Soluble CD40 ligand contributes to blood–brain barrier breakdown and central nervous system inflammation in multiple sclerosis and neuromyelitis optica spectrum disorder

（可溶性 CD40 リガンドは多発性硬化症及び視神経脊髄炎関連疾患患者における血液脳関門破綻と中枢神経の炎症に寄与する）

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Soluble CD40 ligand contributes to blood–brain barrier breakdown and central nervous system inflammation in multiple sclerosis and neuromyelitis optica spectrum disorder

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Abstract

Objective: Soluble CD40 ligand (sCD40L) is reported to be associated with a disruption of the blood–brain barrier (BBB). We aimed to elucidate the role of sCD40L on multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD).

Methods: CSF and serum sCD40L levels were measured in 29 MS, 29 NMOSD, and 27 disease control (DC) patients. Clinical and laboratory profiles including the CSF/serum albumin ratio (Qalb), a marker of BBB breakdown, and interleukin-6 (IL-6) levels were also investigated.

Results: In MS patients, serum sCD40L levels were higher than in DCs (median: 2480 versus 786 pg/mL, $P = 0.046$) and positively correlated with Qalb ($P = 0.044$). CSF sCD40L levels in MS patients were elevated compared to DCs (median: 38.5 vs 4.8 pg/mL, $P = 0.002$) but were not correlated with Qalb ($P = 0.72$). In contrast, in NMOSD patients serum sCD40L levels were not elevated compared to DCs (median: 829 vs 786 pg/mL, $P = 0.87$) and showed no correlation with Qalb ($P = 0.89$). But, CSF sCD40L levels were significantly increased compared to DCs (median: 33.5 vs 4.8 pg/mL, $P = 0.013$), tended to be related with Qalb ($P = 0.056$), and were correlated to CSF cell counts, protein concentrations, and IL-6 levels (P =0.038, 0.018, and 0.015, respectively).

Conclusions: sCD40L could be involved in BBB disruption in MS, whereas it may contribute to CNS inflammation in NMOSD.

Introduction

CD40 ligand (CD40L) is a transmembrane protein that plays an important role in the immune system, helping to produce high-affinity B cells and to promote neutrophil adhesion and migration via the integrin Mac-1.[1,2] Soluble CD40L (sCD40L), which has the same function as CD40L,[3] was reported to be produced by shedding from the surface of activated platelets or activated T cells and exist in trimers.[1] Recent studies have suggested that sCD40L increases the permeability of the blood–brain barrier (BBB).[4]

Helper T (Th) 17 cells were reported to play an important role in the pathogenesis of MS.[5–10] A recent study showed blocking interleukin-6 (IL-6) receptor signaling *in vitro* decreased the monocyte ability to up-regulate Th17 cells in multiple sclerosis (MS) patients.[11] Conversely, IL-6 was also known to be a key molecule in the pathogenesis of neuromyelitis optica spectrum disorder (NMOSD).[12–15]

Previous studies revealed that sCD40L was increased in the sera of patients with autoimmune diseases including systemic lupus erythematosus (SLE)[16,17] and primary Sjögren syndrome.[18] Moreover, increased sCD40L levels were reported in the CSF of MS patients and in the sera of NMOSD patients.[19,20] However, the role of sCD40L in MS and NMOSD have not been identified. Therefore, we examined serum and CSF sCD40L levels in MS and NMOSD patients and investigated the correlation of sCD40L with clinical characteristics or laboratory and IL-6 profiles.

Materials and methods

Patients

We included 29 MS patients, 29 NMOSD patients, and 27 disease control (DC) patients. All MS

patients fulfilled the 2010 revisions of the McDonald criteria[21] and all patients with NMOSD implemented the 2015 international consensus diagnostic criteria,[22] including 19 patients with neuromyelitis optica (NMO).[23] DC patients were as follows; 10 with inflammatory diseases (two CNS lupus, four neurosarcoidosis, and four Behçet disease [one Entero-Behçet disease and three Neuro-Behçet disease]) and 17 with non-inflammatory diseases (one corticobasal syndrome, six motor neuron disease, three multiple system atrophy, five Parkinson's disease, one progressive supranuclear palsy, and one spinocerebellar degeneration).

Standard Protocol Approvals and Patient Consents

The study procedure was approved by the ethics committee of Chiba University School of Medicine. All patients provided informed consent.

