Elevation of Sema4A Implicates Th Cell Skewing and the Efficacy of IFN-β Therapy in Multiple Sclerosis


*J Immunol* 2012; 188:4858-4865; Prepublished online 9 April 2012; doi: 10.4049/jimmunol.1102023

http://www.jimmunol.org/content/188/10/4858

Supplementary Material [http://www.jimmunol.org/content/suppl/2012/04/09/jimmunol.1102023.DC1](http://www.jimmunol.org/content/suppl/2012/04/09/jimmunol.1102023.DC1)

References This article cites 48 articles, 8 of which you can access for free at: [http://www.jimmunol.org/content/188/10/4858.full#ref-list-1](http://www.jimmunol.org/content/188/10/4858.full#ref-list-1)

Subscription Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

Permissions Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Multiple sclerosis (MS) is a demyelinating autoimmune disease of the CNS and a leading cause of lasting neurologic disabilities in young adults. Although the precise mechanism remains incompletely understood, Ag presentation and subsequent myelin-reactive CD4+ T cell activation/differentiation are essential for the pathogenesis of MS. Although semaphorins were initially identified as axon guidance cues during neural development, several semaphorins are crucially involved in various phases of immune responses. Sema4A is one of the membrane-type class IV semaphorins, which we originally identified from the cDNA library of dendritic cell (DC). Sema4A plays critical roles in T cell activation and Th1 differentiation during the course of experimental autoimmune encephalomyelitis, an animal model of MS; however, its pathological involvement in human MS has not been determined. In this study, we report that Sema4A is increased in the sera of patients with MS. The expression of Sema4A is increased on DCs in MS patients and shed from these cells in a metalloproteinase-dependent manner. DC-derived Sema4A is not only critical for Th1 but also for Th17 cell differentiation, and MS patients with high Sema4A levels exhibit Th17 skewing. Furthermore, patients with high Sema4A levels have more severe disabilities and are unresponsive to IFN-β treatment. Taken together, our results suggest that Sema4A is involved in the pathogenesis of MS by promoting Th17 skewing.

Received for publication July 12, 2011. Accepted for publication March 6, 2012.

Y. Nakatsuji and T.O. contributed equally to this work.

Yuji Nakatsuji,* † Tatsusada Okuno,* ††† Masayuki Moriya,* Tomoyuki Sugimoto, ‡ Makoto Kinoshita,* Hyota Takamatsu, † Satoshi Nojima, † Tetsuya Kimura, † Sujin Kang, † Daisuke Ito, † Yukinozaka Nakagawa, † Toshihiro Toyofuku, † Kazuhiro Takata, † Misa Nakano, ‡ Masato Kubo, ‡ Sinobu Suzuki, ‡ Akiko Matsui-Hasumi, ‡† Ayako Uto-Konomi, ‡‡ Ayako Uto-Konomi, ‡‡ Atsushi Ogata, † Hideki Mochizuki, † Saburo Sakoda, ‡‡ and Atsushi Kumanogoh † †

Multiple sclerosis (MS) is a demyelinating autoimmune disease of the CNS and a leading cause of lasting neurologic disabilities in young adults. Although the precise mechanism remains incompletely understood, Ag presentation and subsequent myelin-reactive CD4+ T cell activation/differentiation are essential for the pathogenesis of MS. Although semaphorins were originally identified as axon guidance molecules during neural development, they are currently known to have diverse and important functions in other physiological processes (11), including heart morphogenesis (12), vascular growth (13), tumor progression (14, 15), and immune cell regulation (16, 17). Some semaphorins are crucially involved in immune responses, including Th differentiation (18, 19). Sema4A is a membrane-type class IV semaphorin that we originally identified using a dendritic cell (DC) cDNA library. We previously reported that Sema4A plays critical roles in T cell activation and Th1 differentiation (18, 19). Indeed, the development of myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in wild-type mice can be improved by guest on April 15, 2017 http://www.jimmunol.org/ Downloaded from
by intravenously injecting an anti-Sema4A mAb concurrently with MOG immunization (18). In anti-Sema4A Ab-treated mice, the infiltration of mononuclear inflammatory cells into the spinal cord is diminished, and CD4+ T cells isolated from the draining lymph nodes have markedly decreased responses to the MOG peptide. These previous findings indicate that Sema4A plays crucial roles in EAE. However, the pathological role of Sema4A in human MS has not been determined. Regarding the treatment for MS, there is increasing evidence that immunomodulatory treatment is beneficial to prevent axonal loss and the progression of neurologic disabilities (20, 21). IFN-β treatment is one of the recommended treatments for early-stage MS. However, only two-thirds of patients respond to this therapy, and the disease is exacerbated by IFN-β treatment in some patients (22). The current diagnostic criteria (McDonald’s criteria) are based on clinical characteristics and magnetic resonance imaging results, which favor an early diagnosis (23). These criteria also recommend examination of IgG oligoclonal bands in the cerebrospinal fluid to support the diagnosis, but this procedure does not predict IFN-β responsiveness. IFN-β treatment exacerbates EAE induced by a Th17 cells transfer, and high serum IL-17 levels in MS patients are suggested to be associated with IFN-β nonresponder (24).

