinical signs; 1, loss of tail tone; 2, flaccid tail; 3, impairment of righting reflex; 4, partial hind limb paralysis; 5, complete hind limb paralysis; 6, partial body paralysis; 7, partial forelimb paralysis; 8, complete forelimb paralysis or moribund; 9, death.

**Flow cytometry**

Cells were treated with anti-FcγRII/III (BioLegend, San Diego, CA) to block unspecific binding to Fc receptors and thereafter labeled with fluorescent antibodies for flow cytometric acquisition using a FACScalibur flow cytometer. Data analysis was performed by FlowJo software (Treestar, Ashland, OR). The following antibodies were used for surface staining of splenocytes: Alexa Fluor 488–conjugated anti-CD3 (BioLegend), phycoerythrin-conjugated anti-CD4 (BioLegend), and Alexa Fluor 647–conjugated anti-CD8a (BioLegend).

**Antigen-specific T-cell proliferation assay**

Bone marrow–derived dendritic cells (BMDCs) were prepared according to previously reported methods with slight modifications (79). Bone marrow was isolated from the femurs and tibias of WT and DOCK8<sup>+/−</sup> mice. Bone marrow cells (5 × 10<sup>5</sup>) were resuspended in 10 ml of dendritic cell (DC) medium (RPMI 1640 medium (Nakalai), 10% fetal calf serum, 10 ng/ml granulocyte/macrophage colony-stimulating factor (Pepro-Tech)) and cultured in 10-cm culture dishes at 37 °C. Three days after cell culture, 10 ml of DC medium was added to the culture. Six days after cell culture, 10 ml of the culture was removed and centrifuged, and the cell pellet was resuspended in 10 ml of fresh DC medium and added back to the culture. Nine days after cell culture, BMDCs were harvested, pulsed for 2 h with ovalbumin (100 μg/ml; EMD Millipore), and washed prior to use in in vitro proliferation assays. Additionally, splenocytes of OT-II transgenic mice (80) were isolated and stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; final concentration, 0.5 μM; Dojindo, Kumamoto, Japan). CFSE-labeled OT-II splenocytes (4 × 10<sup>6</sup>) were cocultured with ovalbumin-pulsed BMDCs (1 × 10<sup>5</sup>) in 96-well round-bottom plates and incubated for 3 days at 37 °C. After culture, the cells were stained with an anti-CD4 mAb (1:1500; BioLegend) and analyzed by flow cytometry to detect CFSE dilution of gated CD4<sup>+</sup> OT-II T cells.

**Analysis of complete blood cells**

Peripheral blood was obtained by tail bleeding and transferred into EDTA-coated tubes. The complete blood cell analysis was performed using a Sysmex K-4500 multispecies whole-blood analyzer (Sysmex, Kobe, Japan).

**Multifocal electroretinograms**

Mice were anesthetized by intraperitoneal injection of 87.5 mg/kg sodium pentobarbital. The pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. Multifocal electroretinograms were recorded using a VERIS 6.0 system (Electro-Diagnostic Imaging, Redwood City, CA). The visual stimulus consisted of seven hexagonal areas scaled with eccentricity. The stimulus array was displayed on a high-resolution black and white monitor driven at a frame rate of 100 Hz. The second-order kernel, which is impaired in optic neuritis and optic neuropathy, was analyzed (48, 72, 81).

**Retrograde RGC labeling**

Mice were deeply anesthetized with isoflurane and then placed on a stereotaxic frame before receiving an injection of 2 μl of 1% Fluoro-Gold (FG; Fluorochrome LLC, Denver, CO) dissolved in PBS into the superior colliculus (82, 83). Ten days after FG application, mice were anesthetized, eyes were enucleated, and retinas were isolated for whole-mount preparation. Retinas were fixed in 4% paraformaldehyde in 0.1 M PBS solution for 20 min and mounted on a glass slide with a mounting medium (Vectashield, Vector Laboratories, Burlingame, CA), and RGC density was examined with a fluorescence microscope. The excitation and emission wavelengths for FG were 323 and 620 nm, respectively. Four standard areas (0.1 mm<sup>2</sup>) of each retina at a point 0.5 mm from the optic disc were chosen. FG-labeled cells were manually counted, and the mean number of RGCs per mm<sup>2</sup> was calculated.

**ONI**

Mice were anesthetized with isoflurane before ONI. Optic nerves were exposed intraorbitally and crushed about 0.5-1.0
mm from the posterior pole of the eyeball with fine surgical forces for 5 s (75). Five days after ONI, eyes were enucleated, and isolated retinas were processed for tissue clearing and immunostaining. In some experiments, retrograde RGC labeling was carried out 10 days before ONI.

Immunostaining of human brain

Human samples were handled according to the regulations issued by the Ethics Committee of the Tokyo Metropolitan Institute of Medical Science. This study was conducted in accordance with Declaration of Helsinki principles. CNS tissues were obtained at autopsy from a 54-year-old male (Patient 1), 40-year-old female (Patient 2), and 56-year-old male (Patient 3) whose disease status was relapsing–remitting MS. The tissue was fixed with 20% buffered formalin and embedded in paraffin wax. For immunohistochemistry, 10-μm paraffin sections were immunostained using a rabbit polyclonal antibody against DOCK8 (1:1000). Before antibody incubation, sections were heated using a microwave in citrate-buffered saline (pH 6.0) for 15 min to unmask antigens. Antibody binding was visualized using a labeled streptavidin–biotin immunoperoxidase method.

Statistical analyses

Data are presented as means ± S.E. Data were analyzed by a two-tailed unpaired Student’s t test or Mann–Whitney U test as a nonparametric test, and the p values were not corrected for multiple comparisons. A value of p < 0.05 was regarded as statistically significant. JMP, version 8.0.1 (SAS Institute, Cary, NC) was used for the statistical analyses.

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