Collecting CSF and serum samples

All samples from MS and NMOSD patients were obtained during an attack, the same as a previous report,[24] that is, all samples were obtained within 50 d after the onset of an attack and before treatment. Samples from DC patients with inflammatory diseases were obtained during the acute phase. Serum samples were collected simultaneously at CSF sampling. All serum samples were centrifuged at 3000 rpm for 10 min. All serum samples just after centrifugation and all CSF samples were immediately stored at - 80°C until analysis.

CSF and serum sCD40L measurements

The concentration of sCD40L in the CSF and serum was analyzed using the Bio-Plex ProTM® magnetic bead based immunoassay (Bio-Rad Laboratories, Hercules, CA, USA) with a single

detection according to the manufacturer's instructions. Fluorescence intensity from the immunoassay was measured and analyzed using Bio-Plex TM 200 system software version 6.1 (Bio-Rad Laboratories). The sCD40L concentration (pg/mL) was calculated by a standard curve obtained by standard assays. Values under the dynamic range were replaced by half of the lower limit of sensitivity.

Clinical characteristics and laboratory findings

We reviewed age at disease onset, gender, disease duration to sampling, age at sampling, Kurtzke's Expanded Disability Status Scale (EDSS) at sampling, and treatments at sampling. Laboratory findings including anti-aquaporin-4 antibodies positivity,[25] CSF cell count, CSF protein concentration, CSF/serum albumin ratio (Qalb), IgG index, oligoclonal IgG bands, and platelet count were also reviewed. These items were compared among MS patients, NMOSD patients, and DCs. Correlations between sCD40L levels and other clinical or laboratory items were also investigated.

Comparing sCD40L levels and IL-6 data of a previous report

We previously reported CSF and serum IL-6 levels in MS and NMO patients. Hence, the correlation between sCD40L levels and IL-6 levels previously reported was investigated.[24]

Statistical analysis

Continuous data were compared between disease groups by a Mann-Whitney U test with a Bonferroni correction. Categorical outcomes were evaluated using the chi-square test or Fisher's exact test. A Kendall tau-b correlation was performed to analyze correlations. A P value of <0.05

was considered statistically significant. Statistical tests were conducted using SPSS version 23.0 (IBM Corporation, Armonk, NY, USA).

Results

Demographics, clinical characteristics, and laboratory findings in patients with MS, NMOSD, and DCs

Demographics, clinical characteristics, and laboratory findings in patients with MS and NMOSD are shown in **Table 1**. Age at CSF sampling and EDSS were significantly lower in MS patients than in NMOSD patients ($P < 0.001$, both). Anti-aquaporin-4 antibodies were only positive in NMOSD patients (83%, $P < 0.001$). Long extended cord lesion, Qalb, and the number of patients with any immunomodulating treatment and continuous oral prednisolone were significantly higher in patients with NMOSD than in patients with MS (all P values were <0.001). Meanwhile, the positivity for oligoclonal IgG bands was higher in patients with MS than in patients with NMOSD ($P = 0.027$). The IgG index and platelet count were not different between MS and NMOSD patients. For DCs, 17 patients were female (63.0%), and the median age at CSF sampling was 60.0 years and the interquartile range (IQR) of age at CSF sampling was 14.0 years. The median and IQR of platelet counts in DCs were 233 and 93.5 $\times 10^3/\mu L$, respectively. Platelet count was not different between DCs and MS or NMOSD.

Serum and CSF sCD40L levels in patients with MS, NMOSD, and DCs

Serum sCD40L levels in MS patients tended to be higher than DCs (median: 2480 vs 786 pg/mL, IQR: 2590 vs 1379, $P = 0.046$, **Figure 1A**). However, compared with DCs, the results showed that CSF sCD40L levels were significantly higher in patients with MS (median: 38.5 vs 4.8

pg/mL, IQR: 45.7 vs 23.6, $P = 0.002$) and NMOSD (median: 33.5 vs 4.8 pg/mL, IQR: 38.4 vs 23.6, $P = 0.013$) (**Figure 1B**). No significant difference was found in CSF sCD40L levels between MS and NMOSD.