In this study, the significance of Sema4A in MS was investigated based on studies of patients with MS and in vitro/in vivo experiments. We found that Sema4A was significantly increased in MS patients, in which Th17 skewing of Th cells was observed. In addition, these patients with high Sema4A levels are not IFN-β responders. Materials and Methods

Patients

Serum Sema4A levels were analyzed in 207 patients, including 59 patients with relapsing–remitting multiple sclerosis (RRMS), 22 patients with clinically isolated syndrome (CIS), and 126 patients with other neurologic diseases (OND). The OND patients had various diseases as shown in Supplemental Table I. The 59 MS patients were diagnosed according to McDonald’s criteria. Corticosteroids, immunosuppressants, and immunomodulators except IFN-β1b were not administered to these patients. Patients with secondary progressive MS, primary progressive MS, and neuromyelitis optica (NMO) who tested positive for anti–aquaporin-4 Ab or fulfilled Wingerchuk’s criteria (25) were excluded from the study. Thirty-four OND patients, who were age- and gender-matched to the MS group, are described in Supplemental Table I. This study was approved by the ethical committee of Osaka University Hospital. All patients provided informed consent before enrolling in this study. Blood samples were basically obtained from MS patients during the remitting phase. The blood samples were allowed to clot at room temperature, and then the sera were separated by centrifugation and stored at −80°C until further use. Each assay was performed at least twice.

Sema4A ELISA

mAbs against Sema4A, which recognize both human and mouse Sema4A, were generated as previously described (19). Ninety-six-well polyvinyl ELISA plates (Maxisorb; Nunc) were coated with the monoclonal anti-Sema4A Abs HT302 (2 μg/ml) and 1A2 (2 μg/ml) overnight at 4°C. The patient sera were diluted (1:5), and a biotinylated monoclonal anti-Sema4A Ab 5E3 was used as the detection Ab. One nanogram per milliliter of recombinant Sema4A–Fc protein (18), which was used as a standard, was equivalent to 1 U/ml of serum Sema4A.

Mice

Sema4A-deficient and OT-II TCR transgenic mice on a C57BL/6 background were generated and maintained as previously described (19). Wild-type C57BL/6 mice were purchased from Nippon Clea. All mice used in this study were maintained in a specific pathogen-free environment. All animal experimental procedures followed institutional guidelines.

Cell preparation

Human PBMCs were separated by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Human CD14+ monocytes and CD4+ T cells were isolated from the PBMCs of healthy controls using a MACS sorting kit (Miltenyi Biotec) and then cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS (Life Technologies) and 50 μmol/l 2-mercaptoethanol (2-ME). Human DCs were generated from CD14+ monocytes using GM-CSF and IL-4 (R&D Systems) as previously described (26). THP-1 monocytes and Jurkat cells were grown in RPMI 1640 medium supplemented with 10% FCS and 50 μmol/l 2-ME. Mouse bone marrow–derived dendritic cells (BMDCs) were generated from bone marrow progenitors from wild-type or Sema4A-deficient BMDCs (1 × 106/ml) in the presence of GM-CSF and IL-4 (10 μg/ml) (Th1-skewing conditions), in the presence of IL-4 (1 ng/ml) and anti–IFN-γ (10 μg/ml) (Th2-skewing conditions) or in the presence of IL-6 (20 ng/ml), TGF-β (5 ng/ml), anti–IL-4 (10 μg/ml), and anti–IFN-γ (10 μg/ml) (Th17-skewing conditions) with OT-II peptides (1 μmol/ml; Sigma Genomys) for 5 d. The resulting CD4+ T cells were positively selected using a MACS sorting kit and restimulated with immobilized anti-CD3 Ab (2C11; 10 μg/ml) for 24 h in 96-well plates (1 × 106/ml). The IFN-γ, IL-4, and IL-17A levels were measured using mouse IFN-γ, IL-4, and IL-17A ELISA kits (R&D Systems), respectively.

FACS analysis

For FACS analysis of Sema4A expression, the cells were stained with the following Abs: anti-Sema4A (5E3; MBL), anti-CD4, anti-CD8, anti-CD19, anti–CD14, anti-CD11c, and anti–HLA-DR conjugated with FITC, PE, or biotin (BD Pharmingen) in the presence of normal human serum (for human cells). Streptavidin–allophycocyanin (BD Pharmingen) was used as second-stage reagent for biotinylated Abs. The cells were washed and analyzed using a FACS Canto-2 and Diva software (Becton Dickinson).

Postacquisition analysis was performed using FlowJo software.

For intracellular cytokine analysis, an equal amount of RPMI 1640 supplemented with 10% FCS was added to whole blood samples and then incubated with 5 μg/ml PMA (Sigma), 0.5 μg/ml ionomycin (Sigma), and 10 μg/ml brefeldin A (Sigma) in a 24-well plate for 4 h at 37°C under 5% CO2. After washing with PBS containing 0.1% BSA (0.1% BSA–PBS), the cells were stained with a PerCP– or PE-conjugated anti-CD4 mAb (Becton Dickinson) and incubated on ice in the dark for 15 min. After lysing the red cells with FACS lysing solutions (Becton Dickinson), a FACS permeabilizing solution (Becton Dickinson) was added, and the cells were then incubated for 10 min in the dark. After two washes with containing 0.1% BSA–PBS, the cells were stained with anti–IFN-γ, anti–IL-4, and anti–IL-17A conjugated with FITC-conjugated anti-IFN-γ mAb (Becton Dickinson), PE-conjugated anti–IL-4 (Becton Dickinson), and Alexa Fluor 647-conjugated anti–IL-17A (eBioscience) Abs for intracellular cytokine analyses. After a 30-min incubation on ice in the dark, the percentages of IFN-γ+, IL-4+, and IL-17-producing cells were immediately analyzed by flow cytometry using a Canto II (Becton Dickinson). The analysis gates were first set on lymphocytes based on the forward- and side-scatter properties and then on CD4+ lymphocytes. Postacquisition analysis was performed using FlowJo software.

