Table 2 Laboratory data of rheumatoid arthritis patients treated with adalimumab at the onset of PCP

Pt	WBC (/μl)	Lymphocytes (/μl)	SpO_2 or PaO_2 (Torr) [O_2, l/min][a]	Serum β-D-glucan (μg/ml) [normal range at the institute]	*Pneumocystis jirovecii* PCR
1	7,870	912	SpO_2 96 % [0]	289 [<11]	+
2	5,100	1,989	SpO_2 92 % [0]	30.5 [<11]	+
3	6,300	252	55.1 [0]	1041 [<11]	NA
4	6,200	874	68.0 [0]	25.76 [<11]	+
5	8,050	1,110	60.4 [0]	50.3 [<20]	NA
6	6,400	716	58.9 [0]	37.8 [<6]	+
7	5,660	1,041	71.8 [0]	22.1 [<11]	+
8	6,800	279	31.3 [0]	29 [<11]	+[b]
9	15,900	832	85.7 [3]	79.5 [<20]	+
10	7,500	1,350	65.4 [0]	22.3 [<20]	+
11	8,400	3,696	69.5 [0]	16.4 [<11]	+
12	11,700	1,029	26.1 [0]	21.06 [3.5]	+
13	7,950	1,761	SpO_2 85 % [2]	160 [<5]	+
14	9,580	34	56.7 [0]	13.0 [<11]	NA[b]
15	5,700	1,140	55.1 [0]	13.0 [<11]	–
16	7,000	1,330	56.1 [10]	21.38 [<11]	+
17	3,200	704	52.5 [0]	419 [<11]	+[b]
Median (IQR)	7,000 (5950–8225)	1,029 (710–1340)	Not applicable	Not applicable	Not applicable

PCP Pneumocystis jirovecii pneumonia, *Pt* patient, *WBC* white blood cell, *PCR* polymerase chain reaction, *NA* not assessed, *SpO2* oxygen saturation measured using a pulse oximeter, *IQR* interquartile range

[a] Oxygen therapy during the measurement of PaO_2

[b] *Pneumocystis jirovecii* microscopically detected in bronchoalveolar-lavage fluid

the major components of the cell walls of fungi and a serum maker for PCP [17, 18], were elevated in all patients. Results of sputum culture performed in 14 patients revealed no causative bacteria or fungi.

Chest radiographs and thoracic CT scans were analyzed for all 17 patients. The most common CT finding was ground-glass opacity (GGO) (in 17 patients), either with sharp demarcation by interlobular septa in one patient (type A GGO) (Fig. 1a) or without interlobular septal boundaries in 14 patients (type B GGO) (Fig. 1b). Two patients demonstrated mixed patterns (type C).

Treatment and clinical course of PCP in patients with RA receiving adalimumab

All patients were hospitalized on the same day that PCP was suspected. Fourteen patients (all except for patients 2, 5, and 11) received oxygen therapy on admission. MTX and adalimumab were immediately discontinued in all patients. All patients received therapeutic doses of trimethoprim/sulfamethoxazole (TMP/SMX). Because of adverse drug reactions that included skin eruptions, liver dysfunction, thrombocytopenia, and hyperpotassemia, TMP/SMX was reduced or stopped in eight patients. One patient was changed to pentamidine isethionate. Sixteen patients were concomitantly treated with high-dose corticosteroids within a few days after admission. Eleven patients were empirically treated with antibiotics and four with antifungal agents. Three patients (patients 1, 3, and 8) were intubated on the day of admission because of progressive respiratory failure; two of these patients responded to treatment and were successfully weaned from artificial ventilation. One patient (patient 17) died because of PCP with progressive respiratory failure. Two patients died because of multiple organ failure (patient 12) and gastrointestinal bleeding, cytomegalovirus infection, and multiple organ failure (patient 3) after improvement of PCP.

Case–control study

In order to characterize the PCP group more precisely, we compared demographic information, comorbidities, treatments, and laboratory data at baseline (i.e., at the initiation of treatment with adalimumab) between the PCP and non-PCP groups using a univariate analysis (Table 3). The PCP group was significantly older ($p = 0.003$) and had a more advanced radiographic stage (Steinbrocker's stage III or IV) ($p = 0.010$) than the non-PCP group. Although the rates of patients with preexisting pulmonary diseases and diabetes mellitus in the PCP group were numerically higher, these differences were not statistically significant. There were no differences in disease duration and the dosages of prednisolone and methotrexate between the two groups. None of the patients in the PCP group and fourteen patients in the non-PCP group received prophylaxis for

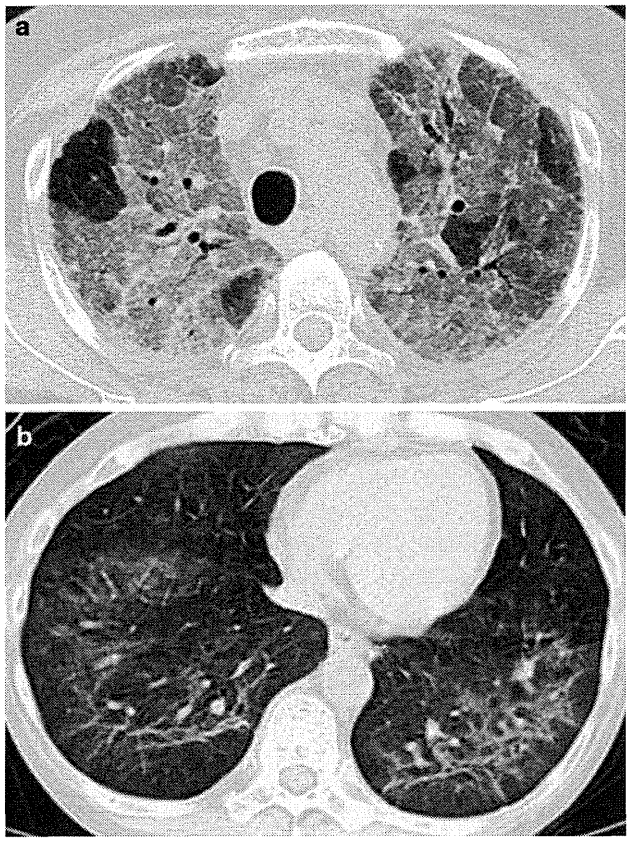

Fig. 1 Representative thoracic computed tomography findings of rheumatoid arthritis patients who developed *Pneumocystis jirovecii* pneumonia while receiving adalimumab. **a** Ground-glass opacity (GGO) with sharp demarcation by interlobular septa (type A) (patient 12). **b** Inhomogeneous GGO without obvious demarcation by interlobular septa (type B) (patient 1)

PCP for at least three months during the observation period. Twelve patients used TMP/SMX and two used aerosolized pentamidine.

Based on the results of the univariate analysis, age, sex, pulmonary comorbidities and Steinbrocker's stage of RA were analyzed as candidate predictors for the development of PCP. The Cox proportional-hazards regression analysis revealed a significant association between advanced radiographic stage (stage III or IV) and development of PCP (hazard ratio (HR) 3.76, 95 % confidence interval (CI) 1.03–7.30, $p = 0.045$). While the hazard ratios of older age and preexisting pulmonary diseases tended to be higher, they did not reach statistical significance (Table 4).

