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Roles of Sema4D–Plexin-B1 Interactions in the Central Nervous System for Pathogenesis of Experimental Autoimmune Encephalomyelitis

Tatsusada Okuno,*†‡ Yuji Nakatsuji,‡ Masayuki Moriya,‡ Hyota Takamatsu,*† Satoshi Nojima,*† Noriko Takegahara,*† Toshihiko Toyofuku,*† Yukinobu Nakagawa,*† Sujin Kang,*† Roland H. Friedel,§¶ Saburo Sakoda,‡ Hitoshi Kikutani,¶ and Atsushi Kumanogoh*†

Although semaphorins were originally identified as axonal guidance molecules during neuronal development, it is emerging that several semaphorins play crucial roles in various phases of immune responses. Sema4D/CD100, a class IV semaphorin, has been shown to be involved in the nervous and immune systems through its receptors plexin-B1 and CD72, respectively. However, the involvement of Sema4D in neuroinflammation still remains unclear. We found that Sema4D promoted inducible NO synthase expression by primary mouse microglia, the effects of which were abolished in plexin-B1–deficient but not in CD72-deficient microglia. In addition, during the development of experimental autoimmune encephalomyelitis (EAE), which was induced by immunization with myelin oligodendrocyte glycoprotein-derived peptides, we observed that the expression of Sema4D and plexin-B1 was induced in infiltrating mononuclear cells and microglia, respectively. Consistent with these expression profiles, when myelin oligodendrocyte glycoprotein-specific T cells derived from wild-type mice were adoptively transferred into plexin-B1–deficient mice or bone marrow chimera mice with plexin-B1–deficient CNS resident cells, the development of EAE was considerably attenuated. Furthermore, blocking Abs against Sema4D significantly inhibited neuroinflammation during EAE development. Collectively, our findings demonstrate the role of Sema4D–plexin-B1 interactions in the activation of microglia and provide their pathologic significance in neuroinflammation. *The Journal of Immunology, 2010, 184: 1499–1506.

Microglia, resident immune effector cells in the CNS, are thought to play a key role in the regulation of neuroinflammation (1). Although activated microglia are known to exert a beneficial role in host defense and tissue repair in the CNS, it has been suggested that they also participate in propagation of inflammation in the CNS through Ag presentation, production of proinflammatory cytokines or chemokines, and NO (2–4). In fact, the mechanisms of how activation of microglia is regulated have been extensively studied. For example, CD40, a member of the TNF receptor family, has been reported to be involved in microglial activation (5, 6). Interactions of CD40 with its ligand (CD154), which is primarily expressed by activated T cells, promote the activation of microglia in the context of enhanced expression of costimulatory molecules and production of proinflammatory cytokines or chemokines and NO (5). Therefore, CD40–CD40 ligand interactions have been implicated in various neurologic disorders such as multiple sclerosis, Alzheimer’s disease, and Parkinson’s disease (5, 7, 8). However, answers are elusive regarding how the immunoregulatory molecules are involved in neuroinflammation.

Sema4D/CD100 is a transmembrane-type semaphorin belonging to the class IV semaphorin subclass. Although semaphorins were initially identified as axonal guidance cues during neuronal development (9, 10), accumulating evidence now indicates that several semaphorins play a crucial role in physiologic and pathologic immune responses (11). The expression of Sema4D is abundantly observed in T cells, but only weakly detected in naive B cells, macrophages, and dendritic cells (DCs); however, its expression is significantly upregulated on cellular activation (12, 13). Regarding its receptor systems, Sema4D has been shown to use two distinct receptors, plexin-B1 in the nervous system and CD72 in the immune system (11, 14, 15). We previously demonstrated the activation of B cells and DCs through the Sema4D–CD72 interactions (15, 16); Sema4D-deficient mice display severe impairments in activation of B cells and DCs, resulting in impaired Ab production and Ag-specific T cell priming (13, 16). In the nervous system, Sema4D participates in axon guidance by regulating activities of RhoA.
through PDZ-p guanine nucleotide exchange factors and leukemia-associated RhoGEF (17). In addition, plexin-B1 has been shown to mediate repellent signals in hypocampal neurons by directly binding Rnd1 and downregulating R-ras activity in response to Sema4D (18). Collectively, these findings indicate the importance of Sema4D in both nervous and immune systems.