Cell surface labeling

Cells were washed with PBS and labeled with 1 ml of PBS containing 50 μg of R-6-giodeaminic acid-N-hydroxysuccinimide ester (Roche) for 15 min. The reaction was stopped by adding 50 μl of 1 mol/ml NH4Cl (pH 7.4) for 15 min, and then cells were washed with ice-cold PBS. For the studies, live cells were resuspended in 2 × 106/ml in GM medium (RPMI 1640 medium supplemented with 10% FCS and 5 μg/ml leupeptin for 1 h on ice. After centrifuging to remove the cellular debris, the lysates were subjected to immunoprecipitation analyses.

Immunoprecipitation and Western blot analyses

To measure Sema4A shedding, culture supernatants and lysates from 2 × 106 biotin-labeled cells were precleared with protein G Sepharose (Protein G Sepharose 4 Fast Flow; GE Healthcare) and then immunoprecipitated with 2.5 μg of the Sema4A mAb 5E3 (MBL) and protein G Sepharose. Immune complexes were eluted by boiling the samples for 5 min in

The Journal of Immunology
Laemmli buffer [125 mmol/ml Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 0.02% bromophenol blue] supplemented with or without 10% 2-ME. The solubilized proteins were separated by SDS-PAGE and electro-transferred onto polyvinylidene difluoride membranes. The blots were incubated with streptavidin–POD (Roche) for 1 h and then visualized by ECL reagents (GE Healthcare). In some experiments, the blots were incubated with an anti-Sema4A rabbit antisemur overnight at 4°C, followed by the secondary Ab for 1 h, and then visualized with ECL.

Gene expression profiling of human blood samples

Total cellular RNA from the peripheral blood of four healthy controls, three MS patients with high Sema4A levels, and three MS patients with low Sema4A levels was extracted with a QIAamp RNA blood Mini Kit (Qiagen) according to the manufacturer’s instructions. Approximately 10 μg of RNA was labeled and hybridized to GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix) according to the manufacturer’s protocols. Expression values were determined using GeneChip Operating Software v1.1.1 and the MAS method. All data analysis was performed using GeneSpring software GX 11.0.1 (Agilent Technologies). Clustering analysis was performed using Cluster 3.0 (developed based on Eisen Lab’s Cluster and Tree View software). The normalized expression values for each gene were calculated and displayed in a heat map, with the gene expression colorimetrically represented from green to red, with green and red denoting low and high expression genes, respectively.

Induction of EAE

EAE was induced in 6- to 8-wk-old wild-type or Sema4A-deficient mice on a C57BL/6 background by s.c. injection of 100 μg pertussis toxin (List Laboratories, except on days 0 and 2. The mice were treated with either Sema4A–Fc (15 μg, Sema4A-deficient n = 6) or control human IgG (15 μg, Sema4A-deficient n = 5) on days 0, 1, 3, 5, 7, 9, and 11. All mice were monitored daily for clinical signs and were scored using a scale of 0–4 as follows: 0, no overt signs of disease; 1, limp tail; 2, complete hindlimb paralysis; 3, complete forelimb paralysis; 4, moribund state or death. The typical disease course of MS is characterized by repetitive relapses and remissions. To investigate whether Sema4A levels are changeable in association with the disease phase, we assayed Sema4A during remitting phase and relapsing phase in the same 23 patients before and after the induction of IFN-β treatment (5128 ± 7581 U/ml versus 4616 ± 6009 U/ml, p = 0.675, Fig. 1B). The black squares show the means. The top and bottom of the box in the end of the whisker represents 1.5 times the interquartile range from the top of the box or the maximum point of all the data.

Results

Serum Sema4A levels are elevated in patients with MS

First, we examined the serum Sema4A levels in RRMS patients by developing a sandwich ELISA. The serum Sema4A titers were significantly higher in MS patients than those in patients with OND (4066 ± 6166 versus 1298 ± 3353 U/ml, p = 0.0020). Among these OND patients, the Sema4A levels were higher in females (2068 ± 4728 U/ml, n = 56, age 51.9 ± 18.9 y) than in males (537 ± 1223 U/ml, n = 70, age 55.2 ± 17.5 y, p = 0.0215), and the titer tended to decrease with age, especially in males [females: Spearman’s rank correlation (rs) = −0.21, p = 0.161; males: rs = −0.34, p = 0.006]. The specificity of the ELISA system for Sema4A was confirmed by a mass spectrometric analysis of the peptides extracted from immunoprecipitates that were obtained with an anti-Sema4A Ab (data not shown). Based on these findings, we compared the serum Sema4A levels in RRMS patients with gender- and age-matched OND patients as controls. MS patients had significantly elevated serum Sema4A levels compared with those in patients with OND (1298 ± 2330 U/ml, p = 0.0021, Fig. 1A). In addition, the serum Sema4A levels in patients with CIS, which is considered as an early stage of MS, were as high as those in MS patients (3619 ± 1263 U/ml) and significantly higher than those of the age- and gender-adjusted OND patients (1218 ± 3353 U/ml, p = 0.0457, Fig. 1A and Supplemental Table II). To exclude the possibility that the higher Sema4A levels in MS patients were due to IFN-β treatment, we assayed the serum Sema4A levels in the patients before and after the induction of therapy. However, there was no significant difference in the Sema4A levels before and after starting IFN-β therapy (5128 ± 7581 versus 4616 ± 6009 U/ml, p = 0.675, Fig. 1B).