Because 14 patients in the non-PCP group received prophylaxis for PCP, we performed the multivariate analysis after excluding these 14 patients, and found a significant association between older age and development of PCP (HR 3.31, 95 % CI 1.09–10.0, $p = 0.034$). The HR of the radiographic stage did not reach statistical significance (HR 2.82, 95 % CI 0.74–10.7) in this model.

Discussion

We accumulated the largest possible number of patients with RA who developed PCP during treatment with adalimumab, and described the clinical and radiologic characteristics of the 17 patients that we found.

Adalimumab is the third TNF antagonist to be approved in Japan. We have already reported the clinical characteristics and risk factors for PCP in RA patients treated with infliximab or etanercept [10–12]. The median interval (range) between the first dose of TNF antagonists and the onset of PCP was 12 weeks (range 4–38) for adalimumab, nine weeks (range 2–90) for infliximab [11], and 14 weeks (range 3–43) for etanercept [12]. PCP developed within six months in the majority of RA patients after the initiation of each TNF antagonist: 90 % for infliximab, 80 % for etanercept, and 76 % for adalimumab.

Previous studies have revealed that patients without HIV infection develop PCP abruptly and progress to fulminating pneumonia with acute respiratory failure [21, 22]. We also reported that RA patients treated with infliximab or etanercept developed PCP rapidly and progressed to severe respiratory failure [10–12]: 18 out of 21 PCP patients using infliximab, all 15 PCP patients using etanercept, and 14 of 17 PCP patients in this study showed severe hypoxemia and required oxygen therapy. The mortalities of the patients with PCP given infliximab (0 %) or etanercept (6.7 %) are numerically lower than the mortality of this study, in which three patients (17.6 %) died. Walzer etal. [23] identified older age, second or third episode of PCP, low hemoglobin level, low PaO_2 breathing room air at admission, pulmonary Kaposi sarcoma, and presence of medical comorbidity as early predictors of mortality of PCP in HIV-infected patients. Although such prognostic factors in non-HIV PCP patients are unknown, all three patients in our study who died were females over 70 years old, and their PaO_2 on admission was less than 60 Torr. Two of these patients had pulmonary comorbidities. One patient had a quite high serum level of BDG, and one was positive for both microscopic detection and the PCR test for the organism. These data would suggest severe pulmonary injury at presentation and a high burden from *P. jirovecii.*

In our study, all patients received therapeutic doses of TMP/SMX. However, eight patients (47.1 %) were obliged to reduce the dosage or stop using the drug due to adverse drug reactions, such as gastrointestinal symptoms and hematological abnormalities. Kameda et al. [24] also reported that more than one-third of the patients could not complete the standard protocol of the TMP/SMX treatment. These data indicate that the optimal dosage and treatment period of TMP/SMX for PCP should be investigated. The clinical benefit of adjunctive corticosteroid

Table 3 Baseline characteristics of patients with rheumatoid arthritis treated with adalimumab

Characteristic	PCP group ($n = 17$)	Non-PCP group ($n = 89$)	p value
Age (years)[a]	68 (48–78)	60 (24–79)	0.003
Female (%)	70.6	80.9	0.255
Disease duration (years)[a]	8.0 (0.7–36)	9.5 (3–40)	0.491
Chronic pulmonary disease (%)	47.1	22.5	0.107
Diabetes mellitus (%)	23.5	7.9	0.074
Steinbrocker's radiographic stage (III or IV) (%)	82.4	48.3	0.010
Steinbrocker's functional class (III or IV) (%)	17.6	19.1	0.596
MTX (%)	100	86.5	0.108
MTX (mg/week)[a]	8.0 (4–10)	8.0 (4–15)	0.119
MTX ≥ 8 mg/week (%)	11.8	28.1	0.228
PSL (%)	76.5	56.2	0.118
PSL (mg/day)[a]	5.0 (3–12)	5.0 (1–17)	0.529
PSL ≥ 5 mg/day (%)	52.9	33.7	0.131
WBC <4,000/μl (%)	0	2.2	0.731
Serum IgG (mg/dl)[a]	1421 (846–1954)	1316 (827–3165)	0.817

PCP Pneumocystis jirovecii pneumonia, *MTX* methotrexate, *PSL* prednisolone, *Chronic pulmonary disease* = interstitial pneumonia, bronchiectasis, chronic obstructive pulmonary diseases, bronchial asthma, middle lobe syndrome, old pulmonary tuberculosis

p values were calculated using the Mann–Whitney test for continuous variables or χ^2 test for categorical variables

[a] Median (range)

Table 4 Cox regression analysis of risk factors for the development of PCP in rheumatoid arthritis patients treated with adalimumab

	Hazard ratio (95 % CI)	p value
Age (≥ vs. <65 years old)	2.38 (0.80–7.05)	0.119
Gender (female vs. male)	0.53 (0.18–1.58)	0.258
Chronic pulmonary disease (yes vs. no)	2.14 (0.79–5.76)	0.133
Steinbrocker's radiographic stage (III/IV vs. I/II)	3.76 (1.03–7.30)	0.045

PCP Pneumocystis jirovecii pneumonia, *CI* confidence interval

Chronic pulmonary disease = interstitial pneumonia, bronchiectasis, chronic obstructive pulmonary diseases, bronchial asthma, middle lobe syndrome, old pulmonary tuberculosis

therapy for PCP patients without HIV infection has not been established [25]. All patients except for one in this study received adjunctive corticosteroid therapy with various treatment durations and dosages, including intravenous methylprednisolone pulse therapy. Nineteen out of 21 PCP patients who used infliximab and nine out of 15 PCP patients who used etanercept used adjunctive corticosteroid therapy as well [11, 12]. Pareja etal. [26] retrospectively analyzed the clinical courses of 30 cases of severe PCP without HIV infection, among which 16 cases who received high doses of adjunctive corticosteroid therapy presented a good clinical outcome. Considering the intense inflammatory response to the organism in non-HIV PCP patients [25] and the favorable effectiveness of adjunctive corticosteroid therapy in previous studies, it is necessary to consider treatment with corticosteroids for PCP patients with RA who show hypoxemia at presentation or during their clinical courses.

In the present study, using the Cox proportional-hazards analysis, Steinbrocker's radiographic stage III or IV was identified as a statistically significant risk factor for the development of PCP in patients receiving adalimumab. Although there was no significant difference in Steinbrocker's functional class, it is plausible that advanced radiographic stages associated with decreased physical function contributed to the development of PCP. Steinbrocker's functional class may be less sensitive to the detection of such differences in physical function. On the other hand, older age was a significant risk factor in another Cox proportional-hazards regression analysis after excluding those who received TMP/SMX or aerosolized pentamidine for prophylaxis at least three months from the non-PCP group. The different results from the Cox proportional-hazards regression analyses can be explained by the fact that nine out of 14 patients given prophylaxis were aged 65 or older. Pulmonary diseases were not significant risk factors for PCP in either Cox proportional-hazards analysis, perhaps because of the small number of PCP cases enrolled.