Regarding neuroinflammation, in which the immune system interacts with the nervous system, it has been suggested that semaphorins are pathogenetically significant. Sema7A expressed in T cells regulates inflammation of experimental autoimmune encephalomyelitis (EAE) (19, 20). In addition, it has been reported that Sema4D is relevant to HTLV-1–associated myelopathy (21). The expression of Sema4D was increased in the cerebrospinal fluid and spinal cords of patients with HTLV-1–associated myelopathy, in which T cell–derived Sema4D impaired immature oligodendrocytes (21). In addition, we previously reported that Sema4D–deficient mice were resistant to the development of EAE because of the impaired Ag-specific T cell priming in the draining lymph nodes (16). Although these facts suggest the relevance of Sema4D in neuroinflammatory diseases, it has not been fully elucidated how and to what extent Sema4D is involved in neuroinflammation.

In this study, we demonstrate enhanced activation of microglia through Sema4D–plexin-B1 interactions. In addition, we find that either plexin-B1–deficient mice or bone marrow (BM) chimera mice with CNS–specific plexin-B1 deficiency were resistant to the development of EAE after adoptive transfer of myelin oligodendrocyte glycoprotein (MOG)–specific T cells. We further present that the treatment with an Ab against Sema4D was effective for EAE blocking, including in the effector phase. These findings demonstrate the significance of Sema4D–plexin-B1 interactions in the inflamed CNS and provide a novel therapeutic target for neuroinflammatory diseases.

Materials and Methods

Mice

Sema4D– and plexin-B1–deficient on the C57BL/6 background were generated and maintained as described previously (13, 22, 23). CD72-deficient mice were provided by Dr. Barnes (Stanford University, Stanford, CA) (24). S7BL/6 (CD45.2 and CD45.1) and SJL mice were purchased from Nippon Clea (Hamamatsu, Japan) and Nippon Charles River (Kanagawa, Japan), respectively. All mice used in this study were maintained in a specific pathogen-free environment. All animal experimental procedures were consistent with our institutional guidelines.

Reagents for cell cultures

Agonistic anti-CD40 Ab (HM40-3), and recombinant mouse interferon–γ (IFN-γ) were purchased from BD Biosciences (San Diego, CA), and Genzyme-Techne (Cambridge, MA), respectively. Recombinant Sema4D, consisting of the extracellular region of Sema4D and the Fc portion of human IgG1 (Sema4D–Fc), was made as previously described (16). Human IgG1, p38 MAPK inhibitor SB203580, MEK inhibitor U0126, and JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA).

Cell cultures and immunocytochemistry

Microglial cell line GML-6-3 (6-3 cells) were grown in MEM (Sigma-Aldrich, St Louis, MO) containing 10% FBS, 0.2% glucose, and 5 μg/ml bovine insulin. Primary microglia were prepared as described (8, 25). Mixed cells prepared from cerebrums of newborn mice were cultured in media (10% FBS-DMEM) for 14 d. Next, microglia were detached by shaking, and the detached cells were replated onto a noncoated dish. After 30 min incubation at 37°C, adherent cells were scraped, centrifuged, and replated onto poly-L-lysine–coated 35-mm dishes (for Western blotting, 2 × 10^5 cells/cm²) or eight-well Lab-Tec chamber slides (for immunostaining, 1 × 10^5 cells/cm²; for measurement of nitrite, 5 × 10^5 cells/cm²).

For inducible NO synthase (iNOS), CD72, or plexin-B1 staining, microglia were fixed with 4% paraformaldehyde for 15 min. After blocking with 2% BSA (Sigma-Aldrich) in PBS containing Fc-block (1:20, anti-CD16/32, 2.4G2; BD Biosciences) for 30 min, cells were incubated with rabbit anti-iNOS (1:100; BD Biosciences), mouse anti-CD72 (1:100; BD Biosciences) or mouse anti–plexin-B1 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) Abs at 4°C overnight, followed by staining with FITC-conjugated goat anti-rabbit or mouse IgG Ab (1:300, Cappel, West Chester, PA). For microglial-staining, PE-conjugated rat anti-CD11b Ab (1:100; BD Biosciences) was used. Images were collected using a confocal microscope (Carl Zeiss) equipped with IMARIS software.