FIGURE 1. Serum Sema4A levels are elevated in MS patients. (A) Serum Sema4A levels were significantly higher in MS and CIS patients than in OND patients. The serum Sema4A levels were measured by ELISA in 59 RRMS patients in the remitting phase, 22 CIS patients, and 34 age- and gender-matched OND patients. *p < 0.05, **p < 0.01. (B) Sema4A levels before and after the induction of IFN-β therapy were assayed in eight MS patients. IFN-β therapy did not affect the Sema4A levels (means 5128 ± 7581 U/ml versus 4616 ± 6009 U/ml, p = 0.675, Welch’s t test). The black squares show the means. The top and bottom of the box in the box–whisker plot indicate the 25th and 75th percentiles, respectively, and the end of the whisker represents 1.5 times the interquartile range from the top of the box or the maximum point of all the data.
MS patients. There was no significant differences between the remitting phase (5199 ± 1509 U/ml) and the relapsing phase (4239 ± 1325 U/ml) (p = 0.249, Supplemental Fig. 1A). Collectively, these data suggest that serum Sema4A is involved in MS pathogenesis even at an early stage but do not reflect the disease activity.

Sema4A is shed from DCs

Sema4A was originally cloned from a cDNA library of mouse DCs (18). To identify the source of increased Sema4A in MS patients, we analyzed Sema4A expression in PBMCs from healthy controls and MS patients by FACS. Although CD4+ T cells, CD8+ T cells, and CD19+ B cells from healthy controls and MS patients expressed very low levels of Sema4A, Sema4A was moderately expressed on CD11c+ HLADR+ cells in healthy controls and was further increased in MS patients (Fig. 2A). These results suggest that monocytes or DCs are likely the main source of soluble Sema4A in MS. Because Sema4A is a transmembrane-type semaphorin, we examined if it can be cleaved from the cell surface, resulting in a soluble form. To evaluate the soluble form of Sema4A that is potentially released from CD14+ monocytes and CD14+ monocyte-derived DCs, the cell surface proteins were biotinylated and incubated for 48 h. The cell lysates and supernatants were collected, immunoprecipitated with an anti-Sema4A Ab, and then analyzed by Western blotting with peroxidase-conjugated streptavidin. Although neither CD4+ T cells nor CD4+ CD14− cells produced detectable levels of soluble Sema4A, soluble Sema4A was detected in the culture supernatants of CD14+ monocytes, and these levels further increased after the cells were differentiated into DCs (Fig. 2B, 2C). Soluble Sema4A was detected in the culture supernatants of wild-type mouse DCs and human monocyte-derived cell line (Fig. 3A, Supplemental Fig. 2). A number of transmembrane proteins such as TNF-α and Sema4D undergo proteolysis and are released from the plasma membrane through a process called ectodomain shedding (16, 27), in which proteinases, including ADAMs and MMPs, have been shown to be involved. To determine whether soluble Sema4A is also secreted through ectodomain shedding, we examined the effects of a set of inhibitors for various protease subclasses. Light metal chelators, EDTA and EGTA, and metalloproteinase inhibitors, GM6001 and phosphoramidon, inhibited Sema4A shedding from monocytes and DCs (Fig. 3A, 3B). Of note, a microarray analysis showed that metalloproteinases such as ADAM10 and MMP-1, -3, -9, -12, and -25, which are reportedly involved in the pathogenesis of MS (28–34), were increased in PBMCs from MS patients with high Sema4A levels compared with those in PBMCs from patients with low Sema4A levels and from healthy controls (Fig. 3C) [the data have been deposited in the Gene Expression Omnibus database and are accessible through GEO Series accession number GSE26484 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26484)]. Taken together, these findings suggest that Sema4A is abundantly expressed on monocytes and DCs in MS patients and proteolytically cleaved from the cell surface of DCs, contributing to the high serum Sema4A levels in a portion of MS patients.

Link between Sema4A levels and Th17 skewing in patients with MS

Sema4A has been shown to be crucial for T cell activation and Th differentiation in mouse experimental systems. To investigate whether high Sema4A levels are relevant to the Th cell-mediated autoimmune pathogenesis in MS, we measured the serum cytokine levels in MS patients. Consistent with our previous report on the role of Sema4A in T cell activation (18), MS patients with high levels of serum IL-2 had significantly higher Sema4A levels (MS patients with more than 1.4 ng/ml IL-2: 5576 ± 8838 U/ml; MS patients with less than 1.4 ng/ml: 1370 ± 1552 U/ml, p = 0.0048, Supplemental Fig. 1B). In addition, we observed that low serum IL-10 levels in MS patients were associated with high Sema4A levels in PBMCs from MS patients with less than 1.7 ng/ml IL-10: 5224 ± 7433 U/ml; MS patients with more than 1.7 ng/ml: 2528 ± 4884 U/ml, p = 0.0078, Supplemental Fig. 1C), which is consistent with our previous finding that Sema4A suppresses IL-10 production (35). Next, we analyzed intracellular cytokine expression in CD4+ T cells. Blood samples were collected from MS patients with high Sema4A levels (>2500 U/ml, n = 7), MS patients with low Sema4A levels (<2500 U/ml, n = 11), and healthy controls (n = 11). Notably, MS patients with high Sema4A levels had a signifi-