None of the 17 patients had received prophylaxis for PCP. Vananuvat etal. [27] conducted a retrospective cohort study for patients with connective tissue diseases (CTD) who were at risk for PCP in order to examine the effectiveness of primary prophylaxis with TMP/SMX and the incidence of adverse drug reactions (ADR) of TMP/SMX. Six patients without and none with prophylaxis developed PCP; the overall incidence rate was 4.3 % and the relative risk reduction was 100 %. Five patients (8.5 %) developed ADR: four had drug eruptions and one had mild hepatitis. These data indicate that TMP/SMX can be used effectively for primary prophylaxis against PCP.

There are definite limitations to our study. First, we included definite and presumptive cases of PCP in our analysis. It has been well documented that the microscopic detection of *P. jirovecii* is difficult in non-HIV PCP [28, 29], as confirmed in this and our previous studies. To increase the specificity of the diagnosis of PCP without detecting the organism microscopically, we utilized composite diagnostic criteria, including clinical symptoms, laboratory tests, radiological findings, and the clinical

course. Kameda etal. found no difference in clinical characteristics of PCP in RA patients between definite PCP (i.e., acute-onset diffuse interstitial lung disease and microscopic positivity for *P. jirovecii* or positivity in both PCR test and BDG) and probable PCP (acute-onset diffuse interstitial lung disease and positivity in either PCR test or BDG) [24]. Their data support the use of composite diagnostic criteria for PCP in patients with RA. Second, we had only 17 RA patients with PCP, which decreased the sensitivity of the Cox proportional-hazards analysis for detecting statistically significant risk factors. Third, a higher incidence of PCP in Japanese RA patients receiving TNF antagonists and their risk factors have gained widespead recognition in the past few years by Japanese rheumatologists who use TNF antagonists; this may have affected the characteristics of the patients who were treated with adalimumab. For example, we found a significant difference in the daily dose of PSL between the PCP and non-PCP groups in our previous two studies, but not in this study.

In summary, the results of this study show that PCP is a serious complication in patients with RA who receive treatment with adalimumab. The majority of the patients developed PCP early in the course of adalimumab treatment and progressed to respiratory failure. Treating physicians should therefore take prophylaxis with TMP/SMX or other agents into consideration in RA patients with a high risk for PCP. Careful monitoring of clinical manifestations and laboratory tests for early diagnosis and treatment of PCP are strongly recommended.

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Review Article

Pathological Role of Interleukin-6 in Psoriatic Arthritis

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Psoriatic arthritis (PsA) is a clinical manifestation of psoriatic disease. Although the pathogenesis of PsA remains unknown, PsA can be managed by treatments similar to those used for rheumatoid arthritis (RA). Because interleukin-(IL-) 6 has been suggested to have a pathogenic role in PsA, a humanized anti-IL-6 receptor antibody tocilizumab treatment for PsA was recently tried. However, the efficacy of tocilizumab for PsA was not favorable. This suggests that the pathogenic roles of IL-6 in PsA and RA are different. In RA, tumor necrosis factor (TNF) primarily contributes to the arthritis effector phase and IL-6 contributes to the arthritis priming phase. In PsA, the TNF-related effector phase is similar to that in RA, but the IL-6-related priming phase might not be critical. This paper discusses the role of IL-6 in PsA.

1. Introduction

Psoriatic arthritis (PsA) was originally designated as inflammatory arthritis associated with psoriasis that was usually negative for the rheumatoid factor and is now considered as a clinical manifestation of psoriatic disease [1]. Although there are no diagnostic tests for PsA, it is a condition that is distinguishable from rheumatoid arthritis (RA); the characteristic features of PsA and RA are slightly different. In PsA, peripheral arthritis evolves with a distinct joint pattern that possibly involves the distal interphalangeal joints. Dactylitis with enthesitis, involving the entire digit, is a characteristic feature of PsA. Furthermore, articular damage assessed by radiographic erosion is more common in RA and typically reveals an asymmetric pattern in PsA. Despite these differences, the therapeutic options, including tumor necrosis factor (TNF) inhibitors, and the methods for assessing the disease activity are mostly the same.

Increased production of interleukin-(IL-) 6 is well known in psoriasis and PsA [2, 3]. Mice with epidermal overexpression of IL-6 (K14-IL-6 transgenic mice) exhibit a psoriasis phenotype [4]. The transcription factor signal transducer and activator of transcription 3 (STAT3) is upregulated in psoriasis. IL-6, which induces STAT3 phosphorylation, is also thought to be a potential therapeutic target [5]. In addition, serum IL-6 levels correlate with PsA disease severity [6]. IL-6 is thought to have similar roles in inflammatory arthritis associated with both RA and PsA. This supports the notion that targeted treatments against IL-6 might be effective [7].

2. Tocilizumab Treatment for Seronegative Spondyloarthritis

A humanized anti-IL-6 receptor antibody, tocilizumab (TCZ), was recently approved for treating RA patients, and its efficacy for these patients has been demonstrated [8]. The clinical applications of TCZ for PsA have not been well described, although there are some reports on the efficacy of TCZ for seronegative spondyloarthritis (SNSA). SNSA is characterized by the absence of the rheumatoid factor and includes diseases such as PsA. Several case reports have shown favorable outcomes with TCZ treatment for reactive arthritis [9] and ankylosing spondylitis (AS) [10–14].

However, a recent larger case series reported that there were unfavorable outcomes with TCZ treatment for AS. Dudler and Aubry-Roziier reported on the efficacy of TCZ for patients with axial spondyloarthropathies [15]. Among 18 cases, three patients had skin psoriasis. No significant

clinical benefits were observed with TCZ for peripheral arthropathies. Del Castillo Piñol et al. reported on five refractory spondyloarthritis (SpA) patients treated with TCZ [16]; a response to TCZ was found in only one of the five severe cases of axial SpA. Lekpa et al. reported on 21 spondyloarthritis patients who were treated with TCZ, for whom anti-TNF-α therapy had failed [17]. Although TCZ decreased acute-phase reactions, TCZ failed to substantially improve axial spondyloarthritis and was inconsistently effective for peripheral spondyloarthritis.

More recently, the results of two randomized control trials (RCTs) that used IL-6 inhibitors were reported. Sieper et al. reported on a phase 2 study of TCZ for AS [18]. They enrolled 102 AS patients, and 51 patients were treated with TCZ for 12 weeks. Although the C-reactive protein (CRP) levels declined, AS symptoms were not improved. The efficacy of TCZ for treating AS was not demonstrated in this RCT. In addition, a phase 2 RCT of another IL-6 receptor antibody, sarilumab, also failed to demonstrate its efficacy in AS patients assessed by their 20% improvement in Assessment of Ankylosing Spondylitis (ASAS20) responses at 12 weeks [19].

3. TCZ Treatment for Psoriatic Arthritis

We recently reported on two PsA patients who were treated with TCZ [20]. The first was a 35-year-old man. He was started on 8 mg/kg every 4 weeks. His clinical course is shown in Figure 1. Before TCZ treatment, his clinical disease activity index (CDAI) was 30.8, and his Psoriasis Area and Severity Index (PASI) was 11.3. After seven TCZ infusions, his CRP levels had not improved (7.20–5.71 mg/dL), suggesting that a 4-week interval between the TCZ infusions was not sufficient to inhibit the IL-6 activity in this patient. After a 2-week interval between infusions, his CRP levels returned to normal. However, both his CDAI and PASI had not improved. Adalimumab was then initiated. Although his CRP levels increased (1.31 mg/dL) and his PASI did not improve rapidly, his CDAI was significantly improved.