Correlations between sCD40L levels and other clinical characteristics or laboratory findings in MS patients

Correlations between sCD40L levels and other clinical characteristics or laboratory findings in patients with MS are shown in **Table 2.** In MS patients, serum sCD40L levels were positively correlated with Qalb (Kendall tau-b = 0.29, $P = 0.044$). In contrast, no correlation was found between CSF sCD40L levels and Qalb in MS patients (Kendall tau-b = - 0.054, $P = 0.72$). Other items were not correlated with serum sCD40L or CSF sCD40L levels in MS patients.

Correlations between sCD40L levels and other clinical characteristics or laboratory findings in NMOSD patients

Correlations between sCD40L levels and other clinical characteristics or laboratory findings in patients with NMOSD are shown in **Table 3**. In NMOSD patients, positive correlations were found between CSF sCD40L levels and CSF cell counts (Kendall tau-b = 0.30, $P = 0.038$) and between CSF sCD40L levels and CSF protein levels (Kendall tau-b = 0.34, $P = 0.018$). IgG index and Qalb tended to be positively correlated with CSF sCD40L levels (Kendall tau-b = 0.28, $P = 0.056$, both). However, serum sCD40L levels were not correlated with Qalb in NMOSD patients (Kendall tau-b = 0.022, $P = 0.89$). No correlations were found between other items and serum or CSF sCD40L levels.

Correlation of sCD40L levels with IL-6 levels

Correlations of sCD40L levels with IL-6 levels in MS and NMOSD patients are shown in **figure e-1**. In MS patients, serum sCD40L levels were correlated with serum IL-6 (Kendall tau-b = 0.51, $P = 0.001$) but not with CSF IL-6 (Kendall tau-b = - 0.035, $P = 0.79$). On the other hand, CSF sCD40L levels showed no correlation with either CSF IL-6 levels (Kendall tau-b = 0.058, $P = 0.68$) or serum IL-6 levels (Kendall tau-b = - 0.027, $P = 0.86$) in MS patients. In patients with NMOSD, a positive correlation with CSF sCD40L levels was found in CSF IL-6 (Kendall tau-b = 0.34, $P = 0.015$) but not with serum IL-6 (Kendall tau-b = 0.30, $P = 0.061$). Meanwhile, serum sCD40L levels showed no correlations with CSF IL-6 (Kendall tau-b = - 0.085, $P = 0.52$) or serum IL-6 (Kendall tau-b = 0.17, $P = 0.28$) levels.

Discussion

Our results suggest a somewhat different role of sCD40L in the pathophysiology of MS and NMOSD. In MS, both serum and CSF sCD40L levels were significantly elevated, but only serum sCD40L levels showed a positive correlation with Qalb, suggesting their involvement in BBB breakdown. By contrast, in NMOSD, sCD40L levels were significantly increased in CSF but not in serum, whereas the CSF sCD40L levels were correlated with CSF cell counts, CSF protein concentrations, and CSF IL-6 levels, indicating a more severe inflammation in the CNS.

Some studies reported sCD40L levels were increased in the sera from patients with SLE and primary Sjögren syndrome.[16–18] Serum sCD40L levels in SLE patients were reported to be correlated with disease severity. That is, sCD40L levels were significantly higher in patients with severe SLE than in patients with moderate and mild SLE.[3] However, the function of sCD40L in these diseases is still unclear.

Meanwhile, previous reports have shown that elevated sCD40L levels could increase BBB permeability.[4,26] In those reports, the authors found increased plasma and CSF sCD40L levels in patients with HIV-associated neurocognitive disorder (HAND). They showed BBB permeability in HAND was dependent on CD40L with CD40L knock out mice.[4] Our study revealed a tendency for increased serum sCD40 levels and a positive correlation between serum sCD40L levels and Qalb in MS patients. On the other hand, in NMOSD patients, our results showed increased CSF sCD40L levels and a positive correlation of CSF sCD40L levels with Qalb. These results may indicate that sCD40L plays an important role in the destruction of the BBB in MS and NMOSD, and that the place where sCD40L plays a role in the destruction of the BBB may be different between MS and NMOSD. That is, sCD40L may affect the BBB from the peripheral side in MS and from the CNS side in NMOSD.

