normal in *RIIB*$^{-/-}$ mice of both genders, while it was augmented in *SLAM*^{129} mice (Figure 6B). The numbers of total GL7^{+}Fas^{+} GC cells and CD19^{+}GL7^{+}Fas^{+} GC B cells were not significantly increased in *RIIB*$^{-/-}$ mice of both genders, while they tended to be increased in female *RIIB*$^{-/-}$*SLAM*^{129} and *SLAM*^{129} animals (Figure 6C, D), these observations being reminiscent of SLAM's important role in adequate B–T cell communication in GCs [22]. Thus, we failed to find marked alterations in *RIIB*$^{-/-}$ mice in terms of the total splenic lymphocyte and GC cell numbers other than slight splenomegaly. Further analysis of other parameters such as plasma cells, antigen-presenting cells and T cells may help to clarify the reason for the production of a small amount of autoantibodies in *RIIB*$^{-/-}$ mice.

Discussion

The exact effect of RIIB deficiency on spontaneous development of autoimmune diseases is controversial. Regarding animal models, *RIIB* gene-targeted mice derived from ES cells of the 129 strain and backcrossed into the B6 genetic background yielded different phenotypes in different laboratories, most probably due to different contributions of the autoimmune-prone *SLAM*^{129} haplotype neighboring the *RIIB* locus [4,5,23,24]. In this study, we, for the first time, established two lines through extensive backcrossing, one line with RIIB deficiency but the non-autoimmune B6 *SLAM* haplotype, and the other with autoimmune-prone *SLAM*^{129} and the intact *RIIB* gene, both having the B6 background and being housed under identical environmental conditions. We found that the RIIB deficiency indeed caused a spontaneous increase of a very small amount of ANAs including anti-DNA autoantibodies, and weak glomerulonephritis with slight IgG-IC deposition in glomeruli in a female-biased manner, while the *SLAM*^{129} line exhibited ANA production accompanying no obvious glomerulonephritis, IgG-IC deposition being minimum and comparable to that in the B6 control.

RIIB is known to be an important suppressor of the production and/or accumulation of autoantibodies in some animal models, in which the diseases were induced experimentally in autoimmune-prone mouse strains or non-autoimmune B6 mice by immunization [15,16,25-28]. A recent report [19] has pointed out the additional role of RIIB, expressed in liver sinusoidal endothelial cells, in the clearance of small size IgG-ICs, and potentially in minimizing the level of pathologic IgG-ICs in the blood. In contrast to well-established feedback immunoregulation, and its crucial role in suppressing autoantibody production and/or accumulation after active immunization of susceptible mice, the role of RIIB in physiological suppression of spontaneous autoantibody production in non-autoimmune strains remained unclear due to the inconsistent phenotypes of RIIB-deficient mice observed in different laboratories [8,16-18], the mice being extremely prone to the production of anti-chromatin autoantibodies and susceptible to the development of fatal lupus [8,21], whereas in others they were not susceptible to the development of lupus nephritis without massive production of autoantibodies [16-18]. To circumvent the difficulty in isolating the effect of RIIB deficiency by backcrossing, Boross et al. generated B6- but not 129-origin RIIB-deficient mice by gene targeting in B6-derived ES cells [17]. They observed that RIIB-deficient mice produced only a small amount of anti-ssDNA antibodies spontaneously even at the age of 10 months, exhibited slight IgG-IC deposition in the kidneys, and increases in urinary albumin and the pathological score of glomerulonephritis, without showing mortality at least up to 12 months of age, indicating that RIIB deficiency only amplifies spontaneous autoimmunity determined by other loci. These phenotypes are mostly the same as those of our *RIIB*$^{-/-}$ mice in this study in terms of the very low ANA level and slight IgG-IC deposition without development of glomerulonephritis. Similar results have also been obtained for congenic lines within the *Nba2* autoimmune susceptibility locus [29]. Thus, our present observations substantially support those reported for B6-based *RIIB*$^{-/-}$ mice [17], and our results also indicate that RIIB prevents the spontaneous production and/or accumulation of a small amount of ANAs.

The IgG-IC deposition, albeit slight, and the increase in the pathological score for glomerulonephritis in *RIIB*$^{-/-}$ mice were unexpected, because these phenotypes were not observed in the *SLAM*^{129} line housed under the same environmental conditions as *RIIB*$^{-/-}$ mice (Figure 5). These observations are also consistent with those for the B6-based RIIB-deficient mice [17]. Interestingly, a recent study employing a cell type-specific RIIB deletion technique has shown that RIIB on myeloid cells and intrinsic renal cells rather than B cells prevents nephrotoxic nephritis, suggesting a significant role of RIIB on myeloid cells and renal mesangial cells in this protection [30]. It has also been shown that neither B cell- nor myeloid-specific deletion of RIIB leads to the development of crescentic glomerulonephritis with a higher incidence than in wild-type mice in an anti-glomerular basement membrane antibody disease model, indicating that RIIB deficiency in either B cells or a subset of myeloid cells alone is not sufficient to increase the susceptibility to the kidney disease [31]. In contrast to *RIIB*$^{-/-}$ mice, *SLAM*^{129} animals did not show IgG-IC deposition in the kidneys, even though they had a comparable level of serum ANAs to that in *RIIB*$^{-/-}$ mice, at least at 24 weeks of age. Thus, the *SLAM*^{129} haplotype in the B6 background does not contribute to the development of glomerulonephritis but to ANA production, due to the lack of a significant role in the kidneys.

Generally, development of autoimmune diseases is biased toward females in humans and in mice, the main

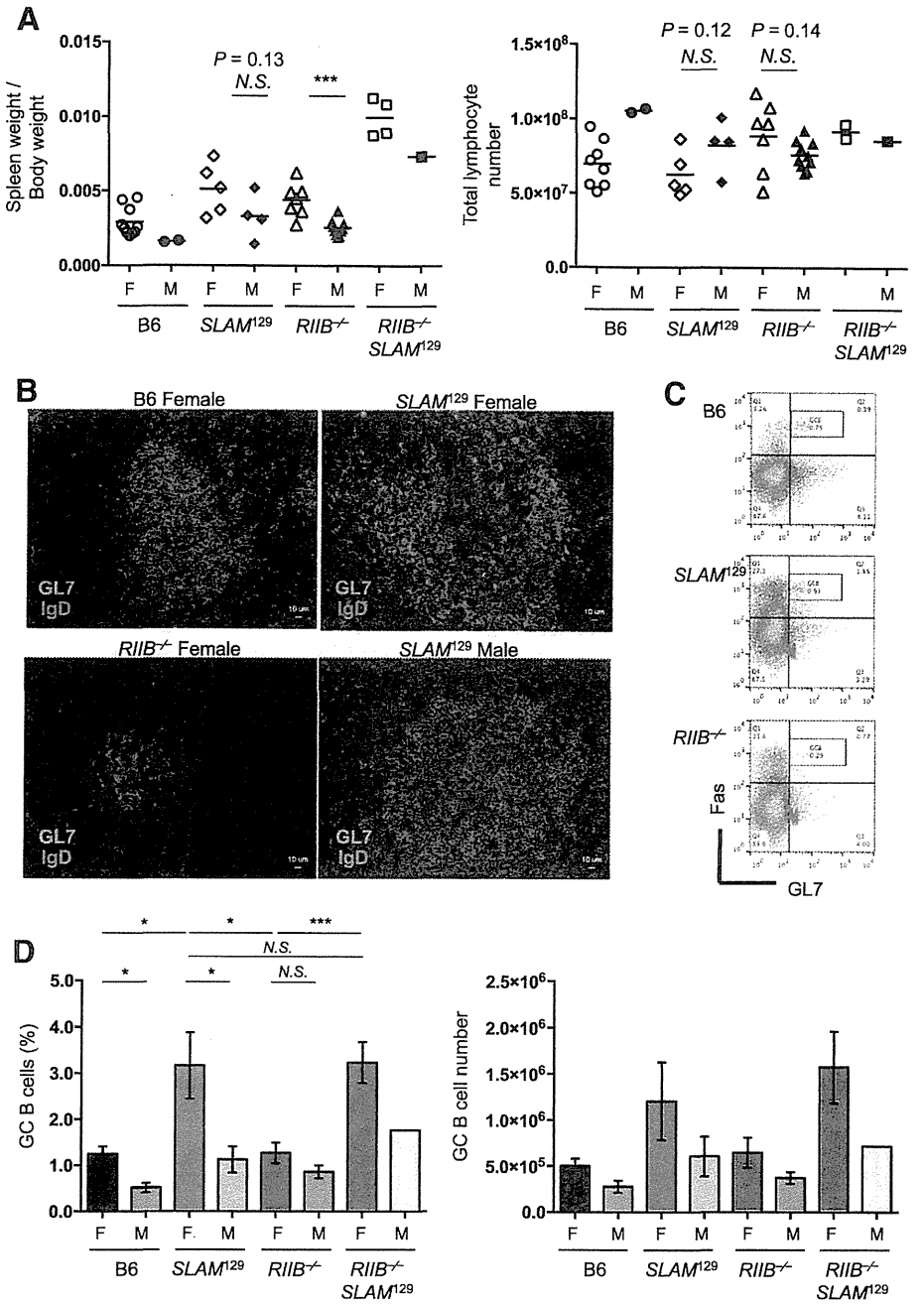

Figure 6 Germinal center B cells are increased in *SLAM*^{129} but not *RIIB*$^{-/-}$ mice. (**A**, *Left*) Slight splenomegaly in female *RIIB*$^{-/-}$ mice. Spleen weights were measured for each line of both genders (*F*, female; *M*, male) and are presented as the ratio of the weight per body weight. Horizontal bars indicate mean values, excepting the plot of *RIIB*$^{-/-}$*SLAM*^{129} male for single determination. ***P <0.001. Student's *t*-test. (**A**, *Right*) Total splenic lymphocytes in each line. Horizontal bar represents the mean number of total splenocytes, excepting the plot of *RIIB*$^{-/-}$*SLAM*^{129} male for single determination. *N.S.*, not significant. Student's *t*-test. **(B)** Germinal center formation in naïve B6, N22 female *RIIB*$^{-/-}$, and female and male *SLAM*^{129} mice at 45 weeks of age. Frozen spleen sections were stained with GL7-Alexa488 and Alexa546-labeled anti-mouse IgD antibodies. Scale bar =10 μm. **(C)** Flow cytometric determination of germinal center (GC) B cells of B6, female *SLAM*^{129} and N22 female *RIIB*$^{-/-}$ mice at 45 week of age. Splenocytes were gated with anti-CD19-APC, and analyzed for anti-Fas-PE and GL7-FITC. **(D)** Total GC cells and GC B cells are not significantly increased in female *RIIB*$^{-/-}$ mice. The percentage of GC B cells in splenic CD19^{+} cells (*Left*), and the absolute number of splenic GC B cells (*Right*) of each line of both genders (*F*, female; *M*, male) are shown. *P <0.05; ***P <0.001; *N.S.*, not significant. Student's *t*-test.

reason for which being the various influence(s) of sex hormones on the immune system and cells [32-34]. The reason for the gender bias in the ANA levels in *RIIB*$^{-/-}$ mice rather than *SLAM*^{129} mice is currently unknown, although one may speculate that antigen-presenting cell–T cell communication could be more prone to be de-regulated in females than B cell–T cell interactions, which could be influenced by *SLAM*^{129}. Further examinations will clarify the differences in gender in splenocytes and lymph node cells, and even in those in the lamina propria.

Conclusion

Separation of the *RIIB*$^{-/-}$ locus from the autoimmune-prone *SLAM*^{129} locus revealed the role of RIIB in maintenance of peripheral tolerance. As judged on assessment under identical genetic and environmental conditions, RIIB deficiency caused slight ANA production and/or its accumulation accompanied by non-lethal glomerulonephritis with a low level of IgG-IC deposition, in contrast to the role of *SLAM*^{129}, which causes ANA production without accompanying glomerulonephritis. The combination of the RIIB deficiency and *SLAM*^{129} synergistically induced substantial ANA production and sub-lethal glomerulonephritis. These results will facilitate development of strategies for targeting RIIB for treating autoimmune disorders involving autoantibody production such as SLE.