Measurement of nitrite

NO production by activated microglia was determined by measuring the amounts of nitrite, a stable oxidation product of NO using Griess reactions in triplicates. An aliquot of the conditioned medium was mixed with an equal volume of 1% sulfanilamide in water and 0.1% N-1-naphthylethenediamine dihydrochloride in 5% phosphoric acid. The absorbance was determined at 550 nm. Statistical significance was analyzed using an unpaired Student's t test, and p ≤ 0.05 was considered significant.

Western blot analysis

Western blot analysis was performed as previously described (26). Cell lysates were lysed with radio-immunoprecipitation assay buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) containing protease inhibitors (20 μg/ml aprotinin, 1 mM phenyl-methylsulfonyl fluoride) and 1 mM sodium orthovanadate. The same amounts of total proteins were loaded onto SDS-PAGE gels and blotted onto nitrocellulose membranes. Membranes were blocked with 10% skim milk–PBS and incubated with primary antibodies overnight at 4°C. After washing with PBS-T, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 h. Signals were detected using chemiluminescent reagents. Nitrite expression was quantified using a chemiluminescent–enhanced image analysis system (LAS1000, GE Healthcare). Densitometric results were normalized to β-actin for equal loading. One representative experiment of at least three independent experiments was shown. All experiments were repeated at least three times with similar results. The results were presented as mean ± SEM of triplicate wells.

FIGURE 1. Sema4D enhances iNOS expression and NO production in microglial cells. A and B, Western blots for the iNOS expression in microglia revealed the enhancement of iNOS expression by Sema4D in a microglial cell line (6-3) (A) and primary microglia (MG) (B). iNOS expression was strongly upregulated by incubation with recombinant Sema4D–Fc proteins. β-Actin was used as an internal control for Western blot analysis. C, Immunocytochemical analysis for iNOS expression in primary MG. The number of iNOS-positive microglia was markedly enhanced by Sema4D–Fc. CD11b was used as a microglial marker. Scale bar, 20 μm. D and E, Measurement of nitrite concentrations in the culture supernatants from microglial cell line (6-3) (D) and MG (E). Nitrite production was significantly increased by addition of Sema4D–Fc in microglia. Data are shown as mean ± SEM of triplicate wells. *p < 0.05. MG or microglial cell line (6-3) were incubated with indicated reagents: human IgG (20 μg/ml), Sema4D–Fc (20 μg/ml), anti-CD40 (0.5 μg/ml), plus IFN-γ (5 U/ml) for 24 h (for Western blot analysis and immunocytochemistry) or 72 h (for measurement of nitrite concentrations). The data presented are representative of three independent experiments.
resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore, MA), and blotted with one of the following Abs at 4°C overnight: rabbit anti-CNTF (1:500, Santa Cruz Biotechnology), mouse anti–plexin-B1 (1:150, Santa Cruz Biotechnology), rabbit anti-inOS (1:300, BD Biosciences), rabbit anti-phospho-ERK1/2 (1:300, Cell Signaling Technology, Danvers, MA), goat anti-mAPK (1:500, Santa Cruz Biotechnology), rabbit anti-total or phospho-JNK (Santa Cruz Biotechnology), rabbit anti-total or phospho-p38 (Santa Cruz Biotechnology) or mouse anti-β-actin (1:8000; Sigma-Aldrich) Abs. They were subsequently incubated with appropriate secondary Abs conjugated with HRP for 60 min and visualized by ECL reagents (Amersham Biosciences, Buckinghamshire, U.K.). The image of each band was captured and analyzed using Image Gauge (Fuji Film, Tokyo, Japan), which allows quantification of the bands. Statistical significance was analyzed using an unpaired Student’s t test; p ≤ 0.05 was considered significant. For adoptive transfer, donor mice were immunized with MOG/CFA in the same fashion as except for no pertussis toxin. Ten days later, spleens and draining lymph nodes were collected, single-cell suspensions were prepared, and RBCs were lysed. Cells (5 × 10^6 cells/ml) were cultured with 40 μg/ml MOG35–55 peptide and 10 ng/ml recombinant mouse IL-12 (R&D Systems, Minneapolis, MN). After 3 d culture, cells were harvested and CD4^+ T cells were isolated by negative selection using Dynabeads (Invitrogen, Carlsbad, CA). Recipient mice irradiated sublethally (500 cGy) received cells i.v.

