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conjugated to anti-Sema4D mAb (A8) as described previously (28).

Induction and assessment of CIA

Eight-week-old male DBA/1J mice were purchased from Oriental Yeast (Tokyo, Japan). Bovine type II collagen (Chondrex, Redmond, WA, USA) was dissolved at 2 mg/ml in 0.05 M acetic acid and emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich). Mice were immunized by intradermal injection at the base of the tail with 100 μl (2 mg/ml) of the emulsion (day 0). Booster injections of 100 μl of emulsion consisting of equal parts of Freund's incomplete adjuvant (Sigma-Aldrich) and 2 mg/ml type II collagen in 0.05 M acetic acid were administered at the other site at the base of the tail on day 21. Mice with CIA received either intraperitoneal injection of 50 mg/kg (n = 6) or 25 mg/kg anti-Sema4D (BMA-12) antibody (30) (n = 8) or isotype control antibodies (Chugai Pharmaceutical, Tokyo, Japan) by intraperitoneal injection on day 28 and day 35. Sera were collected on day 42. Serum levels of IL-6, TNF-α, and IL-1β were determined using the Bio-Plex Pro™ Mouse Cytokine Assay (Bio-Rad Laboratories, Hercules, CA, USA).

The serum titers of anti–type II collagen antibodies were detected by ELISA (Chondrex). Clinical scores of arthritis was determined on a scale of 0–4 for each paw (0, normal; 1, erythema and swelling of one digit; 2, erythema and swelling of two digits or erythema and swelling of the ankle joint; 3, erythema and swelling of three digits or swelling of two digits and the ankle joint; and 4, erythema and severe swelling of the ankle, foot, and digits with deformity). Two paws of each mouse were evaluated histologically. Joint pathology was assessed and quantitated as described previously (38). The paws of mice treated with anti-Sema4D antibody (n = 12), control antibody

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(n = 12), or no antibody (n = 12) were stained with anti-CD31 antibodies. Vessels were counted manually in five 40× fields per paw and averaged. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Osaka University.

Statistical analysis

Data are expressed as means ± SEM. Differences between groups were tested for significance as follows: nonparametric Mann–Whitney U tests were used to compare two groups, and comparisons between three groups were performed using the Kruskal–Wallis test followed by the Mann–Whitney U test. A p-value ≤ 0.05 was considered to indicate statistical significance. The coefficients of correlation (r) between the clinical parameters and serum sSema4D levels were determined using the Pearson correlation test.

Results

sSema4D level was elevated in RA, and Sema4D-expressing cells accumulated in RA synovium.

To investigate the pathological implications of Sema4D in RA, we first measured the serum concentrations of Sema4D in patients with several autoimmune and joint destructive diseases. As shown in Figure 1A, the serum sSema4D levels were significantly higher in RA patients than in healthy individuals (mean ± SD: 5.6 ± 2.7 vs. 11.2 ± 8.4 ng/ml; $p < 0.001$) (Supplementary Table 1). By contrast, serum sSema4D levels were not elevated in patients with OA, AS, or SLE (mean ± SD: 5.2 ± 2.4, 4.9 ± 2.3, and 7.0 ± 2.6 ng/ml, respectively). Notably, Sema4D levels were also elevated

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in the synovial fluid of RA patients, but undetectable in that of OA patients (mean ± SD: 11.8 ± 7.0 vs. N.D. ng/ml; $p < 0.001$) (Figure 1B).

Immunohistochemical staining revealed accumulation of Sema4D-positive cells in RA synovium, in which Sema4D-expressing cells were mainly clustered in follicle-like germinal centers of RA synovium. However, such accumulation and clustering of Sema4D-expressing cells were not observed in OA synovium (Figure 1C). CD3 and CD20 were co-localized in Sema4D-stained follicle-like germinal centers, indicating that the Sema4D-expressing cells were synovial infiltrating lymphocytes (Supplementary Figure 1). By contrast, CD68-positive macrophages faintly express Sema4D. Additionally, the distribution of CD68 positive cells is patchy, of which localization patterns are different from those of T and B cells.

