human neogenin-specific antibody. Alexa Fluor 488-conjugated goat anti-rabbit IgG and 568-conjugated goat anti-mouse IgG (Invitrogen) were used as the secondary antibodies. We determined the fluorescence intensities for neogenin within the contour of each cell, and determined and subtracted the background fluorescence intensity. We determined the average intensities in 50–100 cells from each sample and normalized the relative intensities by using those of the controls.

EAE scoring. We assessed clinical signs of EAE on the basis of the following scale: 0, no abnormalities noted; 0.5, loss of tail tonicity; 1, loss of tail reflex; 2, loss of tail reflex, impaired righting, and paresis of one limb; 3, paresis and paralysis of one limb; 3.5, full hind limb paralysis; 4, front and hind limb paralysis; and 5, moribund or dead. We assigned intermediate scores if the neurological signs were intermediate between two scores. We obtained the mean cumulative score by averaging the total clinical scores of each mouse with EAE after immunization.

Histopathology. In the histological evaluation, we stained PFA-fixed, paraffin-embedded sections of the spinal cord with hematoxylin and eosin to assess inflammation. We examined 20–30 transverse sections from the cervical to thoracic spinal cord per mouse. We scored inflammation (inflammatory index) as follows: 0, no inflammation; 1, cellular infiltration only in the perivascular areas and meninges; 2, mild cellular infiltration in the parenchyma; 3, moderate cellular infiltration in the parenchyma; and 4, severe cellular infiltration in parenchyma[26,27].

Knockdown experiments with siRNA. We synthesized mouse RGMa siRNA (stealth siRNA,

Invitrogen) and mouse neogenin siRNA (Sigma-Genosys). The sense and antisense strands of RGMa siRNA were 5′-AAAGAGGCCGCAGUGAGUGUAGUUG-3′ and 5′-CAACUACACUCACUGCGGCCUCUUU-3′, respectively, and those of neogenin siRNA were 5′-CAAUUCCAUGGAUAGCAAU-3′ and 5′-AUUGCUAUCCAUGGAAUUG-3′, respectively. We transfected RGM siRNA, neogenin siRNA, and nontargeting double-stranded RNA (control mismatch siRNA; Invitrogen) into BMDCs and BMMØs by using Nucleofector (Amaxa, Inc.) according to the manufacturer's instructions. We estimated the transfection efficiency by using BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) at ~50%. We collected the BMDCs and BMMØs on d 7 and washed them twice in PBS. Subsequently, we suspended 1×10^6 cells in 100 μl of nucleofection solution for mouse dendritic cells or macrophages (Amaxa, Inc.) containing 500 pmol of mouse RGMa siRNA, neogenin siRNA, or control siRNA. We transferred the samples into the certified cuvettes and placed them in the Nucleofector device. We accomplished nucleofection of the cells by using the AN-001 program (BMDCs) and Y-001 program (BMMØs). After the transfection, we cultured the cells in complete medium containing RPMI-1640 medium supplemented with glutamine, sodium pyruvate, penicillin, streptomycin, 2-ME, and 10% heat-inactivated fetal bovine serum (FBS).

GFP-labeled cell transfer and trafficking analysis. We performed cell transfer experiments by intravenous injection of 5×10^6 $CD4^+$ T cells derived from MOG-induced EAE C57BL/6-Tg (CAG-EGFP) mice into recipient wild-type mice. We immunized CAG-EGFP mice with MOG, and isolated splenocytes from them on d 7 after immunization, followed by re-stimulation with MOG for 3 d. We treated recipient mice with control or RGMa-specific antibody 3 d before and at the transfer of the restimulated $CD4^+$ T cells. Ten d after adoptive transfer, we transcardially perfused the mice with 4% paraformaldehyde in PBS. We dissected

the brain and cervical spinal cord, fixed them in the same fixatives overnight at 4 °C, and immersed them in 30% sucrose in PBS. Serial sections (20-μm thick) were cut using a cryostat and mounted on MAS-coated slides. We immunostained these sections with a rabbit GFP-specific antibody (Invitrogen) to detect EGFP-labeled cells. We counted the number of the GFP^+ T cells in sections by a standardized protocol for estimating cell density, which involved counting the number of GFP^+ T cells in the forebrain including the lateral ventricle, and the cervical spinal cord. Sections (5–10) with an individual distance of 200 μm were examined in the brain and spinal cord of the normal mice (n = 5) and RGMa-specific antibody-treated mice (n = 5).

Migration assay. We used murine brain-derived capillary endothelial cell line b-End3 (American Type Culture Collection) for this experiment. The culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 2% penicillin plus streptomycin and 10% FBS. The cells were grown to confluence in the upper chamber of a 3-μm-pore 24-well transwell insert (Millipore), shown to induce and maintain blood–brain barrier characteristics *in vitro*. We evaluated barrier function in the cell monolayer by transepithelial electrical resistance measurements performed with Millicell-ERS. The resistance of blank filters as the background resistance was subtracted from the total resistance of each culture insert. At d 11 after EAE induction, we prepared $CD4^+$ T cells from MOG-EAE mice treated with control IgG or RGMa-specific antibody on d 7 and 10 after MOG immunization. We loaded a suspension of 1×10^6 $CD4^+$ T cells ml^{-1} in the upper chamber, in the presence of 10 μg ml^{-1} of RGMa-specific antibody or control antibody. We assessed the ability of $CD4^+$ T cells to cross the monolayer by counting the number of cells that transmigrated to the lower chamber after 18 h.