Additional file

Additional file 1: Tables S1 and S2 and Figures S1 and S2.

Abbreviations

ANA: Anti-nuclear antibody; B6: C57BL/6; ds-: Double-stranded; FcγRIIB: Type IIB low affinity Fc receptor for IgG; FcR: Fc receptor; FcγR: Fc receptor for IgG; GC: Germinal center; HE: Hematoxylin and eosin; RIIB: FcγRIIB; *RIIB*$^{-/-}$: FcγRIIB-deficient; *RIIB*$^{-/-}$SENDAI mice: N28 FcγRIIB-deficient mice; ss-: Single-stranded; SLAM: Signal lymphocyte activation molecule(s); SLE: Systemic lupus erythematosus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YK participated in the design of the study and with HT carried out the analysis of mice and performed the statistical analysis. AST carried out the backcross, genetic analysis, and maintenance of mice, and histological examinations. MI and AN participated in the design of the study. SH provided the mice and participated in the design of the study. TT conceived of the study with AN, and participated in its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Increased serum concentration of BAFF/APRIL and IgA2 subclass in patients with mixed connective tissue disease complicated by interstitial lung disease

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Abstract

B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are known to be crucial for B cell maturation and survival, and increased expression of these factors in various autoimmune diseases has been reported. Human B cells produce two IgA subclasses: IgA1 and IgA2, the latter being abundant in the distal intestine, saliva, colostrum and bronchial fluid. We investigated these parameters in patients with mixed connective tissue disease (MCTD) complicated by interstitial lung disease (ILD+), and compared them with those in MCTD patients without ILD (ILD−). Sixty-three MCTD patients were divided into two groups: 21 ILD+ patients and 42 ILD− patients. In each patient group we analyzed soluble BAFF/APRIL using ELISA, and IgA1 and IgA2 using double immunodiffusion. Furthermore, we analyzed BAFF–APRIL receptors, BCMA, BAFF-R and TACI, using flow cytometry. The ILD+ patients had significantly higher levels of BAFF/APRIL than the ILD− patients. There were significant correlations between BAFF/APRIL, BAFF/KL-6 and APRIL/KL-6. Although there was no significant inter-group difference in the serum IgA1 level, ILD+ patients had a significantly elevated IgA2 level in comparison with ILD− patients. Moreover, although there were no significant inter-group differences in the expression of BCMA, BAFF-R and TACI on B cells, the expression of BAFF-R was significantly decreased in the ILD+ patients. In recent years, relationships between BAFF/APRIL and IgA subclass have been reported. Our results suggest that an elevated level of BAFF/APRIL drives the maturation of B cells, subsequently leading to IgA2 class switching, and possibly to the development of ILD in patients with MCTD.

Keywords

A proliferation-inducing ligand, B cell activating factor, IgA, Interstitial lung disease, Mixed connective tissue disease

History

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Introduction

Mixed connective tissue disease (MCTD) is a systemic autoimmune disorder, accompanied by chronic inflammation involving several organs, which is characterized by the presence of autoantibody against uridine-rich (U1)-RNP polypeptides [1–3]. Interstitial lung disease (ILD) is one of the most important features of MCTD [4–6]. Although the serological features of MCTD are similar to those of systemic lupus erythematosus (SLE), the incidence of ILD differs considerably between the two: up to 60% of patients with MCTD are complicated by ILD [7], whereas the prevalence of ILD in SLE is estimated to be approximately 3% [8,9].

B cell activating factor [BAFF, also known as B lymphocyte stimulator (BLyS)], a member of the tumor necrosis factor (TNF) family, is crucial for B cell maturation and survival. BAFF is also believed to play a role in autoantibody production as well as in T cell activation and differentiation [10]. The related cytokine, a proliferation-inducing ligand (APRIL), is also important for B cell development and function [10]. High serum levels of BAFF and APRIL have been reported in patients with various autoimmune diseases [11–15]. In recent years, a BAFF-blocking antibody, belimumab, has been used for treatment of SLE and other autoimmune diseases.

Immunoglobulin (Ig) A is the major Ig of the healthy intestine as well as respiratory tract, and is thought to be the most important Ig for intestinal and pulmonary defense. Human B cells produce two IgA subclasses – IgA1 and IgA2 – based on differences in their antigenic repertoire [16–18]. It has been demonstrated that dendritic cells directly modulate B-cell growth and differentiation in vitro and stimulate CD40-activated naive B cells to enhance an isotype switch towards IgA1 and IgA2 with the help of TGF-β and IL-10 in a T-cell-independent manner [19]. Whereas systemic B cells produce mostly IgA1, mucosal B cells produce both IgA1 and IgA2. The latter is very abundant in the distal intestine, saliva, colostrum and bronchial fluid [20], and is more resistant than IgA1 to degradation by bacterial proteases [21].

Against this background, the present study was undertaken to investigate the possible alterations of serum soluble BAFF/APRIL, also IgA1 and IgA2, in patients with MCTD complicated by ILD. We also investigated the expression of BAFF/APRIL receptors on B cells. In the sera of MCTD patients with ILD, we found that the levels of IgA2 and BAFF/APRIL were increased, and also that the expression of the BAFF-receptor was altered.

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Table 1. Patient profiles in relation to analysis of BAFF/APRIL and IgA1/IgA2 in serum.

	ILD(+) ($n = 21$)	ILD(−) ($n = 42$)	P value
Sex (F:M)	19:2	38:4	0.126
Age	56.3 ± 16.2	46.9 ± 15.6	0.038*
Duration (year)	13.8 ± 2.35	14.5 ± 1.40	0.792
Lymphocyte (/μL)	1160 ± 122	1067 ± 79.3	0.919
IgG (mg/dL)	1619 ± 119	1640 ± 73.7	0.944
IgA (mg/dL)	319.9 ± 26.4	314.7 ± 19.0	0.551
IgM (dL)	76.0 ± 11.3	80.6 ± 7.02	0.694
RF (IU/ml)	28.3 ± 8.67	27.9 ± 10.5	0.134
KL-6 (U/ml)	1128 ± 835	323 ± 158	0.001*
Therapy			
Corticosteroid (mg/day)	8.95 ± 1.55	6.5 ± 0.89	0.317
Immunosuppressant (n)	7 (33%)	12 (28%)	0.697
Sjögren syndrome	4 (19%)	8 (19%)	0.641
Pulmonary hypertension	2 (10%)	3 (7%)	0.544
ANA pattern			
Homogeneous	4 (19%)	13 (30%)	0.315
Speckled	21 (100%)	42 (100%)	
Centromere	2 (10%)	3 (7%)	0.544
Autoantibodies			
Anti U1-RNP Ab	21 (100%)	42 (100%)	
Anti DNA Ab	5 (21%)	15 (36%)	0.251

Data are shown as mean (± SD). Numbers are percentages of MCTD patients with ILD (ILD+) or without ILD (ILD−). ANA nuclear and/or cytoplasmic HEp2 cell immunofluorescence, titer > 1:80.
*P values are shown for comparisons of mean values by Mann–Whitney U test.

Table 2. Patients profiles in relation to flow cytometoric analysis of BAFF-R, BCMA and TACI.

	ILD(+) ($n = 10$)	ILD(−) ($n = 8$)	P-value
Sex (F:M)	7:3	7:1	0.381
Age	55.3 ± 4.84	41.2 ± 5.46	0.065
Duration (year)	8.50 ± 2.01	12.1 ± 3.89	0.934
Lymphocyte (/μL)	1499 ± 221	1033 ± 144	0.088
IgG (mg/dL)	1469 ± 115	1339 ± 121	0.412
IgA (mg/dL)	302.3 ± 39.1	257.6 ± 26.6	0.715
IgM (dL)	129.4 ± 24.7	67.3 ± 12.1	0.042*
RF (IU/ml)	24.7 ± 9.15	23.3 ± 10.9	0.081
KL-6 (U/ml)	1139.5 ± 258.9	257.3 ± 27.8	0.039*
Therapy			
Corticosteroid (mg/day)	12.2 ± 4.36	25.6 ± 7.86	0.351
Immunosuppressant (n)	8 (80%)	1 (12.5%)	0.007*
Sjögren syndrome	2 (20%)	2 (25%)	0.617
Pulmonary hypertension	2 (20%)	2 (25%)	0.617
ANA pattern			
Homogeneous	1 (10%)	4 (50%)	0.088
Speckled	10 (100%)	8 (100%)	
Centromere	0 (0%)	0 (0%)	
Autoantibodies			
Anti U1-RNP Ab	10 (100%)	8 (100%)	
Anti DNA Ab	1 (10%)	5 (38%)	0.030*

Data are shown as mean (± SD). Numbers are percentages of MCTD patients with ILD (ILD+) or without ILD (ILD−). ANA nuclear and/or cytoplasmic HEp2 cell immunofluorescence, titer > 1:80.
*P values are shown for comparisons of mean values by Mann–Whitney U test.

Patients and methods

Patients

For the analysis of BAFF, APRIL, and IgA1 and IgA2 subclasses, 63 Japanese patients with MCTD were divided into two groups: 21 patients who were complicated by interstitial lung disease (ILD+), and 42 patients who were not (ILD−). Serum samples were collected from these 63 patients, in whom MCTD had been diagnosed according to the classification criteria proposed by the Research Committee of the Ministry of Health and Welfare of Japan [22]. For the analysis of BAFF-R, TACI and BCMA, 18 MCTD patients (10 ILD+ and 8 ILD−) were analyzed. All the patients were treated and followed up at the Department of Rheumatology and Internal Medicine, Juntendo University School of Medicine, Tokyo, Japan. Informed consent was obtained from all the patients. The study was approved by the Ethics Committee of Juntendo University.

Measurement of IgG, IgA, IgM, KL-6, IgA subclass, BAFF and APRIL levels

Serum levels of IgG, IgA and IgM were measured by ELISA, the serum level of KL-6 was measured by CLEIA (chemiluminescence enzyme immunoassay) and serum IgA subclass levels were measured by Ouchterlony double immunodiffusion (The Binding Site Group Ltd., Birmingham, UK). Serum levels of BAFF (R&D

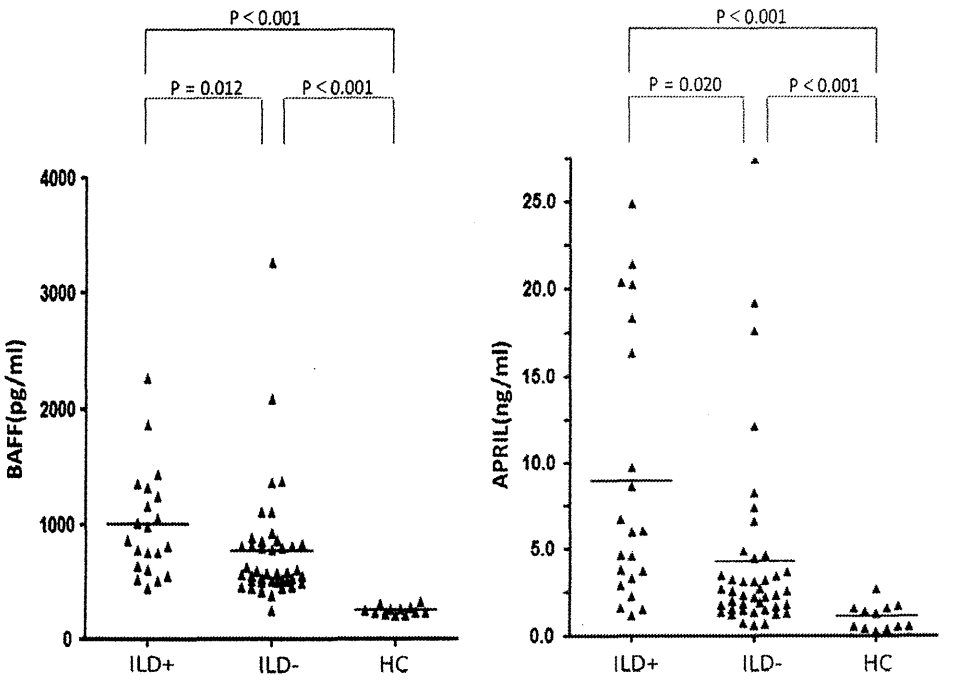

Figure 1. Serum levels of soluble BAFF and APRIL in ILD+, ILD− MCTD patients and healthy controls. Serum levels of soluble BAFF and APRIL were elevated in MCTD ILD+ patients.