Serum sSema4D levels were correlated with RA disease activity and biomarkers

To determine the clinical implications of sSema4D, we examined the correlations between serum sSema4D levels and clinical features. Table 1 summarizes the correlations between patient's clinical features and serum sSema4D levels. There were no apparent correlations between sSema4D and age, sex, disease duration, or medications (data not shown). By contrast, serum sSema4D levels were positively correlated with disease activity markers such as DAS28 ($r = 0.383$, $p < 0.01$), CRP ($r = 0.346$, $p < 0.01$), and RF ($r = 0.328$, $p < 0.01$). In addition, bone metabolic markers such as BAP ($r = 0.255$, $p < 0.05$) and urinary deoxypyridinoline ($r = 0.318$, $p < 0.05$) also correlated with serum sSema4D levels. The scatter plots confirmed the relationships between serum levels of sSema4D and these disease activity markers (Figure 2A). Sequential analysis of serum sSema4D level was performed in RA patients

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treated with bDMARDs (n = 17); specifically, serum levels of sSema4D were evaluated before and 6 months after the initiation of bDMARDs therapy (14 patients were treated with TNF inhibitors, and 3 patients were treated with tocilizumab). A significant decrease in serum sSema4D after bDMARDs treatment was observed in patients who were good responders according to the EULAR response criteria (39) (Figure 2B). By contrast, sSema4D levels were not changed in moderate or non-responder patients (Figure 2B). The post/pre ratio of Sema4D was significantly correlated with the post/pre ratio of DAS28 ($r = 0.799$, $p < 0.001$) (Figure 2C), suggesting the involvement of Sema4D in determining the clinical status of RA. The reductions in serum sSema4D did not differ significantly between patients treated with TNF inhibitors and those treated with an IL-6 inhibitor. Thus, the reduction in serum sSema4D simply correlated with reduction in disease activity.

Shedding of Sema4D in RA patients

Sema4D is abundantly expressed in T cells but weakly expressed in other cell types, such as B cells (24). Sema4D is also expressed on antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs) (32). As shown in Figure 1C, we observed Sema4D-expressing cells in the synovium. Next, we examined Sema4D expression in PBMCs in RA patients and healthy individuals. In healthy individuals, cell-surface Sema4D was expressed abundantly on $CD3^+$ and $CD14^+$ cells, and at lower levels on $CD19^+$ cells (Figure 3A). Furthermore, the cell-surface expression of Sema4D was downregulated in all cells from RA patients, especially in cells positive for CD3 or CD14. By contrast, qRT-PCR revealed that the mRNA level of Sema4D was not reduced in RA patients (Figure 3B), suggesting that the reduction in cell-surface

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Sema4D was due to shedding of Sema4D from the cell surface.

Previous reports showed that although Sema4D is a membrane-bound protein, it can be cleaved from the cell surface by metalloproteinases to yield a soluble form (27). Therefore, we examined the proteolytic cleavage of Sema4D. ADAMs, ADAMTSs, and MMPs are proteolytic enzymes that digest extracellular matrix proteins such as collagen, and process bioactive substances that play a physiological role in RA (40). We incubated Sema4D-expressing cells (THP-1) with recombinant metalloproteinases including MMP-3, MMP-9, ADAM17/ Tumor necrosis factor-α–converting enzyme (TACE), and ADAMTS4, and then measured sSema4D levels in the culture supernatants. ADAMTS4 significantly induced the release of sSema4D into the culture supernatant (Figure3C, Supplementary Figure 2). Consistent with this, we detected elevated levels of ADAMTS4 in serum and synovial fluid in the RA patients examined in this study (Figure 3D, E).