Previous studies reported only activated platelets or activated T cells could release sCD40L. Few studies have investigated whether platelets could migrate into the brain, but a study showed that platelets did not migrate into the brain of Alzheimer disease patients but were concentrated in the blood vessels.[27] Therefore, in the CNS, only T cells could produce sCD40L. In NMOSD, a recent study showed that not only NMO-IgG but also highly encephalitogenic T cells were required to cause severe panencephalitis.[28] Activated pathogenic T cells in the CNS may release sCD40L, leading to a breakdown of the BBB and severe inflammation in the CNS. In addition, IL-6 was reported to contribute to disease severity.[29] Therefore, elevated CSF sCD40L levels and a positive correlation of CSF sCD40L with CSF IL-6 levels, CSF cell count, and CSF protein concentration could lead to severe inflammation through a BBB breakdown in NMOSD. Different from NMOSD, activated platelets or activated T cells may produce sCD40L peripherally, leading to a breakdown of the BBB. Further investigation is needed to clarify the

origin of sCD40L in the pathogenesis of MS and NMOSD.

There are some limitations in our study. First, the sample size was limited. Second, we measured sCD40L levels in serum but not plasma. This may have affected the results. Plasma sCD40L levels are usually higher than serum levels, because 95% of sCD40L is produced from activated platelets in plasma.[30,31] Our assay using serum may reflect sCD40L from T lymphocytes, another source of sCD40L, more than assays using plasma. Second, the molecular weight of sCD40L is much smaller than albumin, resulting in its easier access to the CNS. Therefore, the possibility that the elevated CSF sCD40L levels in MS and NMOSD reflected the serum sCD40L levels cannot be denied. Previously, an anti-CD40L antibody caused thromboembolism in SLE patients.[32] Recently, new anti-CD40L antibodies lacking a functional Fc region were reported not to induce thrombotic events.[33] Our study indicates that a destruction of the BBB may be mediated by elevated serum sCD40L in MS and CSF sCD40L in NMOSD. Therefore, in the future, an anti-CD40L antibody lacking the Fc region could be a potential drug for patients with MS and NMOSD.

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Figure Legends

Figure 1. Comparison of sCD40L levels in MS, NMOSD, and DCs.

(A) Serum sCD40L levels tended to be higher in MS patients than in DCs. No differences were found between MS and NMOSD, NMOSD and DCs. (B) CSF sCD40L levels were increased in patients with MS and NMOSD compared with DCs. In DCs, 10 inflammatory diseases (two CNS lupus, four sarcoidosis, three Neuro-Behçet disease, and one Behçet disease) and 17 non-inflammatory diseases (one corticobasal syndrome, six motor neuron disease, three multi system atrophy, five Parkinson's disease, one progressive supranuclear palsy, and one spinocerebellar degeneration) were included. In DCs, gray and white circles indicate sCD40L levels of inflammatory and non-inflammatory diseases, independently. Grey long-short dashed lines show the medians of each group. DCs = disease control; L.L.D. = lower limit of detection; MS = multiple sclerosis; NMOSD = neuromyelitis spectrum disorder; sCD40L = soluble CD40 ligand.