Systems, Minneapolis, Minnesota, USA) and APRIL (Bender MedSystems GmbH, Vienna, Austria) were measured by ELISA. Samples were analyzed in duplicate.

Flow cytometry analysis

Fluorescein isothiocyanate-conjugated anti-CD20, phycoerythrin-conjugated anti-BAFF-R and phycoerythrin/Cy5-conjugated anti-CD38 were purchased from BD Biosciences (San Jose, CA, USA). Phycoerythrin-conjugated anti-TACI was purchased from eBioscience (San Diego, CA, USA). Phycoerythrin-conjugated anti-BCMA was purchased from R&D Systems (Minneapolis, MN, USA). Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll density-gradient centrifugation and triple-stained with antibodies. Flow cytometric analysis was performed using a FacStation (Becton Dickinson; CA, USA) and the data were processed using the Cell Quest program (Becton Dickinson).

Statistical analysis

Statistical analysis was performed using GraphPad Prism V. 3.03 (GraphPad Software, San Diego, California, USA). For analysis of differences between groups, the Mann–Whitney U test, Fisher's exact test and Pearson correlation analysis were employed. Statistical significance was defined as $P < 0.05$.

Results

Clinical, laboratory and serological data

Clinical and laboratory data are presented in Tables 1 and 2. For analysis of sera, the profiles of the ILD+ and ILD− patients are shown in

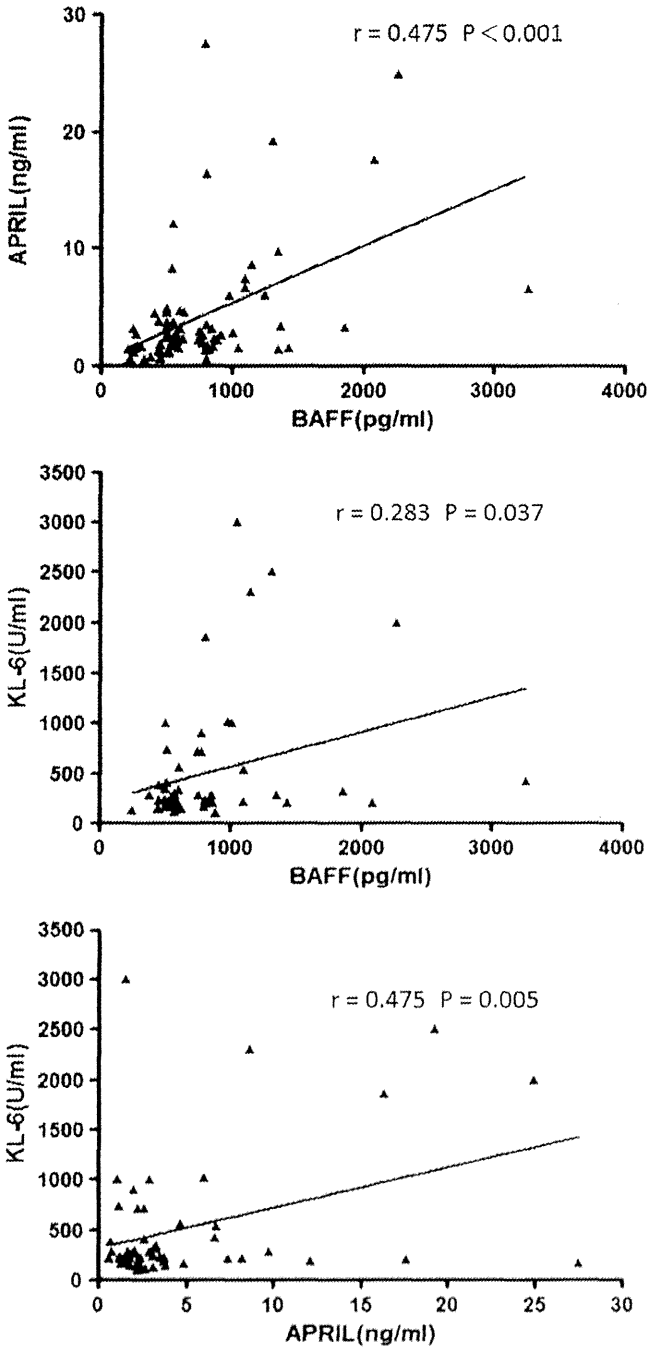

Figure 2. Serum levels of BAFF, APRIL and KL-6 were measured by ELISA and CLEIA. The scatter plot shows the values of BAFF (x axis) versus the values of APRIL (y axis), the values of BAFF (x axis) versus the values of KL-6 (y axis) and the values of APRIL (x axis) versus the values of KL-6 (y axis), along with the regression line. Each point represents a single patient. There were correlations between the values of BAFF/APRIL (Pearson $r = 0.475$, $P < 0.0001$), BAFF/KL6 (Pearson $r = 0.283$, $P = 0.0365$) and APRIL/KL6 (Pearson $r = 0.475$, $P = 0.0052$). Pearson correlation analysis was used to obtain the correlation coefficients.

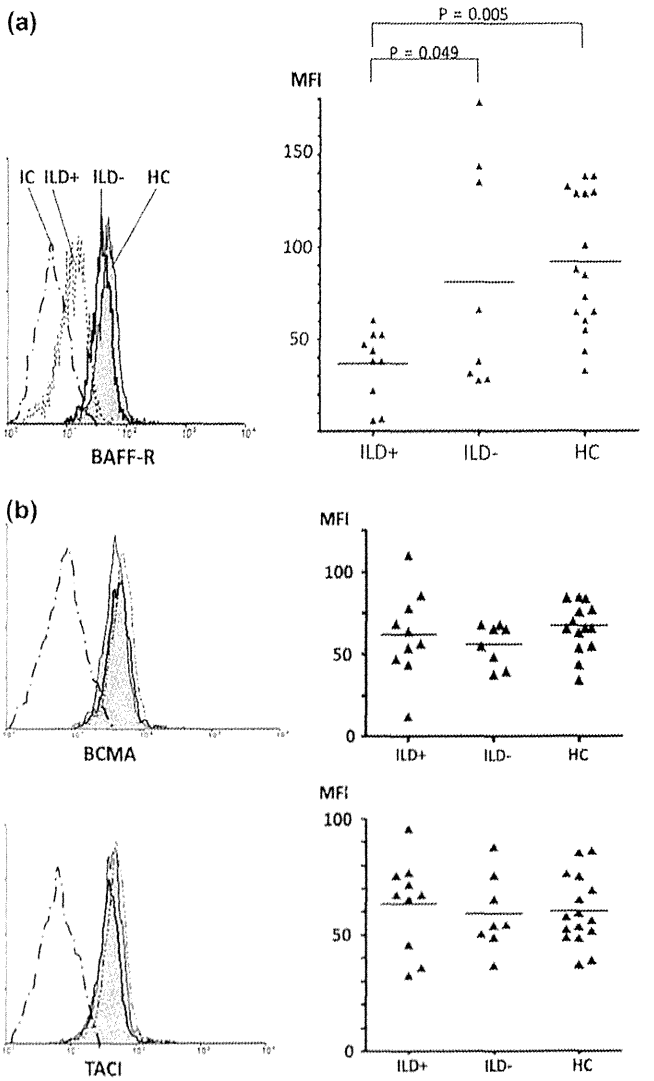

Figure 3. Expression of BAFF-R, BCMA and TACI in ILD+, ILD− MCTD patients and healthy controls. (a) BAFF-R expression on CD20 + B cells was decreased in ILD+ patients relative to ILD− patients or normal controls. ILD, interstitial lung disease; IC, isotype control; MFI, mean fluorescence intensity. (b) There were no significant differences in BCMA or TACI expression among the groups.

Table 1. There were no differences in sex or the numbers of patients using corticosteroids or immunosuppressants. Age at the time of sample-taking was higher in ILD+ patients than in ILD− patients (46.9 + 15.3 years vs. 56.3 + 16.1 years; $p = 0.038$). The concentration of KL-6, a circulating biomarker of interstitial lung disease, was higher in ILD+ patients. For analysis of PBMC, the profiles of the ILD+ and ILD− patients are shown in Table 2. Similarly to the information in Table 1, the ILD+ patients were older and showed a higher KL-6 concentration. Also, a higher proportion of these patients had a nucleolar pattern of anti-nuclear antibody staining.

Serum level of soluble BAFF/APRIL in ILD+/ILD− MCTD patients

First we evaluated the serum levels of BAFF/APRIL in ILD+/ILD− MCTD patients.

ILD+ patients had significantly higher levels of BAFF than ILD− patients (ILD + 990 ± 102 pg/ml vs. ILD − 759 ± 79.5 pg/ml). The level of APRIL was also elevated in ILD+ patients relative to ILD− patients (ILD + 8.96 ± 1.69 pg/ml vs. ILD − 4.27 ± 0.84 pg/ml) (Figure 1).

Serum levels of BAFF, APRIL and KL-6

To confirm the relationship of BAFF and APRIL with ILD activity, we next analyzed the correlations between BAFF/APRIL, BAFF/KL-6 and APRIL/KL-6 separately. Correlations were observed between BAFF/APRIL (Pearson $r = 0.475$, $P < 0.0001$), BAFF/KL6 (Pearson $r = 0.283$, $P = 0.0365$) and APRIL/KL6 (Pearson $r = 0.475$, $P = 0.0052$) values (Figure 2).

Expression of BAFF receptors on B cells of ILD+/ILD− MCTD patients

Next we evaluated the expression of the receptors for BAFF/APRIL. Three kinds of BAFF/APRIL receptors are known: B-cell maturation antigen (BCMA), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), and BAFF receptor (BAFF-R). Details of the expression of these receptors are shown in Figure 3a, b. BAFF-R expression on CD20 + B cells was decreased in ILD+ patients relative to ILD− patients or normal controls (Figure 3a). However, there were no significant differences in BCMA or TACI expression among the groups (Figure 3b). We also conducted immunostaining with anti-CD38 for determination of plasmablasts, but there were no significant differences among the groups (data not shown).

IgA1 and IgA2 subclass concentrations in sera of ILD+/ILD− MCTD patients

APRIL-deficient mice have been reported to show impaired IgA class switching [23]. Accordingly, we next examined the serum concentrations of IgA1 and IgA2 in the ILD+, ILD− patients with MCTD and healthy controls. Although the serum concentration of IgA1 tended to be increased in ILD+ patients relative to ILD− patients, the difference was not significant. However, the serum concentration of IgA2 was significantly higher in ILD+ patients than in ILD− patients. On the other hand, there was no significant inter-group difference in total IgG or total IgA (Figure 4).