**Immunohistochemistry**

Mice were sacrificed followed by transcardiac perfusion with 4% paraformaldehyde in PBS. For Sema4D,plexin-B1, and iNOS labeling, sections (10 μm) were incubated with mouse anti–plexin-B1, mouse anti-Sema4D, or rabbit anti-iNOS Ab (1:50; Santa Cruz Biotechnology) at 4°C overnight, followed by biotin-conjugated secondary Abs (1:200, goat anti-rabbit or mouse; Vector Laboratories, Burlington, CA) for 30 min, then stained with PE-conjugated streptavidin. For double labeling, rabbit anti-IFN-γ Ab (for microglia/macrophage, 1:500; Wako, Osaka, Japan), FITC-conjugated anti–CD3 Ab (for T cells, 1:500; BD Pharmingen), and rabbit anti-IFN-γ Ab (for astrocytes, 1:1000; DakoCytonet, Carpinteria, CA) were used. Images were collected using a confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with IMARIS software (Bitplane AG, Zurich, Switzerland).

**Mononuclear cell isolation from CNS and flow cytometry**

Mice were euthanized with injection of pentobarbital, and spinal cords and brains were dissected. Both tissues were homogenized and strained through a 70-μm nylon filter (Falcon, Franklin Lakes, NJ). After centrifugation, the cell suspension was resuspended in 37% isotonic Percoll (GE Healthcare, Uppsala, Sweden) and underlayed with 70% isotonic Percoll. The gradient was centrifuged at 600 × g for 25 min at room temperature. The interface cells were collected and extensively washed before staining. For flow cytometry, the cells were stained with biotinylated anti-Sema4D, FITC-conjugated anti–CD3, APC-conjugated CD11b mAbs for 30 min at 4°C, washed, and incubated with streptavidin-PE (BD Pharmingen, San Diego, CA) for 15 min. The cells were washed and analyzed using a FACS Canto-2 using Diva software (BD Biosciences). Postacquisition analysis was performed using FlowJo (Tomy Digital Biology, Tokyo, Japan).

**FIGURE 2.** iNOS and NO production by Sema4D is abolished in plexin-B1–deficient but not in CD72-deficient microglia. A and B, Western blot analysis for plexin-B1 (A) and CD72 (B) in microglia. Lysates of the brain or spleen (positive controls) were prepared. C. Immunocytochemical analysis for plexin-B1 and CD72 in microglial cell line (6-3). Both plexin-B1 and CD72 were expressed in microglia. Scale bar, 20 μm. D. Western blot analysis for iNOS expression. The induction of iNOS expression by addition of Sema4D-Fc was abolished in plexin-B1–deficient microglia, but markedly enhanced in either wild-type or CD72-deficient cells. β-Actin was used as an internal control. E. Relative iNOS increase by addition of Sema4D-Fc in microglia from wild-type, CD72-deficient, and plexin-B1–deficient mice. The levels of iNOS expression in microglia incubated with anti-CD40 and IFN-γ plus human IgG without Sema4D-Fc were defined as standards (St). Data are shown as mean ± SEM. p < 0.05. F. Immunocytochemical analysis for iNOS expression in primary microglia. The number of microglia positive for iNOS was not increased by Sema4D-Fc in plexin-B1–deficient mice, but increased in wild-type and CD72-deficient cells. Scale bar, 50 μm. G. Nitrite concentrations in the culture supernatants. The increase of nitrite production in the culture supernatants by addition of Sema4D-Fc was not observed in plexin-B1–deficient microglia. Data are shown as mean ± SEM of triplicate wells. *p < 0.05. For iNOS or NO production, cells were treated with (20 μg/ml) or without Sema4D-Fc in the presence of anti-CD40 (0.5 μg/ml) and IFN-γ (5 U/ml) for 24 h (for Western blot or immunocytochemical analysis) or 72 h (for measurement of nitrite concentrations). The data presented in are representative of at least three independent experiments.
Results