The second case was a 28-year-old man. TCZ was started at 8 mg/kg every 4 weeks. His clinical course is shown in Figure 2. His CRP levels normalized; however, his clinical symptoms as assessed by CDAI and PASI remained unimproved after seven TCZ infusions. In both cases, TCZ treatment resulted in normalized CRP levels, which suggested that TCZ had completely inhibited IL-6 signaling. However, the clinical symptoms of PsA had not improved. TNF inhibitors were efficacious in both cases. These results suggest that TCZ may not be a new therapeutic option for PsA. However, the patient with a history of HLA-B27 positive AS and Crohn's disease from Brulhart et al.'s paper also had psoriasis [10]. While the efficacy of tocilizumab infused at 8 mg/kg monthly was not observed, the infusion frequency to every 15 days induced rapid biological and progressive clinical improvement. It was reported that after 11 months, the patient remained well with no tender and swollen joints with normalization of inflammatory markers and psoriasis skin lesions have completely resolved [10]. In contrast, there are some recent descriptions of patients with RA or adult-onset

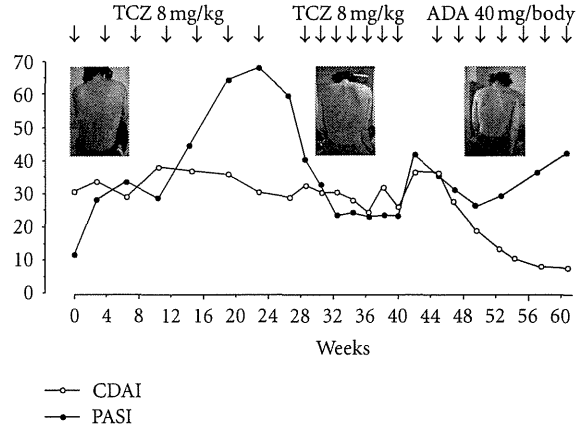

FIGURE 1: Changes in the clinical disease activity index (CDAI) and the psoriasis area-and-severity index (PASI) score for case 1 during tocilizumab and adalimumab therapy.

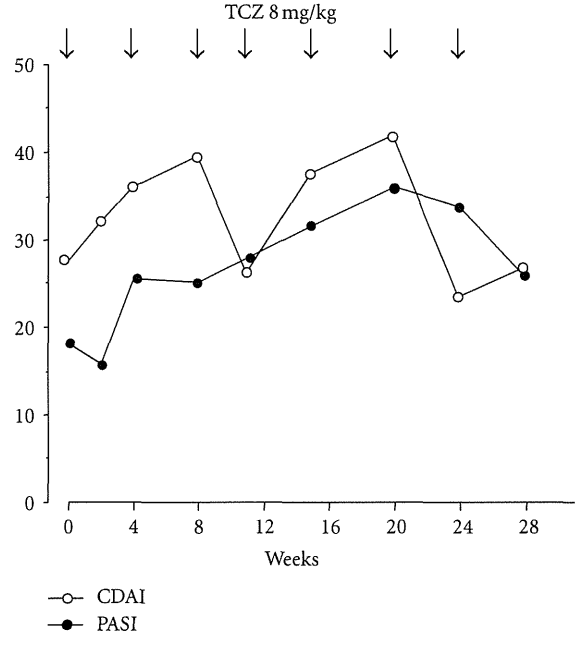

FIGURE 2: Changes in the clinical disease activity index (CDAI) and the psoriasis area-and-severity index (PASI) score for case 2 during tocilizumab therapy.

Still's disease that developed psoriatic skin lesions following treatment with tocilizumab [21, 22]. However, these were only representative cases and randomized controlled trials are needed.

4. Adaptive Immune Responses in PsA

The pathogenic role of the adaptive immune system in PsA is different from that in RA. It is well known that CD4+ T cells are important in RA. In contrast, there is increasing evidence that PsA is an autoimmune disease in which CD8+ T cells play a key role [23]. Indeed, the de novo appearance of PsA in patients with acquired immunodeficiency syndrome

and advanced depletion of CD4+ T cells during the early stages of the human immunodeficiency virus pandemic suggested different roles of T cells in PsA than those in RA and systemic lupus erythematosus (SLE), which are both ameliorated by a loss of CD4+ T cells [24]. A TNF-overexpressing mouse model (TnfΔARE mice) is a model of spondyloarthritic disease that is uniquely associated with Crohn's-like inflammatory bowel disease [25]. This mouse model develops chronic polyarthritis beginning at 5-6 weeks of age and intestinal disease beginning at week 6. A requirement for CD8+ effector functions has been reported for this model [26]. These findings indicate the importance of CD8 T cells in SNSA.

Favorable outcomes with abatacept for PsA were recently reported [27]. Abatacept (CTLA-4-Ig) is a biological agent constructed by genetically fusing the external domain of human CTLA-4 and a fragment of the Fc domain of human immunoglobulin (Ig) G1. As with native CTLA-4, abatacept first binds to CD80/CD86 on antigen-presenting cells and then to CD28 on T cells. Interfering with the CD28 co-stimulation pathway results in inhibiting antigen-dependent T-cell activation [28]. Abatacept treatment inhibits the T- and B-cell activity in the synovial fluid of RA patients by downregulating the expressions of both CD4 and CD8 [27]. Because CTLA-4-Ig inhibits CD8+ T cells' cytotoxic responses [29], abatacept may improve PsA symptoms by inhibiting CD8+ T cell-activation.

In a psoriasis animal model, skin lesions spontaneously developed when symptomless prepsoriatic human skin was engrafted onto AGR129 mice that were deficient in type I and type II interferon receptors and the recombination activating gene 2 [30]. In this model, resident human T cells in prepsoriatic skin underwent local proliferation in the engraftment. Although CD4+ cells were predominantly found in the dermis, CD8+ cells were also located predominantly in the epidermis or the dermoepidermal junction zone. Long-lived, sessile, and resident T cells may be important in the pathogenesis of psoriasis. In addition, human normal skin contains significant numbers of resident T cells, including Th17 cells [31].

Acquired immunity, especially that mediated by the Th17/IL-23 axis, plays an important role in the inflammatory pathology observed in psoriasis and PsA [32]. Th17 cells are a separate lineage of T cells that depend upon IL-23 for their development, survival, and proliferation [33]. Increase in the number of Th17 cells is found in psoriatic lesions [34]. Th17 cells produce the cytokines IL-17, TNF-α, IL-21, and IL-22. IL-22 induces human keratinocyte proliferation [35]. IL-17 is a proinflammatory cytokine that promotes migration of neutrophils into the psoriatic lesions. Injecting IL-23 into the skin of mice induced dermal changes that are seen in psoriasis, and these effects were mediated by IL-22 [36, 37]. In the skin, dendritic cells and keratinocytes produce increased amounts of IL-23, a cytokine that supports the development and proliferation of Th17 cells. IL-6 is also involved in autoimmunity by altering the balance of Th17 cells by inducing the differentiation of Th17 cells from naïve CD4+ T cells [38]. Although IL-6 is a possible inducer of Th17 cells from naïve T cells, IL-23 may contribute to the activation of skin-resident Th17 cells that are already differentiated.