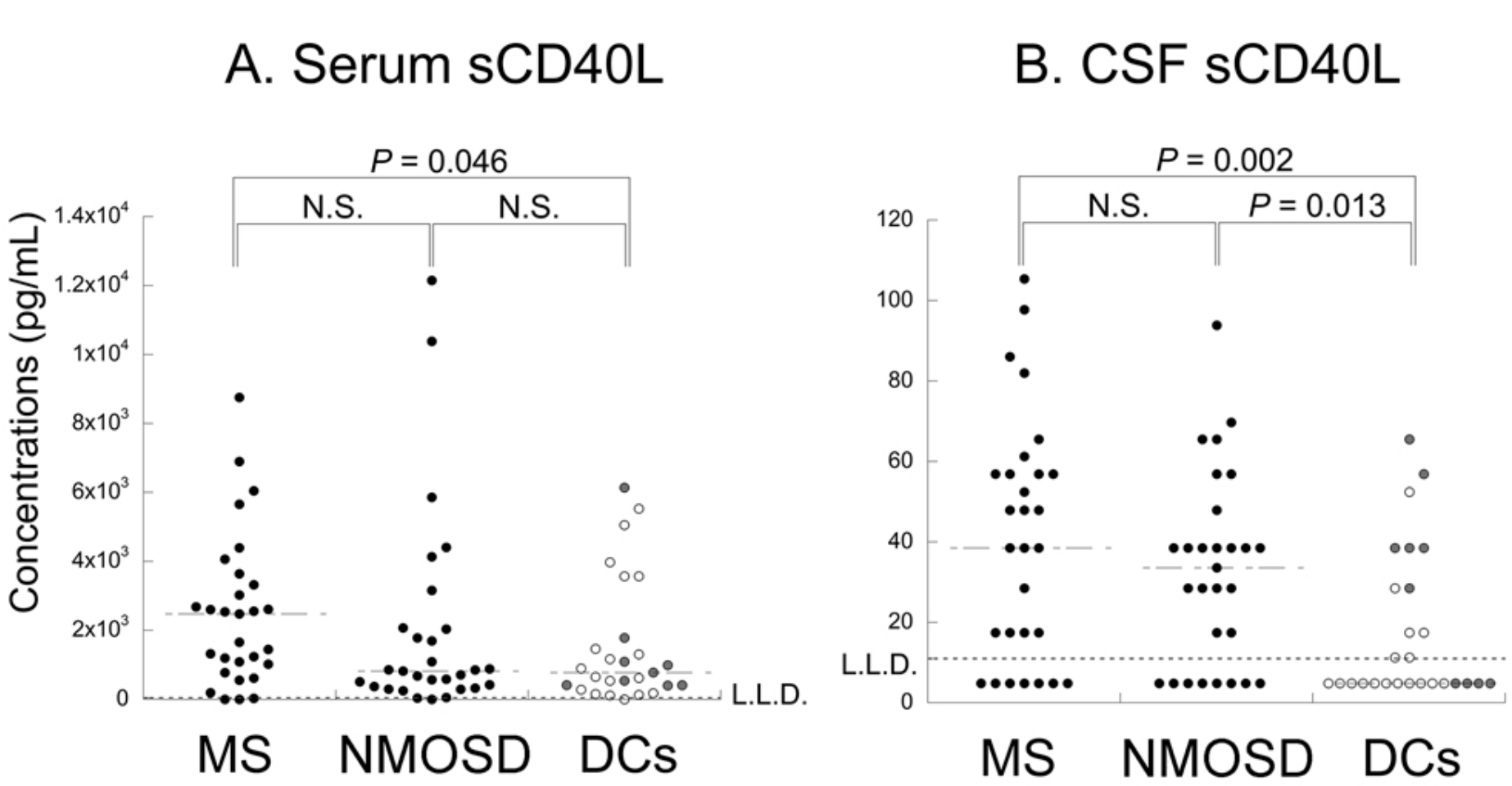
Figure 2. Correlations between sCD40L levels and Qalb in MS and NMOSD.