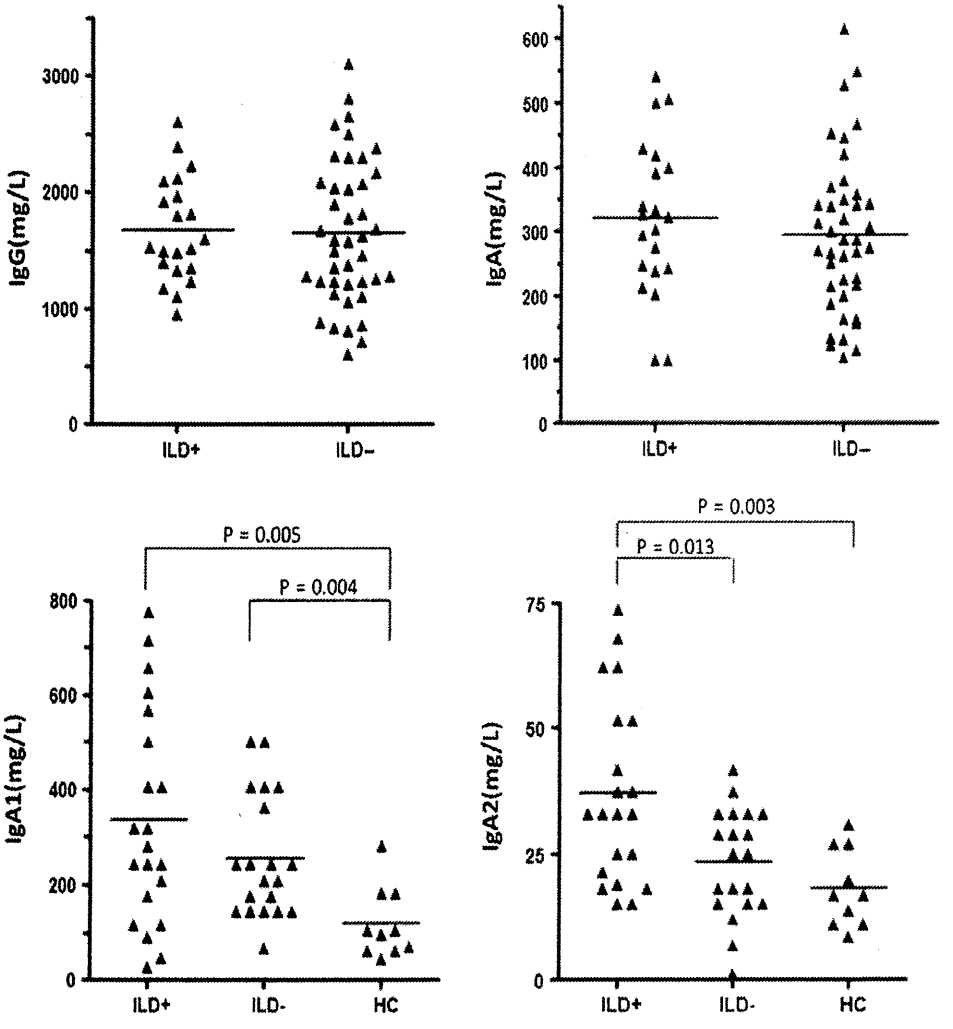

Figure 4. Serum levels of total IgG, IgA and IgA1/IgA2 subclasses. The serum level of IgA2 was significantly elevated in ILD+ patients with MCTD.

Discussion

In this study of patients with MCTD, we found that ILD+ patients had higher serum levels of BAFF/APRIL than ILD− patients. In a study of patients with myositis, Krystufková et al. found that patients with ILD had significantly higher levels of BAFF than patients without ILD, although there was no intergroup difference in the levels of APRIL [10]. They speculated that, in myositis, BAFF was more directly involved in the disease process than APRIL. Also, in a study of patients with systemic sclerosis, Bielecki et al. found increased production of APRIL by PBMC and suggested that this was associated with the presence of scleroderma lung disease [24]. Since our present data indicated significant increases in the serum levels of APRIL as well as BAFF in ILD+ MCTD patients, we speculate that the disease process in such patients is more dependent on APRIL than is the case in myositis.

We also found that the expression of BAFF-R was lower in ILD+ patients than in ILD− patients or healthy controls. A recent report has indicated that the level of soluble BAFF is inversely correlated with the number of B cells and the expression of BAFF-R [25]. In that study, patients with primary antibody deficiency (PAD) were found to have higher BAFF levels than healthy donors. Also, mice constitutively expressing human BAFF were found to have higher concentrations of BAFF in the absence of B cells, than in their presence. The authors speculated that the defects of B cell development and function in PAD patients cannot be compensated by an increased concentration of BAFF. Similarly, our present data indicated that ILD+ patients had an increased level of soluble BAFF and decreased expression of BAFF-R. In this context, there is a possibility that in ILD patients B cells undergo maturation into plasma cells locally in the lung and that the resulting decrease of B cells in peripheral blood triggers the production of BAFF. Further studies are needed to clarify the mechanisms involving BAFF in patients with ILD complicating connective tissue diseases.

In the present study we demonstrated that the level of IgA2 was significantly elevated in the sera of MCTD patients with ILD. In recent years, relationships of BAFF/APRIL to IgA subclass have been reported. Castigli et al. have found that mice with APRIL deficiency show impaired IgA class switching [23], and that mice overexpressing BAFF develop IgA-associated nephropathy [26]. Moreover, BAFF and APRIL have been reported to induce IgA isotype switching in human IgD+ B cells, with BAFF directing IgA1 switching and APRIL directing IgA2 switching [27]. We found a correlation between BAFF/APRIL and KL-6, but the correlation of APRIL to KL-6 was more significant than that of BAFF to KL-6 (Figure 2). Matsushita et al. reported that serum APRIL and BAFF levels were differentially associated in systemic sclerosis; a high APRIL level was applicable as a marker for involvement of pulmonary fibrosis and the BAFF level served as a marker for severe skin sclerosis [28]. Consistent with this result, we showed here that the serum level of APRIL was more significantly elevated than that of BAFF in ILD+ MCTD patients, relative to ILD− MCTD patients.

Our present results suggest that the elevated level of BAFF/APRIL drives the maturation of B cells and subsequently lead to IgA class switching from IgA1 to IgA2 via the effect of APRIL, possibly leading to the development of ILD associated with MCTD.

In recent years, a BAFF-blocking antibody, belimumab, has been used for treatment of SLE and other autoimmune diseases. This is the first new prescription treatment to be approved for SLE in more than 50 years. Our present findings suggest that blockade of BAFF/APRIL might be a useful therapeutic strategy for not only patients with MCTD, but also patients complicated by interstitial lung disease.

Acknowledgements

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Conflict of interest

None.

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RESEARCH PAPER

Autocrine MMP-2/9 secretion increases the BBB permeability in neuromyelitis optica

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ABSTRACT

Objective Pathological breakdown of the blood-brain barrier (BBB) is thought to constitute the beginning of the disease process in neuromyelitis optica (NMO). In the current study, we investigated possible molecular mechanisms responsible for the breakdown of BBB using NMO sera.

Methods We analysed the effects of sera obtained from anti-aquaporin 4 (AQP4) antibody-positive NMO spectrum disorder (NMOSD) patients, multiple sclerosis (MS) patients and control subjects on the production of claudin-5, matrix-metalloproteinases (MMPs)-2/9, and vascular cell adhesion protein-1 (VCAM-1) in human brain microvascular endothelial cells (BMECs). We also examined whether immunoglobulin G (IgG) purified from NMOSD sera influences the claudin-5 or VCAM-1 protein expression.

Results The disturbance of BBB properties in BMECs following exposure to NMOSD sera was restored after adding the MMP inhibitor, GM6001. The secretion of MMP-2/9 by BMECs significantly increased after applying the NMOSD sera. The sera from NMOSD patients also increased both the MMP-2/9 secretion and the VCAM-1 protein level by BMECs. The IgG purified from NMOSD sera did not influence the BBB properties or the amount of MMP-2/9 proteins, although it did increase the amount of VCAM-1 proteins in BMECs. Reduction in anti-AQP4 antibody titre was not correlated with a reduction in VCAM-1 expression.

Conclusions The autocrine secretion of MMP-2/9 by BMECs induced by humoral factors, other than IgG, in sera obtained from NMOSD patients potentially increases BBB permeability. IgG obtained from NMOSD sera, apart from anti-AQP4 antibodies, affect the BBB by upregulating VCAM, thereby facilitating the entry of inflammatory cells into the central nervous system.

INTRODUCTION

Neuromyelitis optica (NMO) is a severe relapsing inflammatory disorder of the central nervous system (CNS), characterised by the development of optic neuritis and longitudinally extensive transverse myelitis (LETM), and is presumed to be a different disease entity from multiple sclerosis (MS).[1] The groundbreaking discovery of an autoantibody against aquaporin 4 (AQP4), which is densely expressed in astrocytic foot processes,[2] with a high sensitivity and specificity for the disease has enhanced our understanding of NMO. Several in vitro and in vivo studies have suggested that these antibodies contribute to the pathogenesis of disease and are crucial for the development of NMO.[3–10] However, high serum levels of anti-AQP4 antibodies are not always accompanied by clinical relapse, indicating that the presence of serum anti-AQP4 antibodies alone is insufficient to induce a clinical relapse and that other factors, including inflammatory mediator(s), are required.[11–13]

The distinctive histological features of NMO are predominantly observed in perivascular lesions, where the blood-brain barrier (BBB) is located.[5] Circulating anti-AQP4 antibodies must penetrate the BBB, which is composed of the tight junctions, and enter the CNS space in order to bind to the AQP4 expressed on astrocytic endfeet. The intravenous or intraperitoneal administration of this antibody in normal animals has, thus far, not successfully induced the development of NMO,[9 10] because, under normal conditions, the BBB does not allow the entry of circulating anti-AQP4 antibodies into the CNS space. Some studies have demonstrated the presence of BBB damage, thus suggesting increased entry of anti-AQP4 antibodies and cytokines into the CNS space, which appears to be associated with the development of NMO[14 15]; however, the molecular mechanism(s) underlying the breakdown of the BBB in patients with NMO are not fully understood.

Previous studies have demonstrated that the serum and cerebral spinal fluid (CSF) concentrations of matrix-metalloproteinase-9 (MMP-9) and adhesion molecules in NMO patients are significantly higher than those observed in patients with MS and healthy controls and are correlated with the clinical and radiological severity of the disease.[16 17] Accumulating evidence also suggests that the BBB disruption induced by MMPs-2/9 is an important step in the development of some inflammatory CNS diseases, including MS[18] and experimental allergic encephalomyelitis (EAE).[19] We thus hypothesised that MMP-2/9 is a candidate molecule causing the breakdown of the BBB in NMO patients. In the current study, we investigated the contributions of humoral factors, particularly MMP-2/9, in the sera obtained from patients with NMO to malfunction of the BBB.

MATERIALS AND METHODS

Sera

This study was conducted in accordance with the Declaration of Helsinki, as amended in Somerset West in 1996, and approved by the ethics committee of the Medical Faculty, Yamaguchi University. We collected sera from 14 patients with NMO spectrum disorders (NMOSD) (all female; mean

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age, 52 years), including 10 patients in the acute phase and four patients with stable disease, who were hospitalised at our institution. Anti-AQP4 antibody assays were conducted according to the methods described in the previous report,[6] and all patients tested positive for anti-AQP4 antibodies and fulfilled the clinical criteria for NMOSD.[20 21] Seven patients with definitive NMO (patients nos. 1, 2, 4, 6, 7, 9 and 10) and three patients with partial NMO, defined as isolated LETM (patients nos. 3, 5 and 8), in the acute phase were enrolled in this study; no patients with isolated optic neuritis were included. Blood samples were obtained within 25 days of the initial attack (mean time from symptom onset to serum sample collection: 13.2 days (SD=5.4)). At the time of sampling, one of 10 patients in the acute phase was being treated with methylprednisolone pulse therapy, although none were taking immunosuppressive drugs, including corticosteroids or azathioprine. Serum samples were also collected from four patients with NMOSD in the stable phase who were being treated with corticosteroids or had been in clinical remission for at least 6 months (patients nos. 5, 8, 9 and 10). Seven of the 10 NMOSD patients were positive for anti-brain microvascular endothelial cell (BMEC) antibodies (patients nos. 2, 4, 5, 6, 8, 9 and 10), while the other three were negative for anti-BMECs antibodies (patients nos. 1, 3 and 7). The method used to detect anti-BMECs antibodies has been previously described.[15] Serum samples were also obtained from 10 patients with conventional MS during the acute phase who fulfilled the revised McDonald criteria[22] (four men, six women; mean age, 35.7 years) and 10 healthy volunteers for comparison. Blood samples were obtained from six patients before treatment with high-dose intravenous methylprednisolone (patients nos. 2, 3, 4, 8, 9 and 10) and from four patients after such treatment (patients nos. 1, 5, 6 and 7) within 28 days of the first appearance of symptoms. Six of the 10 MS patients and one of the 10 healthy controls were positive for anti-BMECs antibodies. All samples were immediately stored at −80°C until the analysis in order to avoid repeated freeze/thaw cycles. We inactivated all samples at 56°C for 30 min immediately prior to the analysis.