FIGURE 2. Soluble Sema4A is secreted from DCs. (A) Increased Sema4A expression in DCs. Sema4A expression was higher in CD11c+ HLADR+ cells in MS patients (red lines) than in the normal controls (filled histograms). (B) Sema4A expression increased when monocytes were differentiated into DCs. Flow cytometric analysis of Sema4A expression in CD14+ monocytes (filled histograms) and monocyte-derived DCs (red line). (C) Sema4A is cleaved from CD14+ monocytes and monocyte-derived DCs. The cell lysates and supernatants of biotinylated cells were immunoprecipitated with an anti-Sema4A Ab and then blotted with streptavidin–peroxidase. Data shown in (A)-(C) are representative of five or three independent experiments.
Sema4A AND MULTIPLE SCLEROSIS

Next, we investigated whether Sema4A expressed on DCs affects the differentiation of naive CD4+ T cells into Th17 cells. Naive CD4+ T cells were derived from OT-II OVA-TCR transgenic mice and then cocultured with wild-type or Sema4A-deficient DCs under Th1-, Th2-, or Th17-skewing conditions in the presence of OT-II peptides. IL-17 production was considerably impaired when the naive CD4+ T cells were cocultured with Sema4A-deficient DCs under Th17-skewing conditions, whereas neither IFN-γ production under Th1-skewing conditions nor IL-4 production under Th2-skewing conditions was affected (Fig. 5A). Even under neutral conditions without cytokines, IL-17 and IFN-γ production was significantly impaired when naive CD4+ T cells were cocultured with Sema4A-deficient DCs. In contrast, IL-4 production was not affected (Fig. 5B, Supplemental Fig. 2D, 2E). Collectively, these findings suggest that DC-derived Sema4A is critically involved in Ag-specific Th17 and Th1 differentiation.

FIGURE 3. Soluble Sema4A is secreted in a metalloproteinase-dependent manner. (A) Soluble Sema4A is produced in the supernatants of a human monocyte-derived cell line (THP-1 cells) through metalloproteinase-dependent shedding, but not in the supernatants of a T cell-derived cell line (Jurkat cells). THP-1 and Jurkat cells were biotinylated and cultured with various protease subclasses for 6 h. The cell lysates and supernatants were immunoprecipitated with an anti-Sema4A Ab and blotted with peroxidase-conjugated streptavidin. (B) Soluble Sema4A is generated in a metalloproteinase-dependent manner. Biotinylated human DCs were incubated with or without EDTA (10 mmol/ml), and then the supernatants were analyzed by immunoprecipitation and Western blotting. Soluble Sema4A production was reduced in the presence of EDTA. (C) A heat map of the normalized expression data of genes encoding various proteinases in PBMCs from MS patients with high Sema4A levels (≥2500, n = 3), MS patients with low Sema4A levels (≤2500, n = 3), and healthy controls (n = 4). The expression levels of MMP-1, -3, -9, -12, and -25 and ADAM10 were higher in MS patients with high Sema4A levels than in MS patients with low Sema4A levels or healthy controls. Data shown in (A) and (B) are representative of three independent experiments.

FIGURE 4. Correlation between Sema4A levels and Th17 skewing in MS patients. FACS analysis for intracellular cytokine expression in CD4+ T cells from MS patients with high (n = 8) and low (n = 11) Sema4A levels and from healthy controls (n = 11). The IFN-γ-1.56, p = 0.0891; and IL-17A-0.98, p = 0.2555 and 5.87 ± 3.97, p = 0.6667, respectively) or healthy controls (2.46 ± 1.56, p = 0.1023 and 8.26 ± 3.97, p = 0.0692, respectively) (Fig. 4A, 4B). These findings suggest that elevated serum Sema4A levels in MS patients reflect the underlying Th17-mediated pathogenesis of MS.

Correlation between DC-derived Sema4A and Th differentiation

To explore the pathogenic implications of Sema4A, recombinant Sema4A proteins were injected into actively immunized wild-type or Sema4A-deficient mice during the course of EAE. Although Sema4A-deficient mice exhibited less severe EAE, administration of recombinant Sema4A exacerbated EAE to a similar degree as that observed in wild-type mice with EAE (Fig. 5C). Furthermore, in the case of IL-17 and IFN-γ production by CD4+ T cells, Sema4A-deficient mice exhibited impaired production of both cytokines, but recombinant Sema4A significantly increased CD4+ T cell activation to produce IL-17 as well as IFN-γ in Sema4A-deficient mice (Fig. 5D, Supplemental Fig. 2F, 2G). However, administration of recombinant Sema4A or anti-Sema4A blocking Abs did not have an influence on the effector phase of the disease course, in which MOG-specific CD4+ T cells were transferred (Fig. 5E). These findings suggest that Sema4A plays an important role in the development of EAE by promoting Th17 and Th1 differentiation in the priming phase rather than the effector phase.