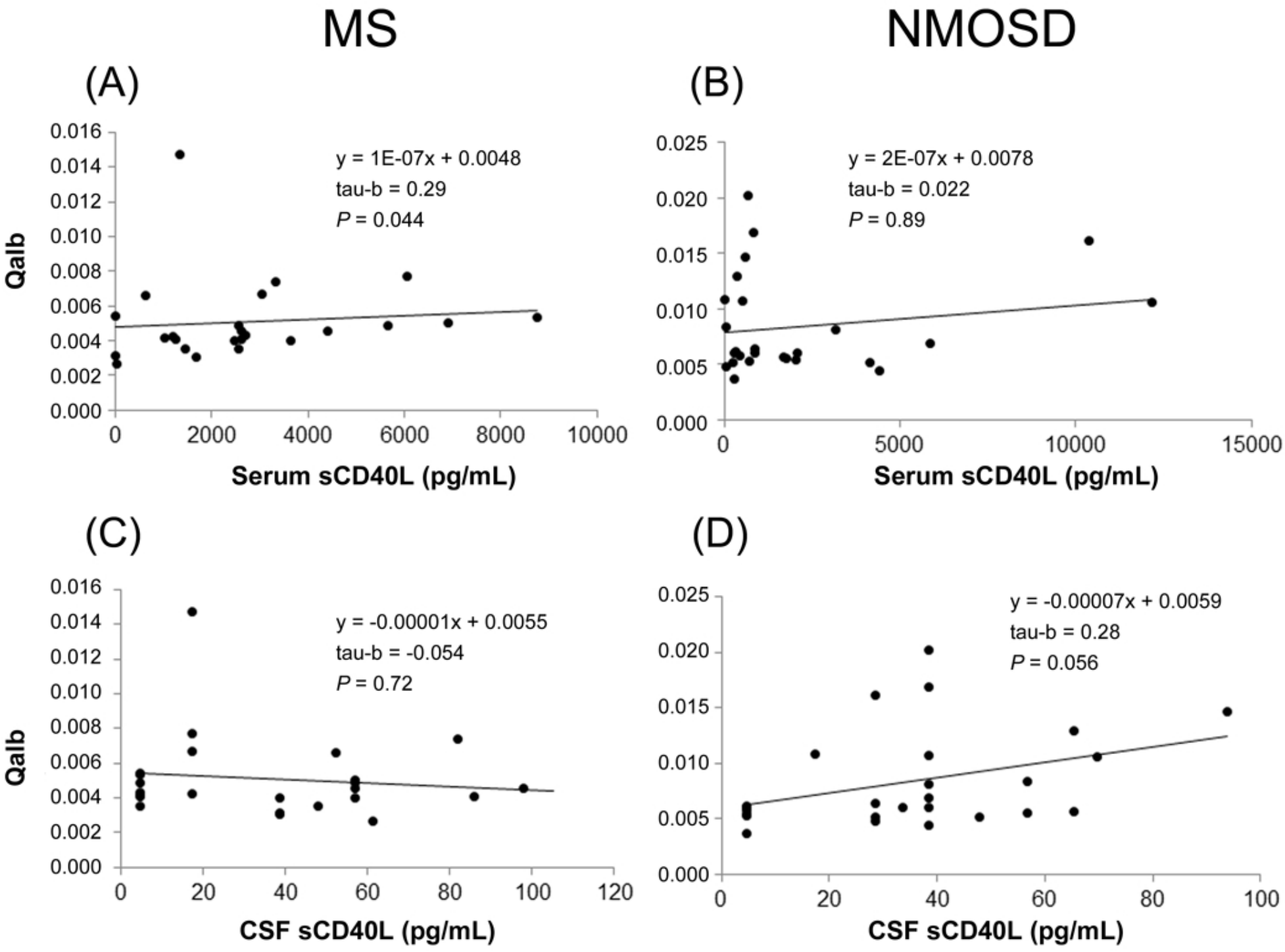
(A) Positive correlation between serum sCD40L levels and Qalb in MS patients. (B) No correlation between serum sCD40L levels and Qalb in NMOSD patients. (C) No correlation between CSF sCD40L levels and Qalb in MS patients. (D) Positive correlations between CSF sCD40L levels and Qalb in NMOSD patients. MS = multiple sclerosis; NMOSD = neuromyelitis spectrum disorder; Qalb = CSF/serum albumin ratio; sCD40L = soluble CD40 ligand.

Figure 3. Correlations between sCD40L levels and IL-6 levels in MS and NMOSD

(A) Positive correlation between serum sCD40L levels and serum IL-6 levels in MS patients. (B) No correlation between serum sCD40L levels and serum IL-6 levels in NMOSD patients. (C) No

correlation between CSF sCD40L levels and CSF IL-6 levels in MS patients. (D) Positive correlations between CSF sCD40L levels and CSF IL-6 levels in NMOSD patients. IL-6 = interleukin-6; MS = multiple sclerosis; NMOSD = neuromyelitis spectrum disorder; sCD40L = soluble CD40 ligand.


A. Serum sCD40L
P = 0.046
N.S.
N.S.
Concentrations (pg/mL)
1.4x10^4
1.2x10^4
1x10^4
8x10^3
6x10^3
4x10^3
2x10^3
0
L.L.D.
MS
NMOSD
DCs
B. CSF sCD40L
P = 0.002
N.S.
P = 0.013
120
100
80
60
40
20
L.L.D.
0
MS
NMOSD
DCs


MS
NMOSD
(A)
(B)
(C)
(D)
Qalb
y = 1E-07x + 0.0048
tau-b = 0.29
P = 0.044
Serum sCD40L (pg/mL)
y = 2E-07x + 0.0078
tau-b = 0.022
P = 0.89
Serum sCD40L (pg/mL)
y = -0.00001x + 0.0055
tau-b = -0.054
P = 0.72
CSF sCD40L (pg/mL)
y = -0.00007x + 0.0059
tau-b = 0.28
P = 0.056
CSF sCD40L (pg/mL)