Cell culture and treatment

We used the conditionally immortalised human cell lines previously described by our group, including microvascular endothelial cells of the brain (BMECs), termed 'TY08,',[23] astrocytes, termed 'hAST-AQP4,'[24] and human blood-nerve-barrier-derived endothelial cells, termed 'FH-BNBs.'.[25] The cell cultures were maintained in fresh medium containing 10% patient or healthy control sera, and 10% fetal bovine serum (FBS) and applied as controls at 37°C in 5% (vol/vol) CO_2/air. The cells were cultured for one day before total mRNA was extracted and the transendothelial electrical resistance (TEER) value was measured. Total proteins were extracted 2 days later.

Reagents

The culture medium was Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, Missouri, USA) containing antibiotics and 10% FBS (Sigma). [23] The polyclonal anti-MMP-9, anti-MMP-2, anti-actin, and anti-vascular endothelial growth factor (VEGF) antibodies were purchased from Santa Cruz (Santa Cruz, California, USA). The polyclonal anti-claudin-5 antibodies were obtained from Invitrogen (Carlsbad, California, USA), and the polyclonal anti-vascular cell adhesion molecule-1 (VCAM-1) antibodies were obtained from R&D systems (Minneapolis, Minnesota, USA). The GM6001 was obtained from Chemicon (Temecula, California, USA), and the MMP-2 and MMP-9 inhibitors were obtained from Santa Cruz.

Quantitative real-time PCR analysis

For real-time RT-PCR, total RNA synthesised from PBS-washed cells and single-stranded cDNA was prepared from 40 ng of total RNA. The sequences of human primers for *MMP-2*, *MMP-9* and *G3PDH* have been previously described.[26] A Stratagene Mx3005P instrument (STRATAGENE, Cedar Greek, Texas, USA) was used to perform the quantitative real-time PCR analyses, and the relative quantity of each molecule was calculated according to the $R_v=R_{Gene}/R_{GAPDH}$ using a software program as previously described.[26]

Western blot analysis

The protein samples (10–20 μg) were fractionated in a 10% gel and electrophoretically transferred onto polyvinylidene difluoride membranes (Amersham, Chalfont, UK), as previously described.[23] The membranes were treated with the primary antibody in PBS-T and 5% milk (dilution 1:100) for 2 h, followed by incubation with the secondary antibody (dilution 1:2000) for 1 h. The bands were visualised with an enhanced chemiluminescence kit (ECL-prime, Amersham, UK). The relative density of each band was measured using the Quantity One software program (Bio-Rad, Hercules, California, USA).

TEER studies

A Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, Florida, USA) was used to measure the TEER values of the cell layers, as previously described.[23] The BMECs were seeded at 1×10^6 cells/insert on the collagen-coated culture inserts and cultured with each flesh medium (the conditioned medium contained 10% patient or healthy control sera) for 24 h.

Permeability studies

Permeability studies with sodium fluorescein were used to determine the degree of paracellular flux across confluent BMEC monolayers, as previously described.[27] The confluent monolayers were prepared on 24-well culture plates. Next, we added 500 μl of culture medium containing sodium fluorescein (10 μg/ml; molecular mass of 400 kDa) on the upper chamber of each well. The lower chamber was sampled after incubation, and the amount of fluorescence that passed through the cell-covered inserts was measured using a MX3000P instrument (Stratagene).

Treatment with MMP inhibitors

GM6001, an MMP-2 inhibitor and an MMP-9 inhibitor were used to inhibit MMP-2 or MMP-9. The serum samples obtained from the patients were preincubated with 25 μM of GM6001, 5 μM of the MMP-2 inhibitor or 5 μM of the MMP-9 inhibitor for 12 h at 37°C. The cells were cultured with the conditioned medium containing sera obtained from either the NMOSD or MS patients or healthy controls; all samples contained an MMP inhibitor.

ELISA

The concentrations of total MMP-2 and MMP-9 observed prior to incubation at 56°C were measured using ELISA (R&D systems). The samples were run in triplicate according to the manufacturer's protocol.

IgG purification and exposure of the cells to purified IgG

Affinity chromatography using a Melon Gel IgG Spin Purification Kit (Thermo Scientific, Rockford, Illinois, USA) was performed to obtain purified immunoglobulin G (IgG) fractions from the sera of five patients with anti-BMEC antibody-positive NMOSD and five healthy individuals. The cells were cultured in medium containing purified IgG (final concentration 400 μg/mL) obtained from FBS as a control (Sigma), the patients or the healthy volunteers.

Absorption of anti-AQP4 antibodies

The methods used to study the absorption of anti-AQP4 antibodies have been previously explained.[15] Sera obtained from two different NMOSD patients (NMOSD1 and NMOSD2) were added to the hAST-AQP4 cells for 150 min and used for the subsequent analyses. In both cases, the titres of anti-AQP4 antibodies were decreased by at least 50% after the 150 min incubation period (anti-AQP4 antibody titres: NMOSD1 before treatment, 1:8 and after 150 min of treatment, 1:4; NMOSD2 before treatment, 1:2048 and after 150 min of treatment, 1:512).[15]

Data analysis

Average values are reported as the mean±SEM. The indicated statistical tests (unpaired, two-tailed Student t tests) were performed assuming significance for $p<0.05$.

RESULTS

The NMOSD sera decreased the barrier function of the BMECs

Figure 1 shows the effects of the NMOSD sera, including those obtained from seven patients with definitive NMO and three patients with isolated LETM, on the expression of tight junctional and adhesion molecules in the BMECs using a western blot analysis. The sera obtained from the NMOSD patients significantly decreased the amount of claudin-5 in the BMECs, whereas the sera obtained from the MS patients and healthy controls did not change the amount of this protein (figure 1A–D). By contrast, the sera obtained from patients with either NMOSD or MS significantly increased the amount of VCAM-1 expressed by the BMECs (figure 1A–C,E), whereas the sera obtained from the healthy controls did not affect the level of this protein. Treatment with the sera obtained from the NMOSD patients significantly showed lower TEER values and greater NaF permeability of BMECs, while the sera obtained from the MS patients and healthy controls did not have this effect (figure 1H,I). There were no significant differences between NMO and isolated LETM samples in terms of the degree of BBB damage following exposure to the sera (figure 1F,G).

The MMP-2/9 inhibitor reversed the BBB damage caused by the NMOSD sera

Figure 2 shows the contribution of MMP-2/9 to the malfunction of the BBB observed in the NMOSD patients. The amount of claudin-5 proteins in the BMECs was significantly increased following exposure to the NMOSD sera pretreated with GM6001, a broad-spectrum MMP inhibitor, regardless of the presence of anti-BMECs antibodies, compared with that observed in cells not exposed to sera pretreated with GM6001 (figure 2A,D). However, the expression of claudin-5 was unchanged following exposure to the sera obtained from the MS patients or healthy controls, regardless of whether the samples were pretreated with GM6001 (figure 2B,C,E,F). Additionally, the TEER values of the BMECs were significantly higher and the NaF permeability was significantly lower following exposure to the NMOSD sera pretreated with GM6001 compared to that in the cells exposed to sera not pretreated with GM6001 (figure 2G, H). The TEER values and NaF permeability were unchanged following exposure to sera obtained from the MS patients or healthy controls, regardless of the presence or absence of GM6001 pretreatment (figure 2G,H). The decreases in the claudin-5 protein levels and TEER values observed in the BMECs were restored by the application of a selective MMP-2 or MMP-9 inhibitor (figure 2I–K), although no significant differences were noted in the degree of BBB disruption following the inhibition of either MMP-2 or MMP-9 alone (figure 2I–K).

Autocrine MMP-2/9 secretion and damage to the BBB caused by the NMOSD sera

Figure 3 shows the contribution of the autocrine secretion of MMP-2/9 by BMECs following exposure to NMOSD sera. No significant differences were observed regarding the serum concentrations of MMP-2 and MMP-9 between the serum samples obtained from the NMOSD and MS patients or healthy controls, as determined using the ELISA method (figure 3A,B). The induction of *MMP-2* and *MMP-9* mRNA in the BMECs was significantly increased following exposure to the NMOSD sera, although it remained unchanged following the exposure of the cells to sera obtained from the MS patients and healthy controls (figure 3C,D). We next examined whether the sera obtained from the NMOSD and MS patients would increase the MMP-2 and MMP-9 protein expression levels in the BMECs (figure 3E–G). The sera obtained from the acute-phase NMOSD patients significantly increased the amount of MMP-2 and MMP-9 secreted by the BMECs, whereas the sera obtained from the MS patients or healthy controls did not have this effect (figure 3H,I). The presence of anti-BMECs antibodies did not influence the MMP-2/9 protein secretion by the BMECs (figure 3J,K). Meanwhile, the amount of claudin-5, MMP-2 and MMP-9 proteins in the BMECs was not affected by incubation with the stable-phase NMOSD sera (figure 3L,M), and the levels of these proteins in the FH-blood-nerve barriers (BNBs) were not influenced by exposure to the acute-phase NMOSD sera (figure 3N,O).

Purified serum IgG increased the VCAM-1 expression

Figure 4A–E shows the effects of the purified serum IgG obtained from the NMOSD patients on malfunction of the BBB. We randomly selected five NMOSD patients (patients nos. 2, 4, 5, 6 and 9), including four patients with definitive NMO and one patient with isolated LETM, all of whom were positive for anti-BMECs antibodies and had not been treated at the time of serum sampling. The amount of claudin-5, MMP-2, MMP-9 and VEGF in the BMECs did not significantly change following a challenge with the IgG obtained from the five NMOSD serum samples with anti-BMECs antibodies, as determined using a western blot analysis (figure 4A,B). The TEER values and NaF permeability of the BMECs were also not affected following exposure to purified serum IgG fractions obtained from the patients with NMOSD (figures 4C,D). By contrast, the amount of VCAM-1 in the BMECs was significantly increased following the application of IgG obtained from the NMOSD sera with anti-BMECs antibodies, and did not change following exposure to purified IgG obtained from the sera of the healthy controls, as determined using a western blot analysis (figures 4A,B).