Indeed, a recent genome wide association study (GWAS) identified a signaling network of adaptive immune responses in psoriasis that involved CD8+ T cells and Th17 cells [39]. In PsA, T-cell activation and inflammatory cytokine production might occur in the dermis with subsequent migration to the joint, or CD8+ cytotoxic T cells might originate in the synovium. In contrast to RA, contribution of the IL-6 priming phase of arthritis may not be important in PsA.

5. Innate Immune Responses in PsA

The previously cited GWAS also identified skin barrier functions and innate immunity responses, involving nuclear factor-kappa B (NFκB) and interferon signaling in PsA [39]. Innate immunity is the first line of defense against invading organisms, which involves activating intracellular regulators such as NFκB. The expressions of numerous target genes involved in the pathogenesis of inflammatory diseases, including TNF, IL-1, and IL-17, are triggered by NFκB. Several factors may exacerbate these manifestations or even trigger the disease, such as traumatic injury to the skin, physical and psychological stress, cold weather, excessive alcohol consumption, and drugs like lithium and beta blockers [40, 41].

The role of physical injury, including trauma, in PsA has also been the subject of some interest. It is intriguing to speculate that the Koebner phenomenon, a recognized feature of skin psoriasis, may also occur in peripheral joints [42]. With regard to the pathogenesis of trauma-induced PsA, the "deep Koebner's phenomenon" has been proposed [43]. Microbial infections have also been known to trigger psoriasis and PsA [44]. In addition, IFN-α is a well-known trigger of PsA [45]. These findings suggest that innate immunity is also involved in PsA. Inflammation derived from the direct activation of innate immunity may result in the production of TNF and the development of arthritis.

6. Pathogenic Role of IL-6 in PsA

The precise mechanisms by which IL-6 blockade leads to improvements in RA are not well understood [46]. IL-6 promotes synovitis by inducing neovascularization, infiltration of inflammatory cells, and synovial hyperplasia. IL-6 also causes bone resorption by inducing osteoclast formation via the induction of the receptor activator of NFκB ligand (RANKL) in synovial cells and cartilage degeneration by inducing the production of matrix metalloproteinases in synovial cells and chondrocytes. Moreover, IL-6 is involved in autoimmunity by altering the balance of Th17 cells by inducing the differentiation of Th17 cells from naïve CD4+ T cells. IL-6 blockade inhibits type II collagen-induced arthritis and requires CD4 T cells, which leads to the production of anti-type II collagen IgG [47]. However, another arthritis model, anti-type II collagen antibody-induced arthritis, bypasses the priming phase of T-cell-dependent antibody generation and is not suppressed in IL-6−/− mice [48]. These findings indicate that IL-6 is involved in the priming

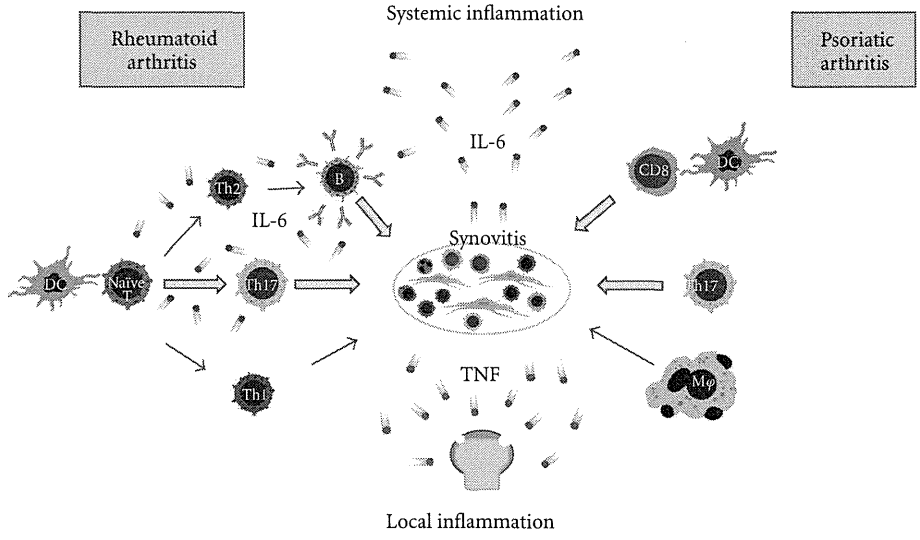

FIGURE 3: Pathogenic roles of IL-6 in RA and PsA. IL-6 contributes to the priming phase of RA. Because an IL-6-independent innate immune mechanism primarily contributes to PsA, the roles of IL-6 in the pathogenesis of PsA are not critical.

phase of RA but not in the effector phase of RA. Therefore, the major mechanism of TCZ is inhibiting the immune activation that leads to the development of RA [49].

IL-1R antagonist (−/−) mice spontaneously develop autoimmune diseases such as arthritis and dermatitis that histologically resemble human psoriasis [50]. In this model, the deficiency of TNF, but not IL-6, suppressed the development of arthritis and skin inflammation [51]. This suggests that TNF, but not IL-6, is important in the pathogenesis of PsA.

A recently proposed model for the pathogenesis of PsA is that the frequent microdamage and tissue repair at normal enthesis attachment sites in healthy joints results in PsA pathogenesis. This model incorporates the concept of autoinflammation in which tissue specific factors, including microtrauma, lead to regional innate immunity activation and persistent inflammation as an alternative to primary immunopathology driven by T- and B-cell abnormalities [52].

The difference in the pathogenesis of RA and PsA are summarized in Figure 3. In RA, CTLA-4-dependent antigen presentation to CD4+ cells and IL-6-dependent Th17 differentiation induces synovitis. In addition, B cells contribute to the pathogenesis of RA. In PsA, IL-23-dependent differentiated skin-resident Th17 cells, activated CD8+ cytotoxic cells, and directly activated macrophages are mainly involved in synovitis development. Because synovitis that produces TNF contributes to the effector phase of arthritis in both RA and PsA, TNF inhibitors are effective for both types of arthritis. IL-6 primarily contributes to the priming phase of synovitis in RA. Systemic inflammation induced by IL-6 is not the main mechanism of TCZ action in treating RA. Although systemic inflammation is inhibited by TCZ, it does not inhibit the associated arthritis.

7. Conclusions

Although TCZ has shown significant efficacy for treating RA, TCZ treatment has not shown favorable outcomes in PsA. This suggests that the roles of IL-6 in RA and PsA are different. Although the exact reason for ineffectiveness of TCZ in PsA is unclear, IL-6-dependent immune response activation may not be important in PsA.

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All the authors agree to the content, presentation, and decision to submit the paper.