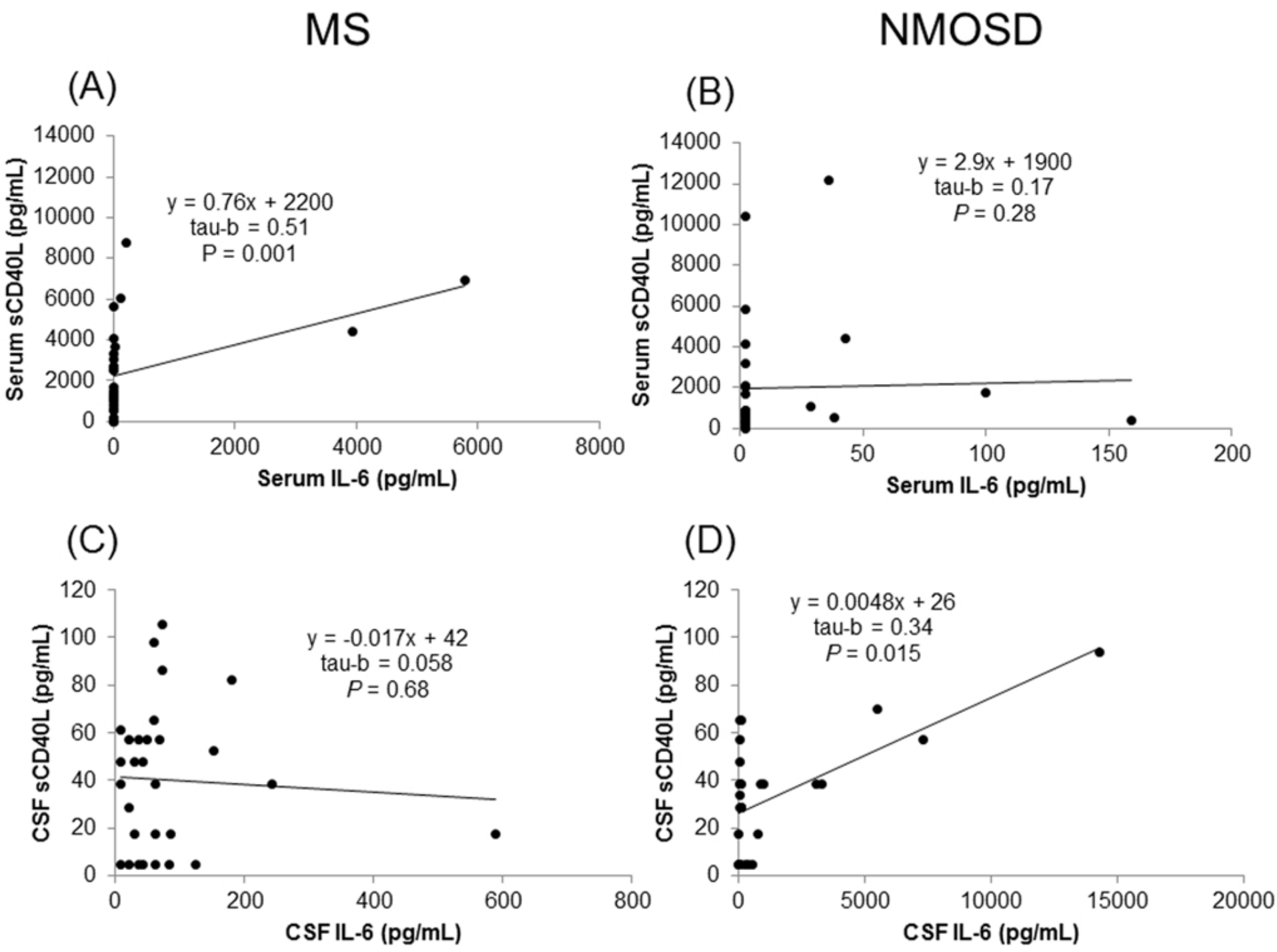

MS
NMOSD
(A)
y = 0.76x + 2200
tau-b = 0.51
P = 0.001
Serum sCD40L (pg/mL)
Serum IL-6 (pg/mL)
(B)
y = 2.9x + 1900
tau-b = 0.17
P = 0.28
Serum sCD40L (pg/mL)
Serum IL-6 (pg/mL)
(C)
y = -0.017x + 42
tau-b = 0.058
P = 0.68
CSF sCD40L (pg/mL)
CSF IL-6 (pg/mL)
(D)
y = 0.0048x + 26
tau-b = 0.34
P = 0.015
CSF sCD40L (pg/mL)
CSF IL-6 (pg/mL)