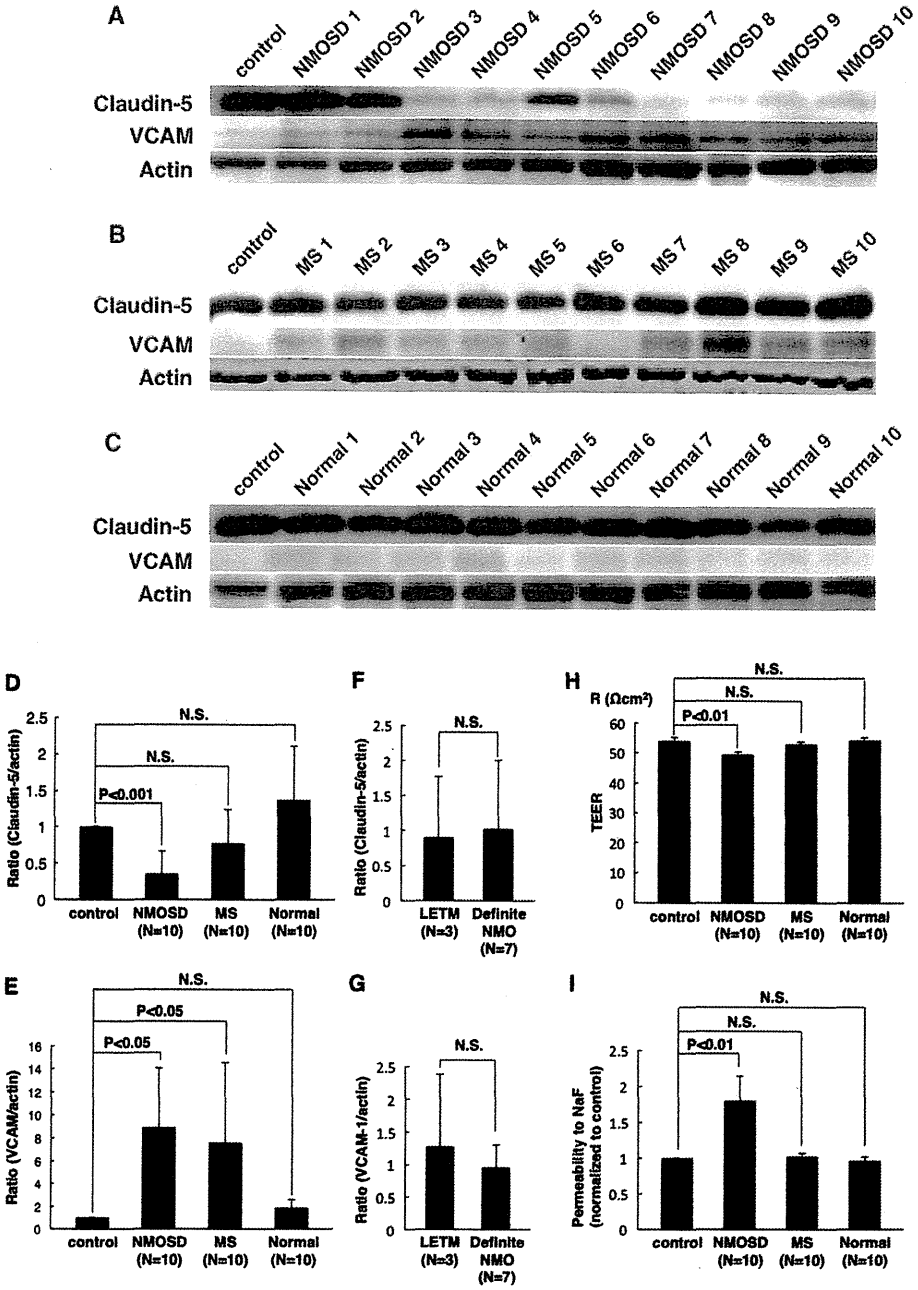


Figure 1 Serum samples were collected from seven patients with definite neuromyelitis optica (NMO) (patients nos. 1, 2, 4, 6, 7, 9 and 10) and three patients with partial NMO, defined as isolated longitudinally extensive transverse myelitis (LETM) (patients nos. 3, 5 and 8). (A–C) The changes in the amount of claudin-5 and VCAM-1 in BMECs were determined after exposure to the sera from patients with NMOSD, multiple sclerosis (MS) or from healthy controls by a western blot analysis. (D, E) The bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=10). The amount of claudin-5 was significantly decreased after the exposure of BMECs to the sera from patients with NMOSD, whereas it was not affected by the sera from patients with MS or from healthy controls. The amount of VCAM-1 expressed by the BMECs was significantly increased after exposure to NMOSD or MS sera, although it was not influenced by the sera from healthy controls. (F, G) No significant differences were observed between the patients with isolated LETM and definite NMO in terms of the amount of claudin-5 and VCAM-1 in the BMECs after exposure to the patients' sera. (H, I) The transendothelial electrical resistance value of BMECs was significantly decreased (H), and the NaF permeability of BMECs was significantly increased (I), after exposure to NMOSD sera, but these parameters were not influenced by the exposure to sera from patients with MS or healthy controls (mean±SEM, n=10, *p<0.01). Control: non-conditioned DMEM containing 20% fetal bovine serum (FBS); NMOSD: conditioned medium with 10% serum from an NMOSD patient diluted with non-conditioned DMEM containing 10% FBS; MS: conditioned medium with a 10% concentration of serum from an MS patient diluted with non-conditioned DMEM containing 10% FBS; normal: conditioned medium with 10% serum from a healthy control diluted with non-conditioned DMEM containing 10% FBS; LETM: conditioned medium with 10% serum from an isolated LETM patient diluted with non-conditioned DMEM containing 10% FBS; definite NMO: conditioned medium with 10% serum from a definite NMO patient diluted with non-conditioned DMEM containing 10% FBS.

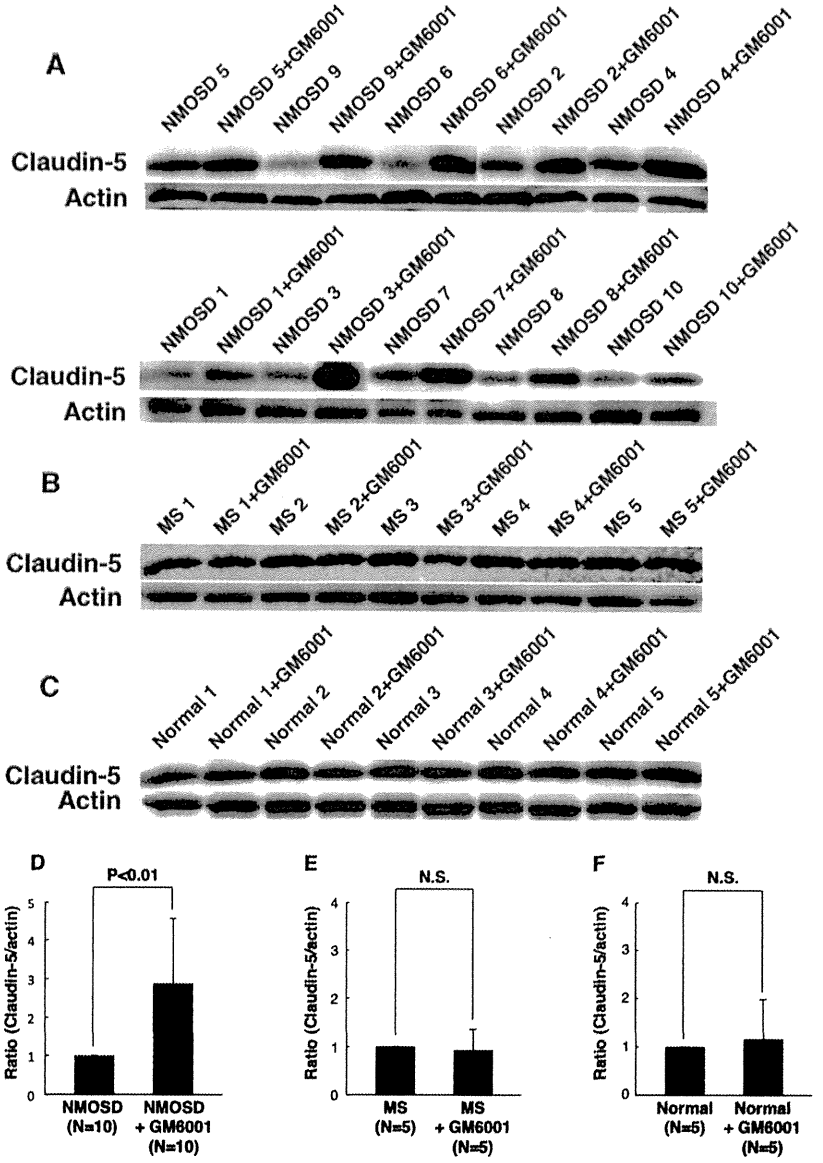


Figure 2 (A–C) The effects of the broad-spectrum matrix-metalloproteinase (MMP) inhibitor, GM6001, on the expression of claudin-5 in brain microvascular endothelial cells (BMECs) after exposure to the sera from 10 NMO spectrum disorder (NMOSD) patients were examined by a western blot analysis. Exposure to sera from NMOSD patients preincubated with GM6001 led to an increase in the amount of claudin-5 protein in the BMECs in comparison with the cells exposed to NMOSD sera without GM6001 pretreatment. (D–F) Each column reflects the combined densitometry data from each independent experiment (mean±SEM, NMOSD n=10, multiple sclerosis (MS) n=5, normal n=5, *p<0.01). (G) (H) The transendothelial electrical resistance (TEER) values of BMECs was significantly increased (G) and the NaF permeability in the BMECs was significantly decreased (H) following the incubation with NMOSD sera that had been pretreated with GM6001 compared to that in the cells exposed to sera without GM6001 pretreatment (mean±SEM, NMOSD n=10, MS n=5, normal n=5, *p<0.01). (I) The effects of the selective MMP-2 or MMP-9 inhibitor on the expression of claudin-5 in BMECs after exposure to the sera from NMOSD patients were investigated by a western blot analysis. Preincubation with a selective MMP-2 or MMP-9 inhibitor led to an increase in the amount of claudin-5 protein in BMECs compared to cells treated with sera without the inhibitor treatment. (J) Each bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=5, MMP-2: p<0.05, MMP-9: p<0.05). There were no significant differences in the amount of claudin-5 following the inhibition of MMP-2 alone or MMP-9 alone. (K) The TEER value of BMECs was significantly increased after the incubation with sera from NMOSD patients pretreated with a selective MMP-2 or MMP-9 inhibitor compared to that in the cells without pretreatment with the MMP-2 or MMP-9 inhibitor (mean±SEM, n=5, MMP-2: p<0.05, MMP-9: p<0.01). No significant differences in the TEER values were observed following the inhibition of MMP-2 alone or MMP-9 alone. NMOSD: conditioned medium with 10% NMOSD patient serum diluted with DMEM containing 10% fetal bovine serum (FBS); NMOSD+GM6001: conditioned medium with 10% NMOSD sera pretreated with GM6001; MS: conditioned medium with 10% MS patient serum diluted with DMEM containing 10% FBS; MS+GM6001: conditioned medium with 10% MS sera pretreated with GM6001; normal: conditioned medium with 10% healthy individual serum diluted with DMEM containing 10% FBS; Normal+GM6001: conditioned medium with 10% healthy individual sera pretreated with a GM6001. NMOSD+MMP-2 inhibitor: conditioned medium with 10% NMOSD sera pretreated with an MMP-2 inhibitor; NMOSD+MMP-9 inhibitor: conditioned medium with 10% NMOSD sera pretreated with an MMP-9 inhibitor.

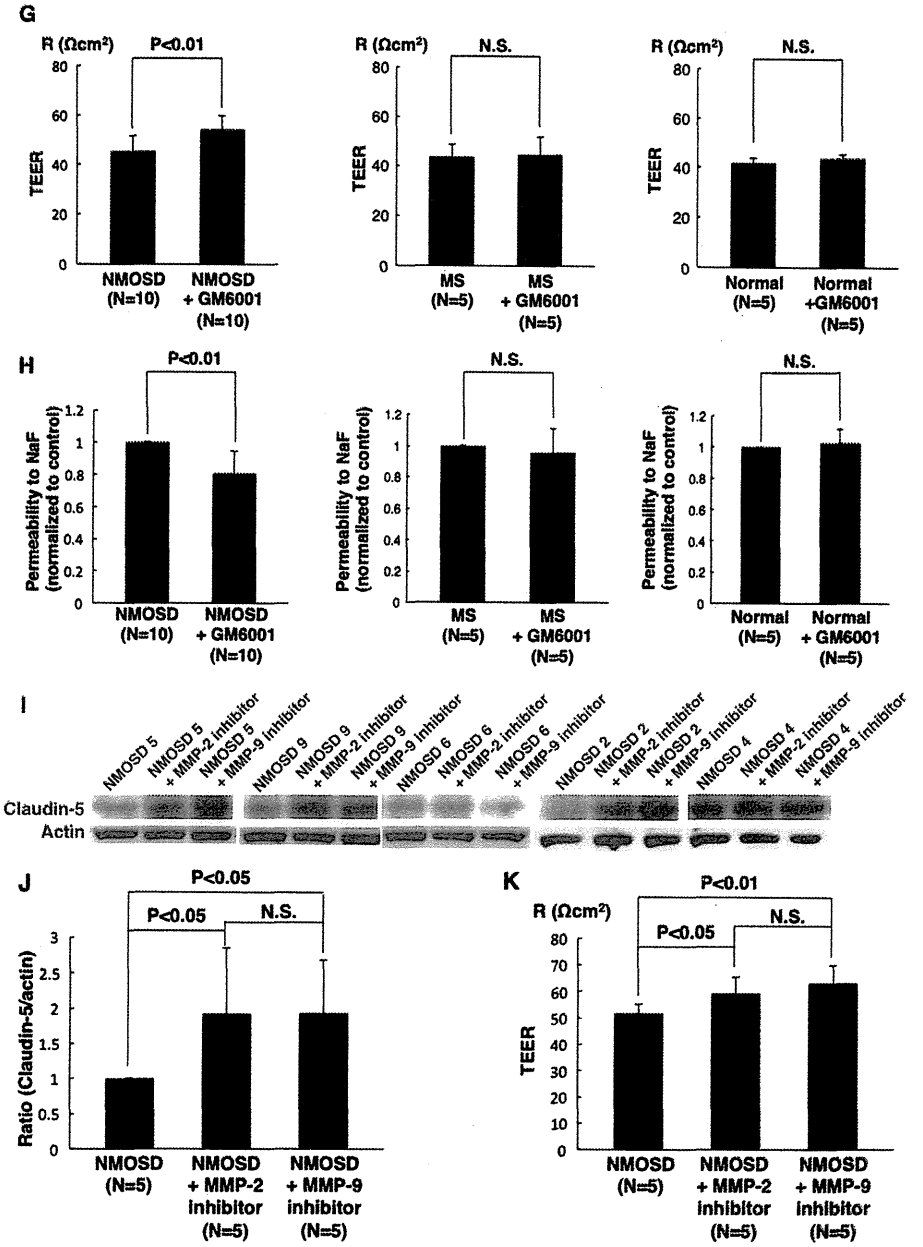


Figure 2 (Continued)