**Sema4D enhances iNOS and NO production in microglia**

To investigate the involvement of Sema4D in activation of microglia, we first examined the effects of Sema4D on the production of iNOS, one of the effector molecules in neuroinflammation (8, 27). Although iNOS production by a microglial cell line 6-3 cells or primary microglia was not detected upon incubation with recombinant Sema4D or control IgG alone (Fig. IA, IB), Sema4D enhanced iNOS production in the presence of anti-CD40 agonistic Ab (Fig. IA, IB). Similar findings were obtained using immunocytochemical analysis, such that iNOS staining was significantly upregulated by the stimulation with Sema4D in the presence of anti-CD40 agonistic Ab (Fig. IC). We then examined NO production by microglia to determine whether iNOS, an NO-synthesizing isoenzyme, is responsible for the increased NO production induced by Sema4D. Consistent with the effects of Sema4D on iNOS expression, the concentrations of nitrite were considerably increased by Sema4D in both 6-3 cells and primary microglia (Fig. 1D, 1E). Collectively, these results indicate that Sema4D has enhancing effects on CD40-mediated microglial activation.

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**Sema4D-induced iNOS and NO production is mediated through plexin-B1**

Because Sema4D uses two receptors, plexin-B1 and CD72 (11), we examined their expression in microglia. As shown in Fig. 2A–C, the expression of plexin-B1 and CD72 proteins was observed in microglia. To address the question of which receptor is responsible for Sema4D-dependent microglial activation, we prepared microglia from plexin-B1−/ or CD72−/− deficient mice and examined their responses to Sema4D. Interestingly, the activating effects of Sema4D on microglia were significantly abolished in plexin-B1−/− deficient but not in CD72−/− deficient cells (Fig. 2D–F). Consistent with this, Sema4D-dependent NO production was also abolished in plexin-B1−/− deficient microglia (Fig. 2G). Collectively, these results strongly suggest that the stimulatory activities of Sema4D on microglia are mediated through plexin-B1.

**Activation of ERK1/2 is responsible for Sema4D-mediated iNOS upregulation**

Previous reports have shown that activation of ERK1/2 plays a key role in iNOS expression in microglia (28). In addition, the activation of other members of MAPK family, JNK and p38, is also shown to be involved in iNOS induction in glial cells (28, 29). We sought to determine whether Sema4D stimulation has an influence on ERK1/2, JNK, and p38 activation in microglia. Following stimulation with anti-CD40 agonistic Ab, phosphorylation of each kinase was observed within 10 min and then gradually declined within 90 min. Sema4D strongly enhanced ERK1/2 phosphorylation, moderately enhanced JNK phosphorylation at 10 min, and sustained ERK1/2 phosphorylation even at 90 min (Fig. 3A, 3B). However, apparent enhancement of p38 phosphorylation was not observed by the incubation with Sema4D (Fig. 3C).

We next examined whether the activation of these kinases induced by Sema4D was relevant to iNOS induction using several kinase inhibitors. Neither SP600125, a JNK inhibitor nor SB203580, a p38 inhibitor, did not inhibit the iNOS-upregulation by Sema4D. However, U0126, an MEK1 inhibitor, displayed an inhibitory effect on Sema4D-induced iNOS and NO production (Fig. 3D, 3E). These results suggest that enhanced activation of ERK1/2 is involved in Sema4D-mediated microglial activation.

**Sema4D–plexin-B1 interactions in the CNS are involved in the pathogenesis of EAE**

The in vitro findings suggest that Sema4D–plexin-B1 interactions are crucially involved in microglial activation. To address the role of Sema4D–plexin-B1 interactions during in vivo pathologic neuroinflammation, we examined the expression of Sema4D and plexin-B1 in pathogenic lesions of EAE. Although the expression of Sema4D was hardly seen in the spinal cords of control mice, it was significantly induced in infiltrating mononuclear cells of the spinal cords of mice with EAE (Fig. 4A, 4B). To determine which cells expressed Sema4D in the CNS, we performed a double immunolabeling and found that CD3+ T cells and a part of IBA-1+ microglia/macrophage populations expressed Sema4D (CD3+ T cells; 41 ± 2.1%, IBA-1+ cells; 25 ± 4.1%, respectively; Fig. 4C, 4D). To further evaluate the expression of Sema4D on the surface of infiltrating mononuclear cells, we prepared mononuclear cell suspension from the brains and spinal cords of mice with EAE and analyzed them by flow cytometry. Consistent with the immunohistochemical analysis, CD11b+ microglia/macrophage and CD3+ T cells in the CNS of mice with EAE expressed Sema4D, whereas those from control mice did not (Fig. 4E, 4F).