Proinflammatory cytokines production by sSema4D

To determine the pathogenic role of elevated sSema4D in RA, we investigated the effect of sSema4D on TNF-α and IL-6 production. Treatment with naturally cleaved sSema4D increased production of TNF-α and IL-6 by $CD14^+$ monocytes in a dose-dependent manner (Figure 4A). In addition, recombinant sSema4D-Fc also dose-dependently induced TNF-α and IL-6 production by PBMCs (Supplementary Figure 3). Moreover, anti-Sema4D antibody suppressed TNF-α and IL-6 production induced by sSema4D (Figure 4B). CD72 is a receptor for Sema4D, and an agonistic CD72 antibody also induced TNF-α and IL-6 production in $CD14^+$ monocytes (Supplementary Figure. 4), in which Sema4D induced dephosphorylation of CD72

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(Supplementary Figure 5).

Because previous reports showed that inflammatory cytokine induce ADAMTS4 in synovial cells (41), we examined ADAMTS4 production in TNF-α and IL-6 stimulated synovial cells. TNF-α and IL-6 treatment increased the mRNA and protein levels of ADAMTS4 (Figure 4C, D). These results not only indicated that sSema4D can induce the production of proinflammatory cytokines, but also implied that such cytokines in turn up-regulate the cleavage of Sema4D by ADAMTS4.

Blocking of Sema4D ameliorates the severity of mouse arthritis

To determine the pathological roles of Sema4D in arthritis, we examined the effect of anti-Sema4D antibody treatment on CIA. The arthritis scores in anti-Sema4D antibody–treated mice were significantly lower than those in control mice in dose dependent manners (Figure 5A, Supplementary Figure 6). Histological analysis revealed that Sema4D blockage in CIA mice also reduced inflammatory infiltration into the synovium, decreased pannus formation, and ameliorated erosion of adjacent cartilage and bone (Figure 5B). The histological scores in the joints of anti-Sema4D antibody–treated mice were significantly reduced (Figure 5C). Furthermore, serum TNF-α and IL-6 levels at day 42 were significantly reduced in anti-Sema4D–treated mice (Figure 5D). We evaluated angiogenesis by antibody staining for CD31, a marker of blood vessel endothelium. Mice treated with anti-Sema4D exhibited poor induction of angiogenesis at inflammatory sites (Supplementary Figure 7A, B). Additionally, the serum level of anti-collagen antibody was reduced in these animals (Supplementary Figure 8).

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Discussion

In this study, we investigated the clinical implications of sSema4D in RA. Levels of sSema4D were elevated in RA sera and synovial fluids. However, sSema4D was not elevated in OA and SLE, suggesting that high sSema4D levels are specifically associated with RA. Serum levels of sSema4D correlated with known clinical and biological markers of RA (Table 1). In particular, serum levels of sSema4D correlated with DAS28, CRP, RF, and urinary deoxypyridinoline in RA patients. Successful treatment of RA reduced serum levels of sSema4D, and reduction in sSema4D level was significantly correlated with reduction in clinical disease activity, as determined by the DAS28 marker. Collectively, these results suggest that sSema4D is a potentially useful biomarker for RA disease activity. In addition, serum levels of sSema4D were correlated with CRP, a well-known acute phase protein induced by proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (42). Because sSema4D induced IL-6 and TNF-α production from $CD14^+$ monocytes, it is possible that sSema4D affects CRP production via induction of IL-6 and TNF-α in RA patients. The titer of RF was also correlated with serum levels of sSema4D. Given that Sema4D has been implicated in activation of B cells and antibody production (28, 31), sSema4D may be directly relevant to RF production.

Recent work showed that Sema4D expressed in osteoclasts inhibits bone regeneration by osteoblast inhibition (15). Consistent with this, we found that some bone metabolic markers were correlated with serum levels of sSema4D in RA (Table 1). The bone formation marker BAP and the bone resorption marker u-DPD were correlated with serum sSema4D. However, other bone formation markers (such as osteocalcin) and bone resorption markers (such as 1CTP and serum NTx) were not

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correlated with serum sSema4D. The relationship between serum levels of sSema4D and bone metabolic markers in RA patients is controversial, although the local concentration of Sema4D may be relevant to joint destruction in RA. Further studies will be required to determine the importance of Sema4D in bone destruction in RA.