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

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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. ***The Journal of Immunology*****, 2012, 188: 4858–4865.**

Multiple sclerosis (MS) is a demyelinating autoimmune disease that predominantly affects the white matter of the CNS and is a leading cause of lasting neurologic disabilities in young adults (1). Although the precise cause of MS is incompletely understood, MS is thought to occur in genetically predisposed individuals after they are exposed to an environmental trigger that stimulates myelin-specific T cells (2, 3). Indeed, genome-wide association studies have shown that the IL-2 receptor and HLA class II are associated with disease susceptibility (4–6). In addition, increasing evidence has shown that Th17 lymphocytes (7–9) and Th1 cells (10) play an important role in the pathology of MS. Thus, Ag presentation and subsequent 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

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Abbreviations used in this article: ANCOVA, analysis of covariance; BBB, blood–brain barrier; BMDC, bone marrow-derived dendritic cell; CIS, clinically isolated syndrome; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability status scale; 2-ME, 2-mercaptoethanol; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMO, neuromyelitis optica; OND, other neurologic disease; RRMS, relapsing–remitting multiple sclerosis.

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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 (Maxisoac; Nunc) were coated with the monoclonal anti-Sema4A Abs HIAT2 (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 Biotech) 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 monocytic cells 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 mice using GM-CSF (R&D Systems) as previously described (19). For the in vitro differentiation of mouse helper T cells with BMDCs, $CD62L^{high}$ $CD4^+$ naive T cells were prepared from OT-II TCR transgenic mice and purified by FACS sorting as previously described (19). $CD62L^{high}$ $CD4^+$ naive T cells (1×10^6 cell/ml) were cultured with wild-type or Sema4A-deficient BMDCs (1×10^6 cell /ml) in the presence of IL-12 (1 ng/ml) and anti–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 Genosys) for 5 d. The resulting $CD4^+$ T cells were positively selected using a MACS sorting kit and restimulated with immobilized anti-CD3 Ab (2C11; 1 or 5 μg/ml) for 24 h in 96-well plates (1×10^5/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-step 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 ng/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% CO_2. 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 0.1% BSA–PBS, the cells were stained with FITC-conjugated anti–IFN-γ (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 D-biotinoyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Roche) for 15 min. The reaction was stopped by adding 50 μl of 1 mol/ml NH_4Cl for 15 min, and then cells were washed with ice-cold PBS. For the shedding experiments, biotinylated cells (2×10^6) were resuspended at a cell density of 10^7/ml in GIT medium (Wako) and then incubated at 37°C for various time periods. When indicated, the reagents were added at the start of the incubation period. At the end of the incubation period, the cell suspensions were centrifuged, and the supernatants were collected. The cell pellets were washed with ice-cold PBS and solubilized in a 1% Nonidet P-40 detergent buffer containing 10 mmol/ml Tris (pH 7.8), 150 mM NaCl, 10 μmol/ml aprotinin, 1 mmol/ml PMSF, 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 2×10^6 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

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 electrotransferred 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 antiserum 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 MAS5 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 mouse/rat MOG_{35-55} peptides (MEVGWYRSPFSPVVHLYRNGK) emulsified in CFA, in addition to two i.v. injections of 100 ng pertussis toxin (List Laboratories) 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 $n = 5$, wild-type $n = 5$) 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 statistical significance was analyzed using an unpaired Student t test, and p values ≤ 0.05 were considered statistically significant.

For adoptive transfer, donor mice were immunized with MOG/CFA in the same fashion 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 MOG_{35-55} peptide and 10 ng/ml of recombinant mouse IL-12 (R&D Systems). After 3 d of the culture, cells were harvested, and $CD4^+$ T cells were isolated by negative selection using Dynabeads (Invitrogen). Recipient mice irradiated sublethally (500 cGy) received cells. The mice were treated with either Sema4A–Fc (15 μg, $n = 5$) or control human IgG (15 μg, $n = 5$) or anti-Sema4A blocking Ab (15 μg, $n = 5$) on days 0, 1, 3, and 5 after transfer.

For the recall assay, $CD4^+$ T cells were purified from the draining lymph nodes by MACS, and 1×10^5 cells were restimulated for 72 h with various concentrations of the MOG peptides in the presence of irradiated (3000 rad) splenocytes (5×10^5). IFN-γ and IL-17A were detected using a mouse IFN-γ and IL-17A ELISA kit (R&D Systems).

Statistical analysis

Continuous variables are expressed as the mean ± SD. Because we were interested in differences and/or trends in the mean values of Sema4A, t tests were consistently used to compare two independent groups. Welch's t test was selected if the SDs of the two groups were highly different. Otherwise, Student t test was used for statistical analyses. To control for the effects of different baseline covariates (age and gender) among patients, we used the matching method and the analysis of covariance (ANCOVA) method. Both methods were used to compare the MS and OND groups. However, the matching method was not used to compare the CIS and OND groups because the sample size was inadequate when comparing the CIS group (22 patients) with the age- and gender-matched OND patients due to the significantly smaller sample size in the CIS group than that in the MS group. To compare the CIS and OND groups, we adopted the ANCOVA model with age and gender as adjusted factors, which simultaneously controls for the effects of between-group age and gender in the linear model and then provides adjusted mean differences. In addition, the ANCOVA method was used to produce the residual (expanded disability status scale; EDSS). The residual (EDSS) represents the EDSS score that was adjusted by estimating the mean effects of gender, age, duration from the last relapses, and the illness duration in the ANCOVA model. All reported p values are two-sided, and p values <0.05 were considered statistically significant. These statistical analyses were conducted using SAS version 9.1 and the R programming language.

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).

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

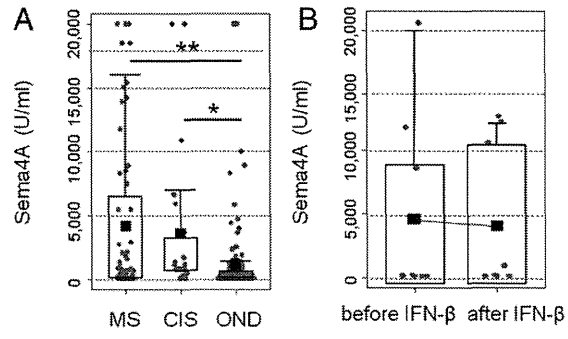

FIGURE 1. Serum Sema4A levels are elevated in MS patients. (**A**) Serum Sema4A levels were significantly higher in MS and CIS patients than in OND patients. The serum Sema4A levels were measured by ELISA in 59 RRMS patients in the remitting phase, 22 CIS patients, and 34 age- and gender-matched OND 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 GES26484 (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 (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-