Regarding plexin-B1, its expression was significantly increased in the lesions of mice with EAE, but not detected in the spinal cords of control mice (Fig. 5A, 5B). Notably, large populations of...
IBA-1–positive microglia/macrophage were positive for plexin-B1 in the lesions of mice with EAE (Fig. 5C), whereas only a small part of CD3- or GFAP-positive cells expressed plexin-B1 (Fig. 5D, 5E). However, the expression of CD72 was not detected in the spinal cords of mice with EAE (data not shown).

We previously reported that Sema4D-deficient mice fail to develop EAE because of impaired T cell priming in the draining lymph nodes (Supplemental Fig. 1) (16). However, it has not been clarified how and to what extent Sema4D–plexin-B1 interactions in the CNS are pathologically significant during the course of EAE development. The fact that the expression of both Sema4D and plexin-B1 was induced in the lesions of EAE (Figs. 4 and 5) led us to investigate the pathologic importance of Sema4D–plexin-B1 interactions in the CNS during the course of EAE.

When we induced EAE by immunizing wild-type or plexin-B1–deficient mice with MOG35–55 peptides, together with pertussis toxin and CFA, plexin-B1–deficient mice displayed a relatively attenuated disease course and delayed clinical onset (Fig. 6A). To exclude a possibility that plexin-B1 is involved in T cell priming in the peripheral lymphoid organs, we examined the Ag-specific...
MOG-specific T cells to these chimera mice. As observed in active immunization of plexin-B1–deficient mice with MOG35–55 peptides, the lack of plexin-B1 expression in CNS resident cells caused a less severe disease course and delayed onset, compared with chimeric mice that express plexin-B1 in CNS resident cells (Fig. 6E).

Sema4D could be detected on IBA-1–positive cells in the spinal cords of mice with EAE (Fig. 4). To exclude a possible contribution of Sema4D expression in non-T cells, we adoptively transferred MOG-specific T cells into wild-type or Sema4D-deficient recipient mice. As shown in Fig. 6F, Sema4D-deficient recipient mice showed severities of EAE comparable to those observed in wild-type recipient mice, indicating that Sema4D expressed in non-T cells is not critical in the progression of EAE. Furthermore, blocking Abs against Sema4D considerably inhibited the development of relapsing EAE induced by an immunization with proteolipid protein PLP139–151 peptides, including when they were administered after priming phases (Fig. 6G). Consistent with the clinical course of EAE, an infiltration of mononuclear cells in the spinal cords of mice treated with anti-Sema4D Abs was markedly attenuated (Fig. 6H). Collectively, these findings strongly support the notion that Sema4D–plexin-B1 interactions in the CNS are pathologically involved in the development of EAE.

Discussion

Sema4D activates microglia through plexin-B1

Activation of microglia has been shown to play a crucial role in inflammation-mediated neurologic disorders, such as multiple sclerosis and Alzheimer’s disease, by producing various kinds of inflammatory effector molecules. In this study, we demonstrate that Sema4D activates microglia by increasing NO production via a plexin-B1–dependent mechanism. Further, T cell-derived Sema4D is crucially involved in the progression of EAE through interactions with plexin-B1 expressed in microglia.

In the immune system, we previously reported that Sema4D enhances CD40 signaling in B cells and DCs (15, 16). Consistent with these previous findings, we found that Sema4D promoted CD40-mediated activation of microglial cells. However, the mechanisms seem to be different between immune cells and microglia. Sema4D is known to use two types of receptors, plexin-B1 in the nervous system and CD72 in the immune system. Plexin-B1 mediates Sema4D-induced axon guidance in the CNS (18), and CD72 mediates Sema4D-dependent modulation of the CD40 pathway in peripheral immune responses (15, 30). Despite the expression of CD72 on microglia, the enhancement of iNOS expression was still observed in CD72-deficient microglia. In contrast, the effects of Sema4D were significantly abolished in plexin-B1–deficient microglia (Fig. 2). These results indicate that enhancement of iNOS expression in microglia by Sema4D occurs in a CD72-independent and plexin-B1–dependent manner. It has been demonstrated that plexin-B1 displays higher affinity to Sema4D than CD72 (31). It thus appears that Sema4D preferentially binds to plexin-B1 in microglia because of its higher affinity to Sema4D rather than CD72 (31).