Because Sema4D is strongly expressed in immune cells (24), we initially assumed that elevated expression of *Sema4D* explained the increase in sSema4D in RA serum and synovial fluid. Contrary to our expectation, however, FACS analysis revealed that levels of Sema4D were actually reduced in lymphocytes and monocytes from peripheral blood of RA patients. Because expression of *Sema4D* mRNA was stable in all cells, it is likely that the relative reduction in cellular levels of Sema4D was due to cleavage and shedding of Sema4D from the cell surface. In support of this notion, a previous study showed that EDTA, an inhibitor of metalloproteases, inhibits sSema4D secretion, suggesting that sSema4D is produced via a shedding mechanism (27). Other reports showed that ADAM17 regulates Sema4D exodomain cleavage on activated platelets (43, 44). Taken together, these studies prompted us to investigate the generation and function of Sema4D.

We examined several proteolytic enzymes as candidate sheddases for sSema4D. Proteolytic enzymes such as ADAMTSs, ADAMs, and MMPs influence inflammation and progression of arthritis (45-47). Here, we showed that induction of sSema4D is dependent on ADAMTS4. Originally, ADAMTS4 was considered to play a key role in the degradation of cartilage proteoglycan (aggrecan) in OA and RA. Consistent with this, the levels of ADAMTS4 were elevated in RA sera, and TNF-α and IL-6 induced the production of ADAMTS4.

In this study, we demonstrated that production of inflammatory cytokines such

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as TNF-α and IL-6 increased upon sSema4D-stimulation. We here showed that sSema4D induced dephosphorylation of CD72 (Supplementary Figure 5). CD72 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic region (48). We have previously reported that Sema4D induces dephosphorylation of CD72 turning off its negative signal in B cells (32). It thus appears that Sema4D is involved in cytokine production in monocyte as well.

Our results indicated that sSema4D induces TNF-α and IL-6 production, and that both TNF-α and IL-6 can induce ADAMTS4, which is involved in generation of sSema4D. Therefore, we hypothesized that the sSema4D/TNF-α and IL-6/ADAMTS4 axis functions as a vicious cycle, triggering an inflammatory loop that contributes to the pathogenesis of RA. It is well known that TNF-α and IL-6 induce osteoclastogenesis through RANKL production, and ADAMTS4 induces cartilage degradation in RA (47). In addition, Sema4D inhibits bone formation (15). Therefore, the autocrine loop involving Sema4D induces cartilage destruction, inhibits bone regeneration, and evokes continuous inflammatory symptoms. This Sema4D loop may play a central role in RA inflammation and the associated joint destruction (Supplementary Figure 9).

We analyzed the effect of anti-Sema4D antibody treatment in a mouse model of inflammatory arthritis. Blockage of Sema4D in CIA exerted favorable therapeutic effects, decreasing destruction of cartilage and bone, cell infiltration into the synovium, and production of TNF-α and IL-6 (Figure 5). To investigate the therapeutic effects of anti-Sema4D antibody on ongoing arthritis, we administered anti-Sema4D antibody after arthritis had already commenced. These observations suggest that Sema4D represents a possible therapeutic target for treatment of RA. Recently developed bDMARDs inhibit TNF-α and IL-6 function; however, these therapies do not inhibit

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cytokine production directly. Therefore, RA flare-ups are often observed after cessation of bDMARD therapy. Thus, it seems likely that direct inhibition of TNF-α and IL-6 production by anti-Sema4D therapy would be useful for RA management. An anti-Sema4D antibody is currently undergoing a phase I clinical trial in human cancer patients (49) (NCT01313065). However, the long-term feasibility of anti-Sema4D antibody is still unknown. Careful and well-designed clinical applications would be required.