Adoptive transfer experiments. For the adoptive transfer of macrophages with neogenin knockdown, we injected CD11b-DTR mice intravenously with 5×10^6 live BMMØs with or without neogenin knockdown. Because CD11b is positive for certain dendritic cells, we injected mice simultaneously with 5×10^6 BMDCs. We then immunized the mice with MOG, injected subcutaneously with 0.2 ml CFA containing 500 μg of *Mycobacterium tuberculosis*. Recipient mice received 200 ng of pertussis toxin intravenously 48 h after the MOG immunization. For ablation of resident macrophages, we injected the CD11b-DTR mice intraperitoneally with 25 ng g^{-1} diphtheria toxin (Sigma) on d –1 and 3 after the MOG immunization.

Intrathecal administration of RGMa-specific antibody in EAE mice. On d 7 after the MOG-EAE induction, we fitted the mice with an osmotic minipump (100 μl solution, 0.5 μl h^{-1}, 7-d delivery; Alzet pump model 1007D) filled with control rabbit IgG (22.3 μg kg^{-1} d^{-1} over 1 week; Sigma-Aldrich) or RGMa-specific antibody (22.3 μg kg^{-1} d^{-1} over 1 week). This antibody dose has been shown to be effective in inhibiting the effect of RGMa in the CNS[10]. We placed the minipump under the skin of the animal's back and connected a Silastic tube to the minipump under the dura of the thoracic cord. We sutured the tube just caudal to the laminectomy site to anchor it in place. Afterward, we sutured the muscle and skin layers.

Animal model of spinal cord injury and behavioral analysis. We anesthetized female C57BL/6 mice with sodium pentobarbital (40 mg kg^{-1}) and performed laminectomy at the T9–T10 vertebral level. The mice then received a moderate (60 kdyn) contusion injury with an Infinite Horizons impactor. We intraperitoneally administered 400 μg of RGMa-specific or control antibody (rabbit IgG; Sigma-Aldrich) to the mice on d 0 and 3 after the injury. To assess their functional recovery after the injury, two blinded observers tested the mice by

using the open-field Basso Mouse Scale (BMS)[28] on d 1, 3, and 5, and once a week thereafter until the end of the study. We plotted the mean scores per treatment group as a function of the time post-injury. We also performed manual bladder expression twice a day until reflex bladder emptying was established. We had assessed the animals before the injury to ensure the absence of deficits in hind limb function and expose them to an open-field testing environment.

PBMC isolation and stimulation. We isolated all PBMCs freshly by using standard Ficoll-Hypaque centrifugation within 24 h of venipuncture and immediately applied them to the following assays. We pretreated PBMCs with 10 μg ml^{-1} of mouse monoclonal human RGMa-specific antibody, which we generated, or with control IgG for 30 min and then stimulated them with PMA (10 ng ml^{-1}) and ionomycin (1 μg ml^{-1}) (both from Sigma-Aldrich) in RPMI-1640 medium supplemented with glutamine, sodium pyruvate, penicillin, streptomycin, 2-ME, and 10% heat-inactivated FBS. We estimated the cell proliferation by measuring the BrdU incorporation and estimated the production of *IL-2*, *IFN-γ*, *IL-17*, *IL-4*, *IL-10*, and *TGF-β* by RT-PCR analysis.

Biodistribution of RGMa-specific antibody. We administered RGMa-specific antibody (400 μg intraperitoneally) to mice on d 0 and 2. We anesthetized the mice and removed their spleen, lymph node, spinal cord, and brain tissues on d 7 for biodistribution analysis of RGMa-specific antibody. We lysed the tissues and subjected them to Western blotting with horseradish peroxidase-conjugated rabbit IgG antibody (Cell Signaling Technology). We also administered RGMa-specific antibody by intraperitoneal injection to the mice on d 7 and 10 after the MOG immunization and assessed the biodistribution on d 11.

Intracellular staining. We analyzed intracellular cytokine expressions in freshly isolated splenocytes stimulated with CD3-specific antibody in the presence of 10 μg ml^{-1} Brefeldin A (Sigma-Aldrich) for 6 h. After staining the cells with a phycoerythrin-conjugated CD4-specific antibody (BD Biosciences), we fixed and permeabilized (Cytofix/Cytoperm and Perm/Wash buffer; BD Biosciences) and stained them with FITC-conjugated IL-4- or IL-10-specific (BD Biosciences) antibody, and then analyzed the results fluorocytometrically (FACSCalibur; BD Biosciences).

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