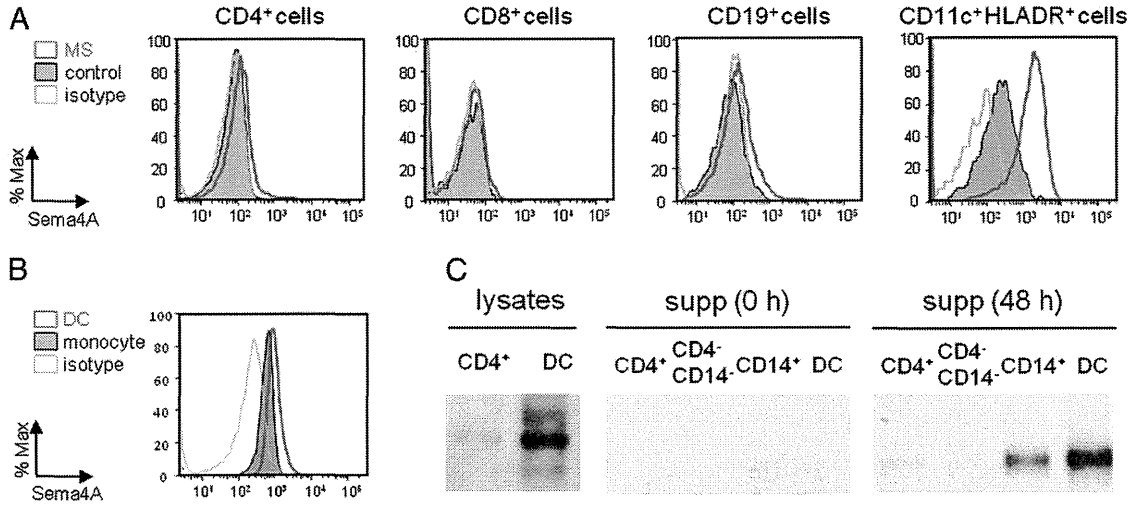

FIGURE 2. Soluble Sema4A is secreted from DCs. (**A**) Increased Sema4A expression in DCs. Sema4A expression was higher in $CD11c^+$ $HLADR^+$ cells in MS patients (red lines) than in the normal controls (filled histograms). (**B**) Sema4A expression increased when monocytes were differentiated into 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.

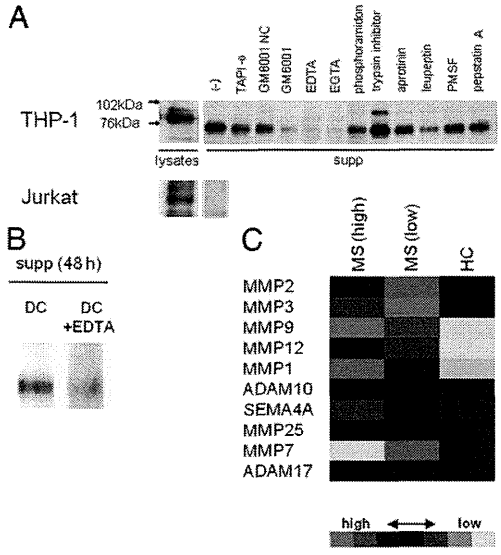

FIGURE 3. Soluble Sema4A is secreted in a metalloproteinase-dependent manner. (**A**) Soluble Sema4A is produced in the supernatants of a human monocyte-derived cell line (THP-1 cells) through metalloproteinase-dependent shedding, but not in the supernatants of a T cell-derived cell line (Jurkat cells). 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.

cantly higher ratio of IL-17–producing cells among $CD4^+$ T cells (6.74 ± 3.74) than that of patients with low Sema4A levels (2.05 ± 1.08, $p = 0.0011$) or healthy controls (2.30 ± 1.07, $p = 0.0017$, Fig. 4C). However, there is no direct correlation between the serum Sema4A levels and the percentage of IL-17^+ cells (rank-correlation coefficient = 0.107, $p = 0.8397$), suggesting that Sema4A may determine the threshold of Th17 differentiation. Less than 0.5% of $CD4^+$ T cells were positive for both IL-17 and IFN-γ. The ratios of IL-4– and IFN-γ–producing cells in patients with high Sema4A levels (1.39 ± 0.58 and 5.18 ± 1.51) were not statistically different from those of patients with low Sema4A levels (1.88 ± 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

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.

Pathogenic implications of Sema4A in EAE

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 support 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.

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

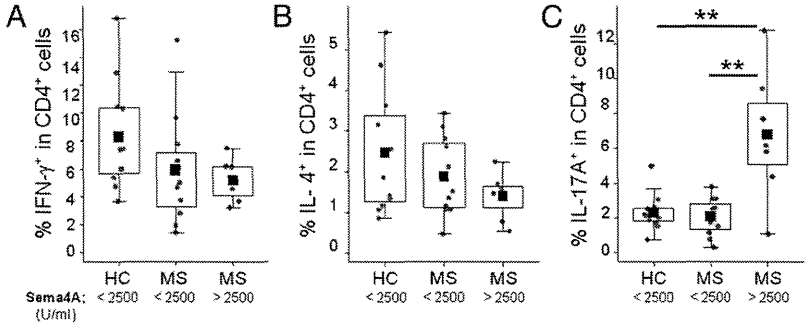

FIGURE 4. Correlation between Sema4A levels and Th17 skewing in MS patients. FACS analysis for intracellular cytokine expression in $CD4^+$ T cells from MS patients with high ($n = 8$) and low ($n = 11$) Sema4A levels and from healthy controls ($n = 11$). The IFN-γ^+ (**A**), IL-4^+ (**B**), and IL-$17A^+$ (**C**) cells among $CD4^+$ T cells were determined by intracellular cytokine staining. $^{**}p < 0.01$.