In summary, we demonstrated that serum sSema4D levels are well correlated with known markers of clinical features and laboratory findings. The critical roles of Sema4D in RA pathogenesis suggest that Sema4D is potential novel target for RA treatment.

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Author contributions

All authors were involved in drafting the article or critically revising the important intellectual content, and all authors approved the final published version. Y. Yoshida and A. Ogata had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Correlations between soluble Sema4D levels and clinical features in RA patients. n = 101.

Category		r	p
Clinical disease activity	DAS28	0.383	< 0.01
Autoantibodies	RF	0.328	< 0.01
	ACPA	−0.041	N.S.
Biomarkers	CRP	0.346	< 0.01
	MMP-3	0.055	N.S.
Bone turnover markers	BAP	0.255	< 0.05
	Osteocalcin	0.092	N.S.
	u-DPD	0.318	< 0.05
	1CTP	0.091	N.S.
	Serum NTx	0.026	N.S.
DEXA	X-ray of spine	−0.145	N.S.

r = correlation coefficient; DAS28, disease activity score 28; RF, rheumatoid factor; ACPA, anti-citrullinated peptide antibody; CRP, C-reactive protein; MMP-3, matrix metalloproteinase 3; BAP, bone alkaline phosphatase; u-DPD, urinary deoxypyridinoline ; 1CTP, pyridinoline cross-linked carboxyterminal telopeptide of type collagen; NTx, N-terminal telopeptide, DEXA, dual-energy X-ray absorptiometry

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Figure Legends

Figure 1. The level of soluble Sema4D in RA sera and synovial fluids, and *Sema4D* expression in RA synovium.

A) sSema4D levels of sera from healthy individuals and 101 patients with rheumatoid arthritis (RA), 10 with osteoarthritis (OA), 10 with ankylosing spondylitis (AS), and 34 with systemic lupus erythematosus (SLE). B) Levels of sSema4D in synovial fluids of seven patients with RA and ten with OA. C) H&E staining and immunohistochemical staining for Sema4D, CD3, CD20, and CD68 in synovial tissue of patients with OA or RA. Representative images are shown from seven RA patients and ten OA patients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. Correlations of serum levels of soluble Sema4D with RA disease activities.

A) Serum levels of sSema4D were positively correlated with disease activity score 28 (DAS28), C reactive protein (CRP), urinary deoxypyridinoline (u-DPD), and rheumatoid factor (RF). B) Serum sSema4D levels before and after bDMARDs treatment in nine good responders and eight moderate responders or non-responders, according to the DAS improvement criteria. C) Correlation of serum sSema4D reduction (post-biologics/pre-biologics) with DAS28 improvement (post-biologics/pre-biologics) $n = 17$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Sema4D expression, and soluble Sema4D production with ADAMTS4 as the sheddase.

Sema4D in rheumatoid arthritis.

A) Histogram of cell-surface expression of Sema4D in peripheral blood in $CD3^+$, $CD19^+$, and $CD14^+$ cells. Results shown are representative findings from five RA patients and five healthy individuals. B) mRNA expression of *Sema4D* in peripheral blood $CD3^+$, $CD19^+$, and $CD14^+$ cells. Results from five RA patients and five healthy individuals were shown. C) Levels of sSema4D in culture supernatant of THP-1 cells cultured with recombinant MMP3, MMP9, ADAM17, and ADAMTS4 (n = 5 per group). Representative results of three independent experiments are shown. D) Serum levels of ADAMTS4 in 20 RA patients and 16 healthy individuals. E) Synovial fluid levels of ADAMTS4 in seven RA patients and ten OA patients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Inflammatory cytokine production induced by Sema4D, and elevated ADAMTS4 expression by inflammatory cytokines

A) TNF-α and IL-6 levels in culture supernatant of $CD14^+$ monocytes from RA patients stimulated with naturally cleaved sSema4D for 72 hours. Representative results of three independent experiments are shown. B) TNF-α and IL-6 levels in culture supernatant of $CD14^+$ monocytes from RA patients stimulated with naturally cleaved sSema4D for 48 hours, with or without anti-Sema4D antibody. Representative results of three independent experiments are shown. C, D) Elevated ADAMTS4 mRNA and protein levels in primary cultures of synovial cells from RA patients. Data were compiled from five independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5. Blocking Sema4D ameliorates the severity of collagen-induced arthritis (CIA) in mice.