Pathogenic implications of Sema4A in EAE

MS patients with high Sema4A levels exhibit more severe clinical course

Finally, we examined whether high serum Sema4A levels, which implicate underlying Th17 pathology, are linked to the clinical
severity and/or responsiveness to IFN-β treatment. The 59 MS patients were divided into two groups based on a Sema4A threshold of 2500 U/ml. Patients with higher Sema4A levels had a significantly more severe EDSS score (36) ($p = 0.042$), and their relapse rate tended to increase, although this difference was not significant (Fig. 6A, Table I). Furthermore, when the MS patients under IFN-β treatment were classified into two groups based on a serum Sema4A threshold of 2500 U/ml, the difference in the EDSS score became more apparent ($p = 0.00064$, Fig. 6B), suggesting that IFN-β exacerbates the neurologic disabilities of MS patients with high Sema4A levels. Thus, MS patients with high Sema4A levels tended to have more severe disability, and IFN-β treatment was not beneficial to these patients.

Discussion
In the current study, we highlighted the clinical implications of Sema4A in MS. Serum Sema4A is significantly elevated in patients with MS, and about one-third of these patients exhibit remarkably high serum Sema4A levels. Those patients with high Sema4A levels have underlying Th17 pathogenesis. Furthermore, we...
showed that high Sema4A levels were associated with severe disabilities and unresponsiveness to IFN-β therapy.

Sema4A, which is a transmembrane-type semaphorin, was originally identified as a molecule expressed in mouse DCs. We extended the findings in human studies and showed not only that Sema4A is expressed in human DCs but also that Sema4A is upregulated on the surface of DCs in MS patients. These findings suggest that DCs are potentially a major source of increased serum Sema4A. Of note, high levels of soluble Sema4A were accompanied by increased MMP levels, which suggests that Sema4A is proteolytically cleaved from the cell surface by MMPs to produce soluble Sema4A in the sera of MS patients. It is also possible that Sema4A contributes to these increased MMP levels. Further studies will be required to determine the pathologic relationship between soluble Sema4A and MMPs.

MS is thought to occur in genetically predisposed individuals after they are exposed to an environmental trigger that stimulates myelin-specific T cells (6, 37). In addition, the aberrant differentiation of Th cells and their subsequent transmigration across the blood–brain barrier (BBB) have been implicated in the pathogenesis of MS (37). We previously reported that Sema4A plays critical roles in Th cell activation and Th1 differentiation during the course of EAE (18, 19). In this study, we show that patients with high serum Sema4A levels had Th17 skewing. We further confirmed that Sema4A is involved in Th17- as well as Th1-mediated pathogenesis in our experimental models. The involvement of Sema4A in Th1 or Th17 differentiation may depend on aspects of each situation, including Ag doses and DC numbers. In addition, there may be differences between human and mice regarding the Th1/Th17 preference. Recent reports suggest that Th17 cells play vital roles in the pathogenesis of MS (7–9, 37), in which Th17 cells have been shown to contribute to the disruption of BBB and to infiltrate the CNS (38). The increased number of Th17 cells in MS patients with high Sema4A levels suggests that Sema4A contributes to MS pathogenesis by promoting BBB permeability. In addition, Th17 cells have been shown to enhance CNS inflammation by inducing the expression of inflammatory chemokines and IL-6 in astrocytes (39–41). In fact, patients with NMO, who generally have higher IL-17 and IL-6 levels in the cerebrospinal fluid than those of MS patients, tend to exhibit severe pathological changes (42–45). The clinical severities in MS patients with high Sema4A levels may be partially explained by the increased permeability of the BBB and subsequent infiltration of Th17 cells into the CNS. In addition, we showed that PBMCs from MS patients with high levels of Sema4A had increased MMP-9 mRNA levels. Because MMP-9 is increased in the sera of MS patients and involved in BBB disruption (30, 31), Sema4A may also contribute to the increased permeability of the BBB via increasing MMP-9 expression.

Notably, the difference in the EDSS scores between patients with high Sema4A levels and those with low Sema4A levels became more apparent when the patients were further subclassified into patients who had received IFN-β therapy. Our findings suggest that IFN-β therapy does not ameliorate but rather exacerbates MS in patients with high Sema4A levels. Therefore, these findings suggest that IFN-β therapy is not clinically beneficial for MS patients with high Sema4A levels. Consistent with our findings, IFN-β administration was reported to exacerbate EAE that was induced by adoptively transferring Th17 cells (24). In addition, NMO patients, who are thought to have greater Th17 pathology than MS patients, have been shown to deteriorate after receiving IFN-β therapy (46, 47). More notably, consistent with an inhibitory role of Sema4A in IL-10 production (35), we found that patients with high serum Sema4A levels had low serum IL-10 levels. Because IL-10 has been linked to the beneficial effects of IFN-β treatment (24, 48), it is plausible that high Sema4A levels result in decreased IL-10 levels, leading to the unresponsiveness to IFN-β therapy.

Further studies would be important to elucidate the mechanism for the increase in Sema4A expression on DCs in MS patients and to develop Sema4A-targeted immunotherapy.