Reducing the anti-AQP4 antibody level did not affect the VCAM-1 expression

Figure 4E,F shows that the reduction of the anti-AQP4 antibody titres in the sera did not influence the expression level of VCAM in the BMECs. Following incubation with the hAST-AQP4 cells, a more than 50% decrease was observed in the anti-AQP4 antibody titres in the sera obtained from two different NMOSD patients (see Methods).[13] The expression of VCAM-1 in the BMECs was unchanged following treatment with the serum samples with a diminished anti-AQP4 antibody titre (figure 4E,F) compared to that observed following exposure to the untreated samples, suggesting that a 50% or 70% decrease in the anti-AQP4 antibody titre in NMOSD sera is not correlated with a reduction in the VCAM-1 expression level in BMECs.

DISCUSSION

Several lines of evidence suggest that most anti-AQP4 antibodies are produced not intrathecally, but rather peripherally, in patients with NMOSD.[6 28] In a recent animal study, the systematic injection of serum IgG obtained from NMO patients was insufficient to induce the formation of NMO-like lesions in normal rats, although it was sufficient to cause the disease in animals with a pre-existing inflammatory state in the CNS, such as that which occurs in experimental autoimmune encephalomyelitis (EAE) models mediated by encephalogenic T-cells, or in mice following exposure to Freund's adjuvant.[10 29] Saadoun *et al*[30] also reported that only direct co-injection of IgG obtained from NMO patients with human complement into the murine brain can induce characteristic histological features of NMO. These findings indicate that BBB damage is required for

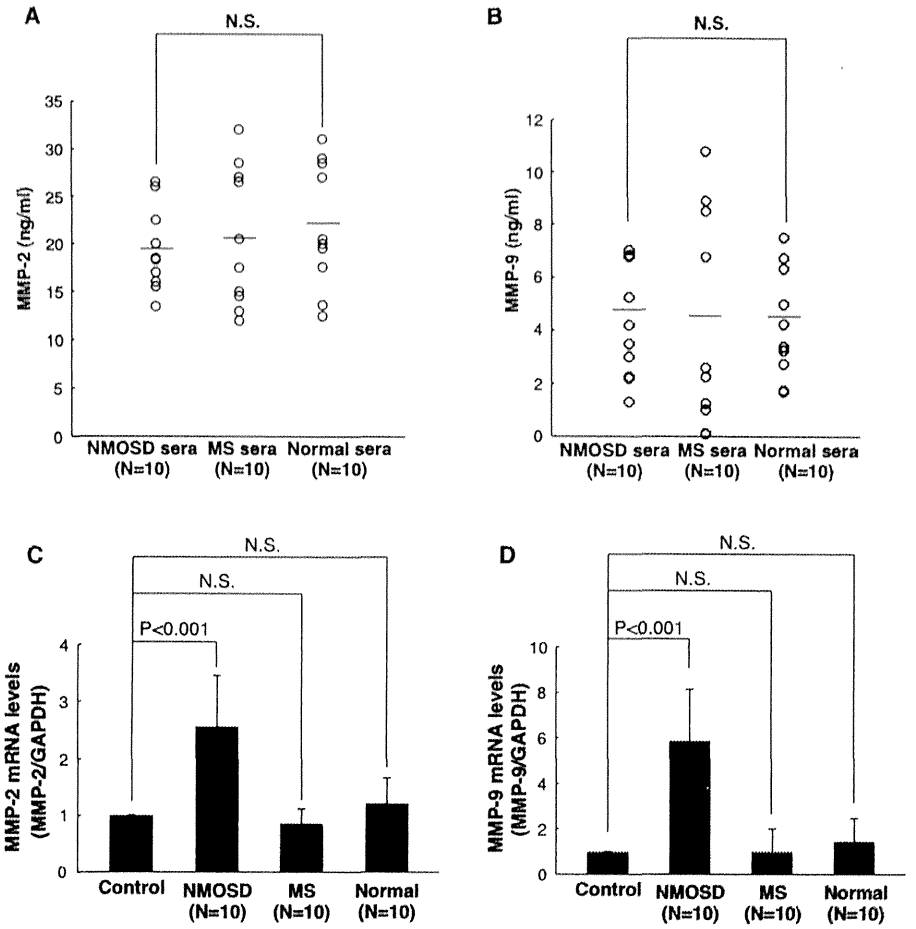
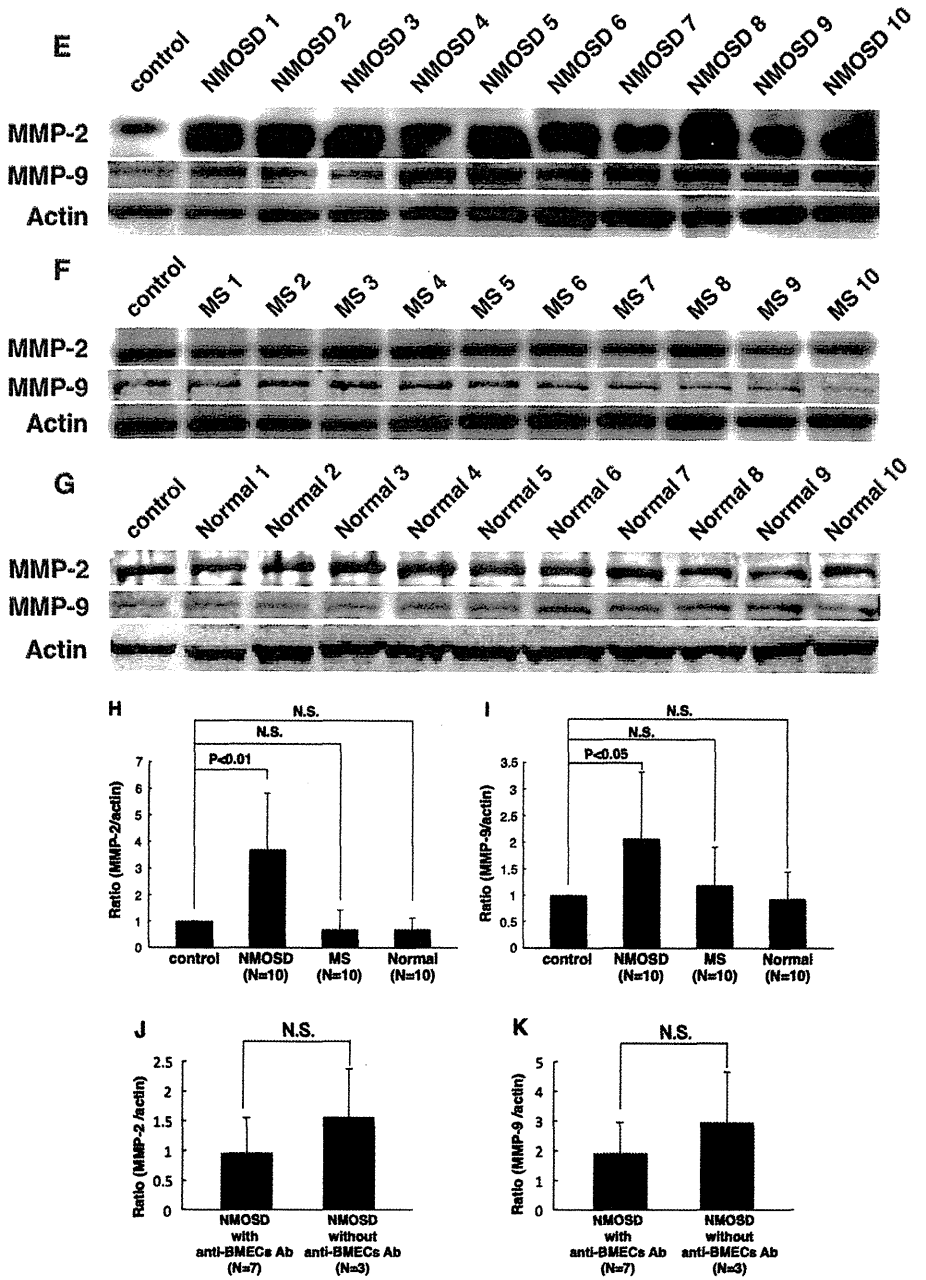


Figure 3 (A)(B) The MMP-2/9 concentration was analysed in the sera from patients with NMO spectrum disorder (NMOSD) or multiple sclerosis (MS), or from healthy control subjects. The bars indicate the mean of each group. There were no significant differences among the three groups. (C) (D) The *MMP-2/9* expression in brain microvascular endothelial cells (BMECs) after exposure to sera from NMOSD patients was determined by relative quantification with a real-time RT-PCR analysis. The expression level of *MMP-2* (C) and *MMP-9* (D) in the BMECs was significantly upregulated after the application of sera from NMOSD patients, although it was unchanged after incubation with the sera from MS patients or healthy controls (mean±SEM, n=10, *p<0.001). (E)(F)(G) The changes in the MMP-2 and MMP-9 protein level in BMECs after treatment with sera from NMOSD patients were determined by a western blot analysis. Treatment with the sera from NMOSD patients significantly increased the amount of MMP-2 and MMP-9 protein (mean±SEM, n=10) (E), although the sera from MS patients or healthy controls did not have these effects (mean ±SEM, n=10, *p<0.01) (F, G). (H, I) The bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=10, MMP-2: p<0.01, MMP-9: p<0.05). (J, K) The presence of anti-BMECs antibodies did not influence the MMP-2 or MMP-9 protein levels secreted by BMECs. (L) Sera collected during the stable phase of NMOSD (patients nos. 5, 8, 9 and 10) did not influence the amounts of claudin-5, MMP-2 and MMP-9 protein in the BMECs. (M) Each bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=4). (N) The amounts of claudin-5, MMP-2 and MMP-9 protein in the human blood-nerve barrier (BNB)-derived endothelial cell lines were not changed after exposure to the acute-phase NMOSD sera. (O) Each bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=10). Control: non-conditioned DMEM containing 20% FBS; NMOSD: conditioned medium with 10% serum from an NMOSD patient at the acute phase diluted with non-conditioned DMEM containing 10% FBS; MS: conditioned medium with a 10% concentration of serum from an MS patient diluted with non-conditioned DMEM containing 10% FBS; normal: conditioned medium with 10% serum from a healthy control diluted with non-conditioned DMEM containing 10% FBS; NMOSD with anti-BMECs Ab: conditioned medium with 10% serum from an NMOSD patient with anti-BMECs antibodies diluted with non-conditioned DMEM containing 10% FBS; NMOSD without anti-BMECs Ab: conditioned medium with 10% serum from an NMOSD patient without anti-BMECs antibodies diluted with non-conditioned DMEM containing 10% FBS; stable NMOSD: conditioned medium with 10% serum from an NMOSD patient at the stable phase diluted with non-conditioned DMEM containing 10% FBS. MMP, matrix-metalloproteinase.