Sema4D in rheumatoid arthritis.

A) Average arthritis scores of CIA mice. Anti-Sema4D or control antibody (50 mg/kg) was administrated intraperitoneally on days 28 and 35 (arrow) (n = 6 per group). Data are representative of three independent experiments. B) Sections of a mouse ankle joint on day 42 after first immunization. Sections were stained with H&E and Safranin O. C) Average pathological scores of paw sections on day 42 (n = 12 per group). D) Serum levels of IL-6 and TNF-α on day 42 (n = 6). Data are means ± SEM, and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

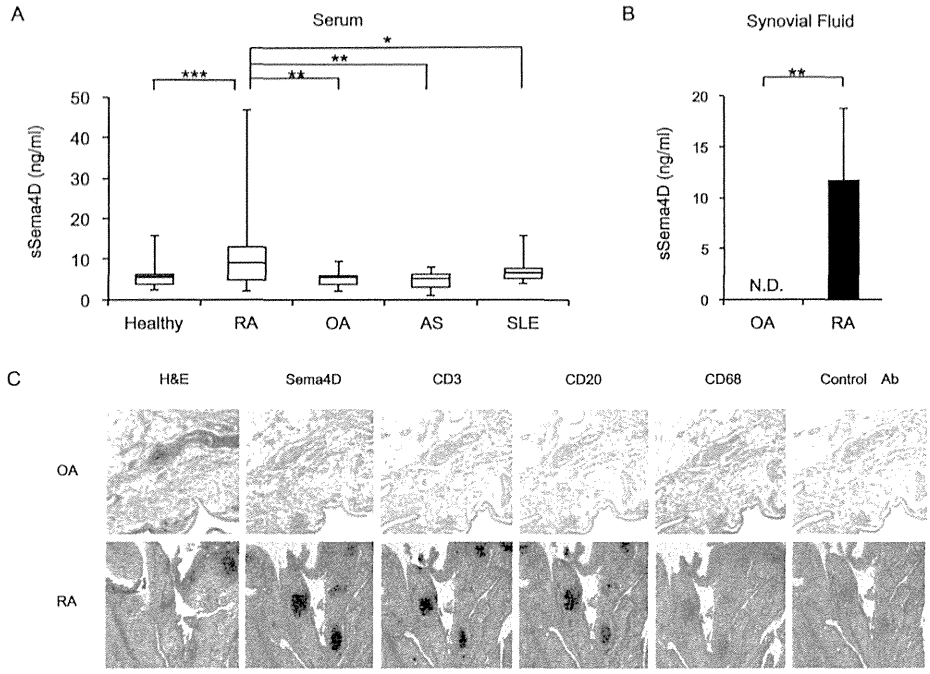

Figure 1. The level of soluble Sema4D in RA sera and synovial fluids, and Sema4D expression in RA synovium.
A) sSema4D levels of sera from healthy individuals and 101 patients with rheumatoid arthritis (RA), 10 with osteoarthritis (OA), 10 with ankylosing spondylitis (AS), and 34 with systemic lupus erythematosus (SLE). B) Levels of sSema4D in synovial fluids of seven patients with RA and ten with OA. C) H&E staining and immunohistochemical staining for Sema4D, CD3, CD20, and CD68 in synovial tissue of patients with OA or RA. Representative images are shown from seven RA patients and ten OA patients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

230x170mm (150 x 150 DPI)