Table I. The characteristics of patients with higher and lower Sema4A levels based on a Sema4A threshold of 2500 U/ml

<table>
<thead>
<tr>
<th>Sema4A, U/ml</th>
<th>Number of Patients (F/M)</th>
<th>Age, y</th>
<th>Duration of Illness, mo</th>
<th>Relapse Number/2 y</th>
<th>Mean EDSS Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2500</td>
<td>40 (35/5)</td>
<td>38.7 ± 11.3</td>
<td>91.8 ± 104.1</td>
<td>1.84 ± 1.29</td>
<td>2.27 ± 1.94</td>
</tr>
<tr>
<td>≥2500</td>
<td>19 (16/3)</td>
<td>35.8 ± 11.5</td>
<td>72.6 ± 40.2</td>
<td>2.44 ± 2.3</td>
<td>3.34 ± 1.7*</td>
</tr>
<tr>
<td>&lt;2500</td>
<td>30 MS Patients Receiving IFN-β Therapy</td>
<td>36.7 ± 11.9</td>
<td>81.1 ± 108.4</td>
<td>2.27 ± 1.4</td>
<td>2.34 ± 2.4</td>
</tr>
<tr>
<td>≥2500</td>
<td>14 (13/1)</td>
<td>33.3 ± 7.9</td>
<td>67.2 ± 31.6</td>
<td>2.62 ± 2.5</td>
<td>3.74 ± 1.6**</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01.
Acknowledgments
We thank T. Yazawa and R. Yasui for valuable technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure Legends

Supplementary Figure 1.

(A) Sema4A levels between the remitting and relapsing phases. There were no significant differences in the Sema4A levels between the remitting (4,239 ± 1,325 U/ml) and relapsing (5,199±1,509 U/ml) phases in the same 23 patients with RRMS (p=0.249, paired t-test). (B) Correlation between serum Sema4A titers with IL-2 levels. Fifteen MS patients whose serum IL-2 had been assayed were divided into two groups based on an IL-2 titer of 1.4 ng/ml. MS patients with more than 1.4 ng/ml IL-2 had higher Sema4A levels (5,576 ± 8,838 U/ml, n = 6) than those with less than 1.4 ng/ml IL-2 (1,370 ± 1,552 U/ml, n = 9, p = 0.0048). (C) Correlation between serum Sema4A titers with IL-10 levels. Thirty-seven MS patients whose serum IL-10 levels had been assayed were divided into two groups based on an IL-10 titer of 1.7 ng/ml. MS patients with less than 1.7 ng/ml IL-10 had higher Sema4A levels (5,224 ± 7,433 U/ml, n = 17) than those with more than 1.7 ng/ml IL-10 (2,528 ± 4,884 U/ml, n = 20, p = 0.0078). The black squares show the means. The top and bottom of the box in the box–whisker plot indicate the 25th and 75th percentiles, respectively, and the end of the whisker represents 1.5 times the interquartile range from the top of the box or the maximum point of all the data.

**p<0.01
Supplementary Figure 2.

(A) Flow cytometric analysis of Sema4A in mouse BMDCs. The cells were stained with a FITC-conjugated anti-Sema4A antibody. Sema4A was abundantly expressed on BMDCs (red line). (B) Soluble Sema4A was detected in the supernatants of mouse BMDCs. BMDCs from wild-type or Sema4A-deficient mice were biotinylated and cultured for 6 h. The cell lysates and supernatants were immunoprecipitated with an anti-Sema4A antibody and then blotted with peroxidase-conjugated streptavidin. Although soluble Sema4A was not detected in Sema4A-deficient BMDCs, an apparent band for soluble Sema4A was detected in the supernatants of wild-type BMDCs. The data are representative of three independent experiments. (C) Sema4A-deficient DCs failed to induce naïve CD4⁺ T cells to express ROR alpha and ROR gamma in Th17 skewing condition. CD62L^{high} CD4⁺ naïve T cells prepared from OT-II TCR transgenic mice were co-cultured with wild-type or Sema4A-deficient DCs in the presence of OT-II peptides under Th17-skewing condition. After five days of culture, CD4⁺ cells were positively selected by MACS and the levels of ROR alpha and ROR gamma mRNAs were analyzed by quantitative RT-PCR. *p < 0.05. (D) Sema4A-deficient BMDCs failed to differentiate naïve CD4⁺ T cells into Th1 and Th17 cells under non-skewing
conditions. CD62L\textsuperscript{high} CD4\textsuperscript{+} naïve T cells (5×10\textsuperscript{5}) prepared from OT-II T cell receptor transgenic mice were co-cultured with wild-type or Sema4A-deficient BMDCs (2.5×10\textsuperscript{5}) in the presence of various concentrations of OT-II peptides (0.2–5 μmol/ml). After 5 days of culture, CD4\textsuperscript{+} cells were positively selected by MACS and the resulting cells were restimulated with immobilized anti-CD3. The cytokine levels in the culture supernatants were measured by ELISA. (E) FACS analysis for intracellular cytokine expression in CD4\textsuperscript{+} T cells. CD62L\textsuperscript{high} CD4\textsuperscript{+} naïve T cells (5×10\textsuperscript{5}) prepared from OT-II T cell receptor transgenic mice were co-cultured with wild-type or Sema4A-deficient BMDCs (2.5×10\textsuperscript{5}) in the presence of 1 μmol/ml OT-II peptides. After 5 days of culture, IFN-γ and IL-17A positive cells among CD4\textsuperscript{+} T cells were determined by intracellular cytokine staining. (F) Sema4A-deficient mice with EAE had impaired Th1 cell generation, which was partially restored by administering Sema4A–Fc. CD4\textsuperscript{+} T cells were purified from the draining lymph nodes and spleen of immunized mice and restimulated with MOG\textsubscript{35-55} peptide. (G) FACS analysis for intracellular cytokine expression in CD4\textsuperscript{+} T cells from draining lymph nodes. Wild-type or Sema4A-deficient mice were immunized with MOG\textsubscript{35-55} peptides in CFA. Cells from the draining lymph nodes were stimulated with PMA and ionomycin 7 days after immunization, then IL-17A and IFN-γ positive cells among CD4\textsuperscript{+} T cells were analyzed.
Supplementary Table 1: Characteristics of patients with relapsing–remitting MS (RRMS) and clinically isolated syndrome (CIS) and other neurological diseases (ONDs)