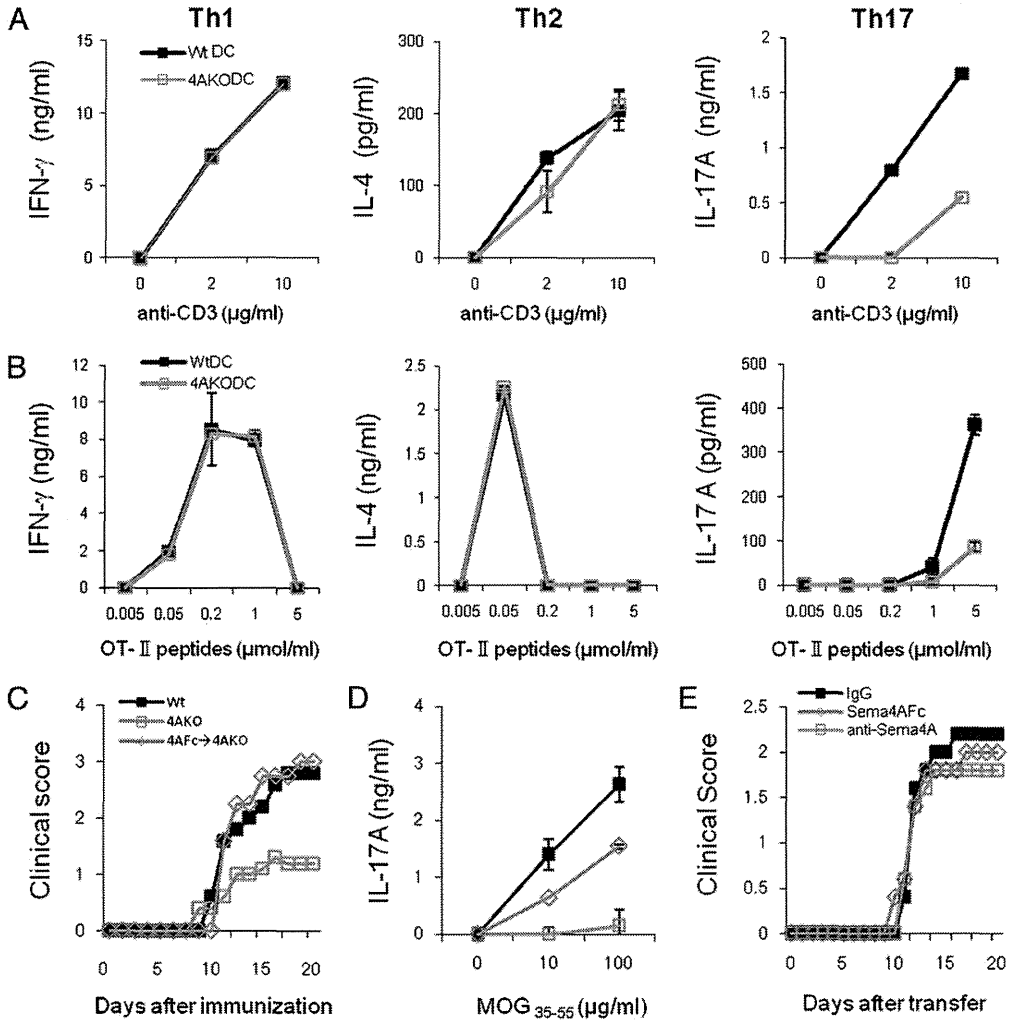

FIGURE 5. Pathological correlation between DC-derived Sema4A and Th17 differentiation. (**A**) Sema4A-deficient DCs fail to induce naive $CD4^+$ T cells to differentiate into Th17 cells. $CD62L^{high}$ $CD4^+$ naive T cells prepared from OT-II TCR transgenic mice were cocultured with wild-type or Sema4A-deficient DCs in the presence of OT-II peptides under Th1-, Th2-, or Th17-skewing conditions. After 5 d of culture, $CD4^+$ 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. (**B**) Sema4A-deficient BMDCs fail to differentiate naive $CD4^+$ T cells into Th17 cells under nonskewing conditions. $CD62L^{high}$ $CD4^+$ naive T cells prepared from OT-II TCR transgenic mice were cocultured with wild-type (closed squares) or Sema4A-deficient BMDCs in the presence of various concentrations of OT-II peptides (0.005–5 μmol/ml). After 5 d of culture, $CD4^+$ 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. (**C**) Administration of Sema4A–Fc exacerbates EAE. EAE was induced in six wild-type and 10 Sema4A-deficient mice. Sema4A–Fc was injected into five Sema4A-deficient mice. Human IgG was administered to the remaining five Sema4A-deficient EAE mice and six wild-type mice. (**D**) Sema4A-deficient mice with EAE have impaired Th17 cell generation, which is restored by administering Sema4A–Fc. $CD4^+$ T cells were purified from the draining lymph nodes and spleen. (**E**) The clinical course of passively immunized EAE is not altered by administering recombinant Sema4A–Fc or an anti-Sema4A blocking Ab. EAE was induced by adoptively transferring MOG-specific T cells into sublethally irradiated wild-type C57BL/6 mice. Human IgG (n = 6, black squares) or recombinant Sema4A–Fc (n = 6, green rhombuses) or an anti-Sema4A blocking Ab (n = 6, red squares) was administered on days 0, 1, 3, and 5 after transfer. There were no significant differences between the three groups. Cytokines in the culture supernatants were measured by ELISA. The data are representative of three independent experiments in (A)–(E) and are shown as the means ± SEMs of triplicate wells in (A), (B), and (D).

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

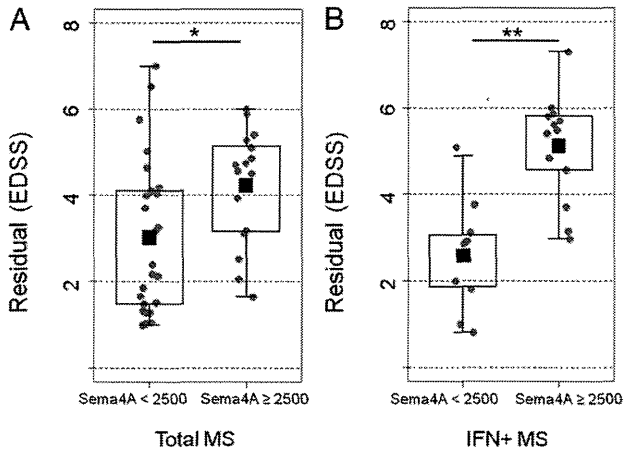

FIGURE 6. Comparison of IFN-β efficacy between MS patients with high and low Sema4A levels. (**A**) Correlation between the serum Sema4A levels and neurologic disabilities (EDSS score). Fifty-nine RRMS patients were divided into two groups based on a Sema4A titer of 2500 U/ml. MS patients with higher Sema4A levels had greater EDSS scores than those of patients with lower Sema4A levels. (**B**) Correlation between serum Sema4A levels and the EDSS scores of MS patients who were treated with IFN-β. MS patients with higher Sema4A levels had higher EDSS scores than those of patients with lower Sema4A levels. The top and bottom of the box in the box–whisker plot indicate the 25th and 75th percentiles, respectively, and the ends of the whisker represent 1.5 times the interquartile range from the box or the most extreme points of all the data. $*p < 0.05$, $**p < 0.01$ (ANCOVA).

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 pathological 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

Sema4A, U/ml	Number of Patients (F/M)	Age, y	Duration of Illness, mo	Relapse Number/2 y	Mean EDSS Score
		59 RRMS Patients			
<2500	40 (35/5)	38.7 ± 11.3	91.8 ± 104.1	1.84 ± 1.29	2.27 ± 1.94
≥2500	19 (16/3)	35.8 ± 11.5	72.6 ± 40.2	2.44 ± 2.3	3.34 ± 1.7*
		30 MS Patients Receiving IFN-β Therapy			
<2500	16 (14/2)	36.7 ± 11.9	81.1 ± 108.4	2.27 ± 1.4	2.34 ± 2·4
≥2500	14 (13/1)	33.3 ± 7.9	67.2 ± 31.6	2.62 ± 2.5	3.74 ± 1.6**

$*p < 0.05$, $**p < 0.01$.

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Disclosures

The authors have no financial conflicts of interest.

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Clinical Medicine Insights: Arthritis and Musculoskeletal Disorders

EXPERT REVIEW

Safety and Efficacy of Tocilizumab for the Treatment of Rheumatoid Arthritis

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Abstract: Because of the pathological role of IL-6 in rheumatoid arthritis (RA), tocilizumab (TCZ), a humanized anti-IL-6 receptor monoclonal antibody, was expected to improve inflammation and joint destruction of RA. Indeed, randomized clinical trials demonstrated the clinical efficacy of TCZ as monotherapy or combined with methotrexate (MTX) for RA patients with inadequate responses to disease-modifying antirheumatic drugs, MTX or tumor necrosis factor (TNF) inhibitors. Although long-term tolerability for TCZ is superior to that for TNF inhibitors, information regarding the potency of drug free remission of TCZ is limited at present. In terms of its safety profile, the general risk of infection when using TCZ is comparable to that of TNF inhibitors. TCZ has some advantage in RA patients who can not use MTX and are non-responders to TNF inhibitors. In conclusion, TCZ is one of the most prospective next generation biologics for the treatment of RA.

Keywords: rheumatoid arthritis, interleukin-6, tocilizumab

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