NO production by Sema4D is mediated via ERK pathways

An activation of MAPK family members, such as ERK and p38, has been shown to play a critical role in the regulation of iNOS and TNF-α in microglia (28, 32). It has been reported that CD40 stimulation in microglia results in an activation of Ras-MAPK pathway via phosphorylation of Src family proteins Lck and Lyn, leading to the production of proinflammatory cytokines such as TNF-α (33). Similarly, Sema4D was reported to activate Ras-MAPK pathway downstream of plexin-B1 in neuronal cells and endothelial cells...
Interactions between Sema4D and plexin-B1 in the CNS are crucial for the progression of EAE

Consistent with our in vitro data that Sema4D–plexin-B1 interactions were involved in activation of microglia (Figs. 1 and 2), we further found that plexin-B1–deficient mice or BM chimera mice with a deficiency in plexin-B1 expression in the CNS were resistant to the development of EAE after an adoptive transfer of MOG-specific T cells (Fig. 6). It is well known that Sema4D is abundantly expressed on T cells (12). Indeed, in the pathologic lesions of EAE, Sema4D was expressed in infiltrating T cells in the spinal cords (Fig. 4), whereas plexin-B1 was expressed in microglia (Fig. 5). It thus appears that CNS-infiltrating Sema4D-positive T cells can interact with plexin-B1–positive, CNS-resident microglia, resulting in activation of microglia during EAE progression. It is possible that BM-derived macrophages have some contribution to the neuroinflammation in EAE, because Sema4D is also expressed on cells other than T cells, such as IBA-1–positive microglia/macrophages in the spinal cords of mice with EAE (Fig. 4). However, there were no significant differences in the severity of EAE between Sema4D-deficient and wild-type recipient mice when transferred with wild-type MOG-specific T cells. This finding implies that T cell-derived Sema4D is primarily responsible for the pathogenesis of EAE through interactions with plexin-B1–expressing microglia. It is also possible that T cell-derived Sema4D has some influence on other CNS cells such as oligodendrocytes. In fact, Giraudon et al. (21) reported that T cell-derived Sema4D induces collapse of...
process extension in immature oligodendrocytes and death of immature neural cells, resulting in compromised remyelination in the inflamed brain. However, plexin-B1–deficient mice displayed delayed onset and decreased severities of EAE even at the early phase, which is difficult to explain simply with improved remyelination at the later phase of EAE. In addition, major populations of plexin-B1–positive cells were also positive for IBA-1, but negative for the oligodendrocyte marker OLIG-1 (data not shown). Collectively, these findings support the conclusion that attenuated development of EAE in plexin-B1–deficient mice is primarily due to impaired Sema4D-mediated microglial activation. However, we cannot completely exclude a possibility that Sema4D may directly injure oligodendrocytes in EAE. In addition, a possible protective effect of Sema4D in neuroinjury was recently suggested (37), although our experimental system could not reproduce such results.

In conclusion, we demonstrate that Sema4D–plexin-B1 interactions are crucially involved in activation of microglia. We also present that Sema4D is expressed in infiltrating T cells in the spinal cord of mice with EAE, whereas plexin-B1 is expressed in microglia and participates in the pathogenesis of EAE in the CNS. Furthermore, blocking Abs against Sema4D significantly inhibits neuro-inflammation during EAE development. Together with our previous data that MOG-specific T cell priming is impaired in Sema4D–deficient mice (16), a blockade of Sema4D would be a valuable therapeutic target for neuroinflammatory diseases including EAE, because it can prevent the generation of encephalitogenic T cells and ameliorate inflammation even after clinical onset.

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Disclosures
The authors have no financial conflicts of interests.

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