<table>
<thead>
<tr>
<th></th>
<th>patients No. (F/M)</th>
<th>age (yr)</th>
<th>duration of illness (Mo)</th>
<th>relapse No./2yrs</th>
<th>EDSS score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RR-MS</strong></td>
<td>59 (51/8)</td>
<td>37.7±11.4</td>
<td>85.1±87</td>
<td>2.02±1.7</td>
<td>2.60±1.9</td>
</tr>
<tr>
<td><strong>CIS</strong></td>
<td>22 (17/5)</td>
<td>33.1±7.8</td>
<td>28.1±47</td>
<td>1.02±0.9</td>
<td>1.55±2.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ONDs</th>
<th>No. of patients (age and gender-matched)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkinson’s disease and syndrome</td>
<td>29 (2)</td>
</tr>
<tr>
<td>Encephalitis</td>
<td>14 (4)</td>
</tr>
<tr>
<td>Myositis</td>
<td>12 (4)</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>12 (3)</td>
</tr>
<tr>
<td>Spinocerebellar degeneration</td>
<td>11 (4)</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>10 (3)</td>
</tr>
<tr>
<td>Dementia</td>
<td>9 (2)</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>6 (1)</td>
</tr>
<tr>
<td>Behçet disease</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Normal pressure hydrocephalus</td>
<td>3 (0)</td>
</tr>
<tr>
<td>Muscular dystrophy</td>
<td>3 (1)</td>
</tr>
<tr>
<td>Cervical /lumbar spondylosis</td>
<td>3 (1)</td>
</tr>
<tr>
<td>Myotonia</td>
<td>2 (0)</td>
</tr>
<tr>
<td>CNS lymphoma</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Cervical cord tumor</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Leukoencephalopathy</td>
<td>1 (1)</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td><strong>126 (34)</strong></td>
</tr>
</tbody>
</table>

Characteristics of 126 patients with OND (56 females and 70 males with an average age of 53.9 ±18.1 years). Characteristics of 34 patients with OND who were age- and gender-matched to the MS group are shown in parentheses (29 females and 5 males with an average age of 38.7±16.3 years).
Supplementary Table 2: The ANCOVA method was used to compare the serum Sema4A levels in CIS or MS patients to the levels in 126 OND patients

<table>
<thead>
<tr>
<th>Factor</th>
<th>Unadjusted differences (U/ml)</th>
<th>95%CI</th>
<th>Adjusted differences (U/ml)</th>
<th>95%CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIS vs OND (OND)</td>
<td>2401</td>
<td>[651, 4150]</td>
<td>1946</td>
<td>[37, 3855]</td>
<td>0.0457</td>
</tr>
<tr>
<td>Age (10 years old)</td>
<td>-397</td>
<td>[-743, -50]</td>
<td>-201</td>
<td>[-584, 172]</td>
<td>0.2824</td>
</tr>
<tr>
<td>Gender (female)</td>
<td>-1258</td>
<td>[-2517, 1.0]</td>
<td>-510</td>
<td>[-1828, 806]</td>
<td>0.4440</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>Unadjusted differences (U/ml)</th>
<th>95%CI</th>
<th>Adjusted differences (U/ml)</th>
<th>95%CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS vs OND (OND)</td>
<td>2976</td>
<td>[1574, 4377]</td>
<td>2995</td>
<td>[1230, 4661]</td>
<td>0.0010</td>
</tr>
<tr>
<td>Age (10 years old)</td>
<td>-501</td>
<td>[-910, -90]</td>
<td>-99</td>
<td>[-534, 337]</td>
<td>0.6556</td>
</tr>
<tr>
<td>Gender (female)</td>
<td>-2122</td>
<td>[-3471, 772]</td>
<td>-864</td>
<td>[-2431, 705]</td>
<td>0.2784</td>
</tr>
</tbody>
</table>

The ANCOVA method was used to compare the serum Sema4A levels in CIS or MS patients with the levels in 126 OND patients, taking into account the differences among the various baseline covariates. Age and gender were incorporated as adjusted factors in the ANCOVA model. The mean differences represent the change from the reference or 1 unit in each factor, which were estimated by the beta-coefficients of the ANCOVA model. The adjusted mean differences were estimated using the ANCOVA model and include all factors, while the unadjusted results were estimated by the single models and each included only one factor.
Supplementary Figure 1
Supplementary Figure 2