circulating anti-AQP4 antibodies to enter the CNS and may be involved in the pathogenesis of NMO. This role of BBB damage is also suggested clinically by the finding of increased albumin CSF/serum ratios in anti-AQP4 antibody-positive NMOSD patients.[31] The present study demonstrated that sera obtained from NMOSD patients, including both those with definitive NMO and isolated LETM, decreases the BBB function, as determined using our newly established human BMECs. Our previous study showed that sera obtained from definitive NMO patients reduce the BBB function by upregulating VEGF in BMECs.[15] Because IgG against BMECs was found in the sera obtained from the NMO patients, we speculated that anti-BMEC antibodies derived from NMO patients cause BBB damage. However, another molecule other than anti-BMECs



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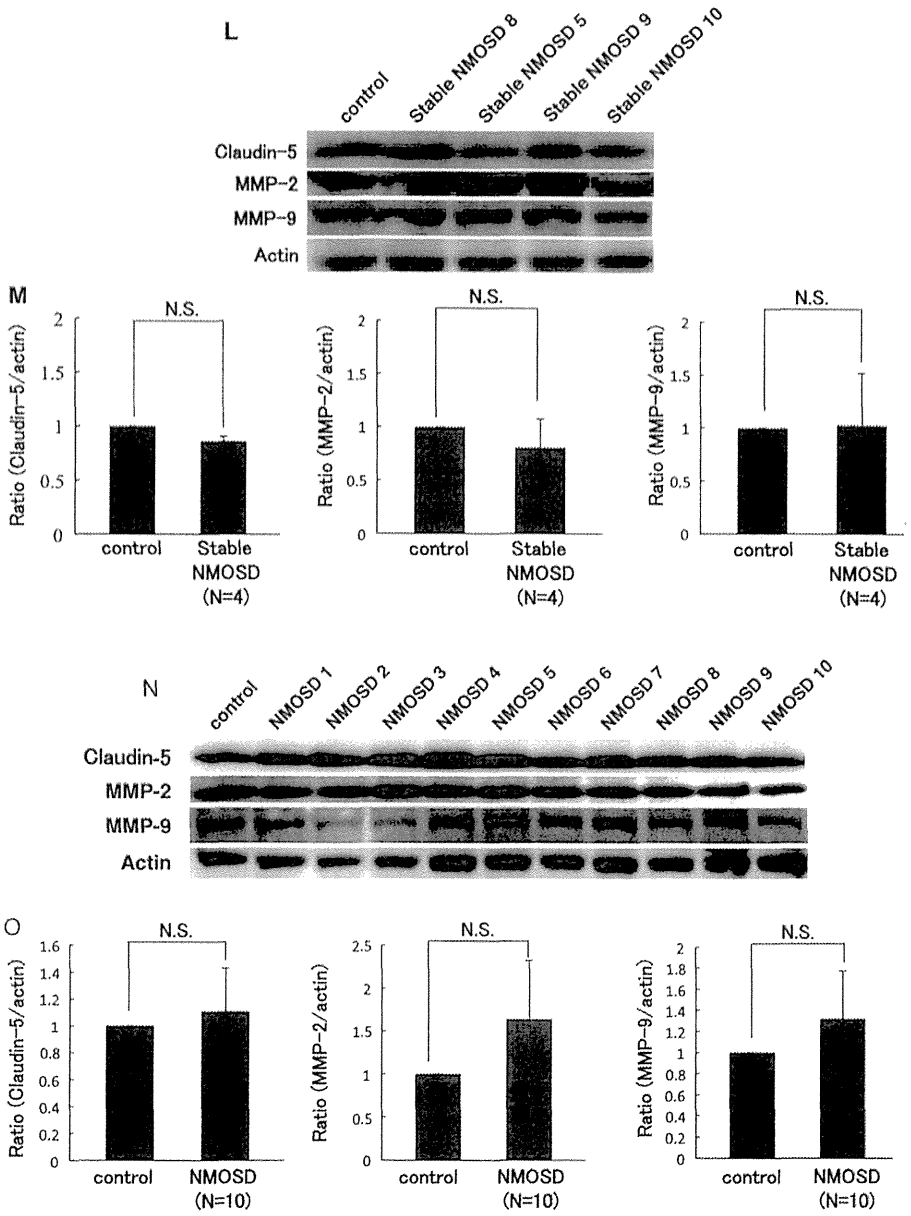
antibodies that induce BBB damage may be present in NMO sera, because the sera obtained from the NMO patients without anti-BMECs antibodies also decreased the BBB function.[15]

We hypothesise that the MMP-2 and/or MMP-9 present in NMOSD sera partly contribute to increasing permeability of the BBB. The present study demonstrated that BBB damage following exposure to NMOSD sera was prevented when the cells were pretreated with GM6001, a broad-spectrum MMP inhibitor. Further supporting this possibility, the mRNA expression and protein levels of MMP-2/9 were increased in the cells treated with NMOSD sera, regardless of whether anti-BMEC antibodies were present. However, there were no significant differences in the serum concentrations of MMP-2/9 between the NMOSD patients and healthy controls. These findings suggest that humoral factors other than anti-BMECs antibodies present in the sera obtained from NMOSD patients induce MMP-2/9 secretion by BMECs via an autocrine mechanism, thus inducing BBB damage due to autodegradation of tight junction proteins, including claudin-5. Therefore, even minimal secretion, which does not influence the serum concentration, may have a significant effect. Our previous report demonstrated that the sera obtained from patients with Bickerstaff's brainstem encephalitis, but not Miller Fisher syndrome, induce BBB damage in the same in vitro BBB model.[26] The present study also showed that sera obtained from stable-phase NMO patients did not influence the amount of claudin-5 or MMP-2/9 or the TEER values in this in vitro BBB model. Additionally, the sera obtained from the acute-phase NMO patients did not affect the amounts of



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these proteins in our in vitro BNB model. These results suggest that the BBB damage observed following exposure to NMO sera due to the autocrine secretion of MMP-2/9 may be a specific and essential event that occurs in the BBB only during the acute phase of NMO. The current study also demonstrated that GM6001 has therapeutic potential for restoring BBB disruption in NMOSD patients. Previous reports have suggested that GM6001 repairs BBB damage in setting of EAE and inhibits the development of clinical EAE.[32] However, treatment with GM6001 is not recommended for clinical use at present, because the drug does not appear to be sufficiently selective, which may result in undesirable side effects, and more selective MMP inhibitors have been proposed.[33] Novel approaches for restoring the BBB using more selective MMP inhibitors during the acute stage of the disease might achieve promising therapeutic benefits in patient with NMO.

Several studies have demonstrated that the administration of IgG obtained from the sera of NMO patients alone is insufficient to induce the formation of NMO lesions in the absence of inflammation and/or complement.[8 10 27] We thus examined whether purified IgG obtained from NMOSD sera alone without complement has a direct influence on the properties of the BBB. In agreement with the findings of previous studies, the present study demonstrated that IgG derived from NMOSD sera alone does not influence the amount of claudin-5, MMP-2/9 or VEGF proteins, the TEER values or NaF permeability in the BBB. Taken together, these results indicate that unknown humoral factor(s), other than IgG, in the sera obtained from NMOSD patients may disrupt the BBB by inducing the autocrine secretion of MMP-2/9 and VEGF in BMECs; however, we were unable to identify which factor(s) in the NMOSD sera caused these effects, or to clarify whether IgG causes BBB

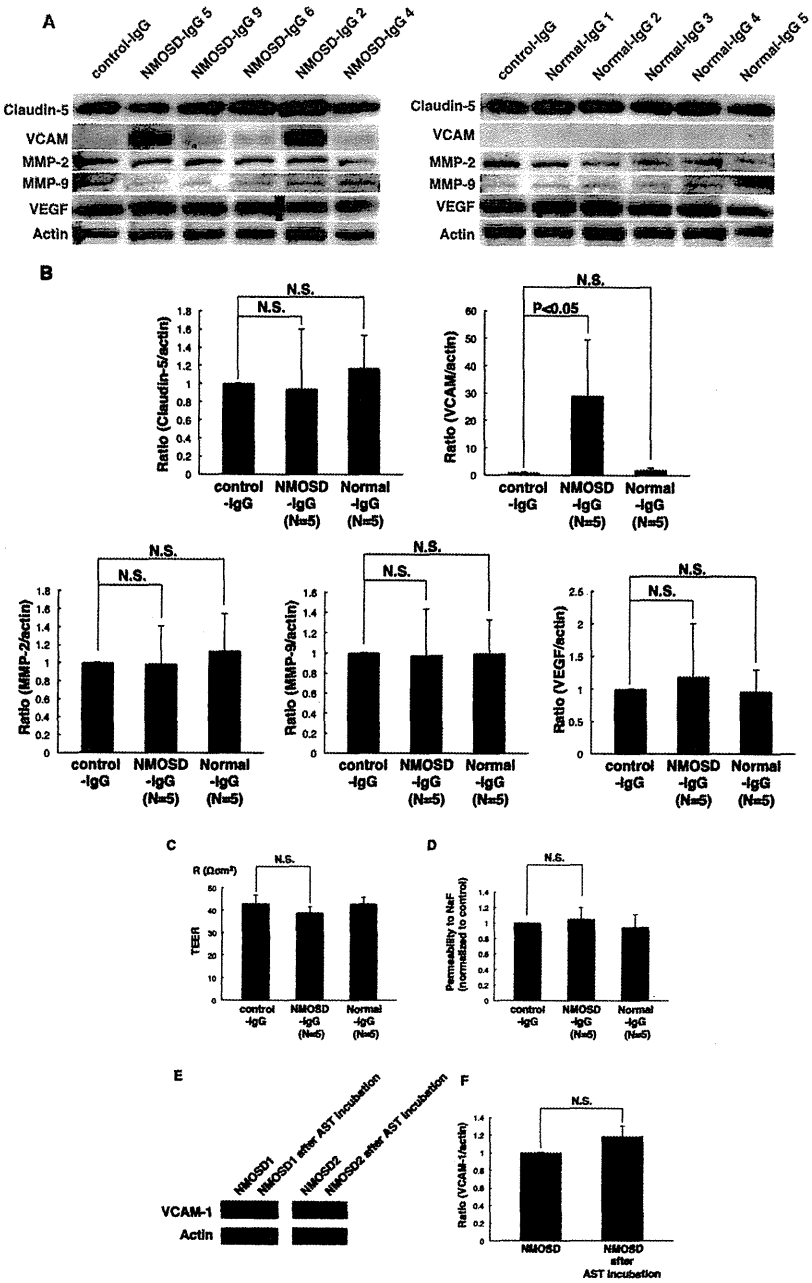

Figure 4 Five NMO spectrum disorder (NMOSD) patients (patients nos. 2, 4, 5, 6 and 9), including four patients with definite neuromyelitis optica (NMO) and one patient with isolated LETM were randomly selected. (A) The effects of the purified serum IgG from these NMOSD patients with anti-BMECs antibodies on the expression levels of VCAM-1 and claudin-5 in human BMECs was analysed by a western blot analysis. The amount of VCAM-1 protein in the BMECs was significantly increased after exposure to the purified IgG fractions of sera from NMOSD patients, whereas it was not affected by the purified IgG fractions from healthy controls, as determined by a western blot analysis. The amounts of claudin-5, MMP-2/9 and VEGF protein in BMECs was unchanged after the application of the purified IgG fractions of sera from NMOSD patients, as determined by a western blot analysis. (B) Each bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=5, *p<0.05). (C, D) The transendothelial electrical resistance value (C) and the NaF permeability (D) of BMECs did not change after exposure to the purified IgG fraction from NMOSD patients. (E) The effects of reducing the anti-AQP4 antibody titre on the expression of the VCAM-1 protein in BMECs. The reduction of the anti-AQP4 antibody titre did not influence the expression of VCAM-1 in BMECs. (F) Each bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=3). Control-IgG: conditioned medium containing purified IgG fractions obtained from fetal bovine serum (FBS); NMOSD-IgG: conditioned medium containing purified IgG fractions obtained from the sera of NMO patients; normal-IgG: conditioned medium containing purified IgG fractions obtained from the sera of healthy individuals; NMOSD: conditioned medium with 10% serum from an NMOSD patient diluted with DMEM containing 10% FBS; NMOSD after AST incubation: conditioned medium with 10% NMOSD sera after a 150 min incubation with astrocytes-expressing AQP4.