Table 1. Demographic and clinical characteristics, laboratory findings and treatment in MS and NMOSD patients

	MS (n = 29)	NMOSD (n = 29)	*P*
Demographic and clinical features			
Female (%)	23/29 (79.3%)	27/29 (93.1%)	0.25
Age at CSF sampling (years)	36.0 [13.0]	52.0 [22.5]	<0.001*
Disease duration (months)	49.0 [132.5]	65.0 [169.0]	0.40
Days from attack to CSF sampling	11.0 [18.5]	11.0 [15.0]	0.59
EDSS	2.5 [2.0]	6.0 [3.0]	<0.001*
Laboratory findings			
Positive anti- aquaporin-4 antibodies	0/29 (0%)	24/29 (82.8%)	<0.001*
CSF cell count (/μL)	11.0 [25.0]	8.0 [14.0]	0.45
CSF protein concentration (mg/dl)	32.0 [10.0]	36.0 [28.0]	0.18
Qalb (/10^{-3})	44.1 [13.9]	60.8 [53.3]	<0.001*
IgG index	0.73 [0.40]	0.63 [0.22]	0.069
Positive oligoclonal IgG bands	16/27 (59.3%)	7/26 (26.9%)	0.027*
Platelet count (10^3/μL)	257 [88]	255 [110]	0.74

Treatments			
Any immunomodulating treatment	2/29 (6.9%)	15/29 (51.7%)	<0.001*
Continuous oral prednisolone	0/29 (0%)	14/29 (48.3%)	<0.001*
Azathioprine	0/29 (0%)	1/29 (3.4%)	1.0
Interferon-β	2/29 (6.9%)	1/29 (3.4%)	0.49
Fingolimod	0/29 (0%)	0/29 (0%)	-

Data are presented as median [interquartile range] or number (%).

*$P < 0.05$.

CSF: Cerebrospinal fluid; EDSS: Kurtzke's Expanded Disability Status Scale; MS: multiple sclerosis; NMOSD: neuromyelitis optica spectrum disorder.

Table 2. Correlation between sCD40L and clinical and laboratory findings in patients with MS.

	Kendall tau-b Serum sCD40L	*P*	Kendall tau-b CSF sCD40L	*P*
EDSS	-0.004	0.98	-0.026	0.87
CSF cell count (/μL)	0.084	0.58	0.085	0.59
CSF protein concentration (mg/dl)	0.21	0.17	-0.009	0.96
Qalb (/10-3)	0.29	0.044*	-0.054	0.72
IgG index	-0.062	0.96	-0.008	0.96
Serum sCD40L	1.0	NA	-0.067	0.62
CSF sCD40L	-0.067	0.62	1.0	NA

*$P < 0.05$. CSF: Cerebrospinal fluid; EDSS: Kurtzke's Expanded Disability Status Scale; sCD40L; soluble CD40 ligand; MS: multiple sclerosis.

Table 3. Correlation between sCD40L and clinical and laboratory findings in patients with NMOSD.

	Kendall tau-b Serum sCD40L	*P*	Kendall tau-b CSF sCD40L	*P*
EDSS	-0.034	0.81	0.23	0.10
CSF cell count (/μL)	-0.13	0.36	0.30	0.038*
CSF protein concentration (mg/dl)	0.046	0.74	0.34	0.018*
Qalb (/10-3)	0.022	0.89	0.28	0.056
IgG index	-0.062	0.96	0.28	0.056
Serum sCD40L	1.0	NA	0.12	0.38
CSF sCD40L	0.12	0.38	1.0	NA

*$P < 0.05$. CSF: Cerebrospinal fluid; EDSS: Kurtzke's Expanded Disability Status Scale; sCD40L; soluble CD40 ligand; NMOSD; neuromyelitis optica spectrum disorder.

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