signed-rank test), respectively. These results indicate that a significant reduction occurred in both indices of disease activity (the DAS28-ESR and the SDAI) in both treatment groups after 24 weeks of administration.

Interestingly, in the abatacept treatment group, the percentage of pSyk-positive cells among CD19+ B cells diminished from week 0 to week 24 from a mean ± SD 21.4 ± 30.9% to 3.3 ± 3.8% ($P = 0.0341$, by Wilcoxon's matched-pairs signed-rank test), whereas in those treated with TNF inhibitors, this percentage increased from 30.0 ± 23.1% to 42.0 ± 34.8% from week 0 to week 24 ($P = 0.1255$, by Wilcoxon's matched-pairs signed-rank test). These results indicate that Syk phosphorylation in B cells was significantly decreased in the abatacept treatment group, whereas no change was observed in the TNF inhibitors group after 24 weeks of administration (Figures 2B and 3A). Although 2 different subsets of TNF inhibitor–treated patients were observed, one in which the pSyk levels increased and another in which the pSyk levels decreased after treatment with TNF inhibitors, the background features were similar in the 2 groups. However, the change in Syk phosphorylation in B cells after treatment was not correlated with the response to treatment (Figure 3B).

We next assessed the mechanism of abatacept-induced inhibition of Syk phosphorylation in B cells. For this purpose, we examined the proportion of Th1 cells (CD4+CXCR3+ cells) and Tfh cells (CD4+CXCR5+PD-1+ cells), which are CD4+ T cells that play important roles in the maturation and differentiation of B cells (17). Preliminary data (not shown) indicated that there was a significantly higher percentage of Tfh cells among CD4+ T cells in RA patients compared to healthy controls. However, there were no differences in the percentage of Th1 cells between the 2 groups (results not shown). Although the proportion of Th1 cells was not changed, treatment with abatacept significantly reduced the proportion of Tfh cells, from a mean ± SD 5.7 ± 5.7% at week 0 to 3.4 ± 4.7% at week 4 ($P = 0.0206$, by Wilcoxon's matched-pairs signed-rank test) (Figure 3C). In contrast, treatment with TNF inhibitors did not change the proportion of Tfh cells after 4 weeks of administration. Examination of the direct effect of abatacept on B cells showed that abatacept did not change the expression levels of the costimulatory molecules CD80 and CD86 on B cells (results not shown).

DISCUSSION

In this study, we revealed that Syk phosphorylation is enhanced in the peripheral blood B cells of patients with RA compared to healthy subjects, and we found that Syk phosphorylation was increased in RA patients strongly positive for ACPAs. We also found that treatment with abatacept resulted in inhibition of Syk phosphorylation in B cells, whereas treatment with TNF inhibitors did not produce the same effects. Treatment with abatacept also significantly reduced the proportion of Tfh cells.

Rituximab was approved for treatment of RA in 2006 in the US. The positioning of rituximab as the second-line biologic product that follows TNF inhibitor therapy has been established, and B cells are assumed to be the therapeutic target in RA. Whereas several studies have shown no abnormalities in peripheral blood B cells in patients with RA (34–37), others have identified B cell abnormalities in patients with RA, including a high proportion of IgD−CD27− double-negative memory B cells (38). In this regard, there is an increased likelihood of RA relapse in patients whose proportion of memory B cells increases after rituximab administration (39), and abnormalities of chemokine receptors in B cells are often detected in RA patients (40). In this study, we found a significant increase in Syk phosphorylation in peripheral blood B cells of RA patients compared to healthy subjects, suggesting that B cells are abnormal in RA patients.

How could this abnormality affect the pathologic processes of RA? Our results showed that the increased level of phosphorylation of Syk in B cells correlated with the production level of ACPAs, but not with the severity of disease activity, in patients with active RA (Figure 1 and Table 3). Consistent with these results, we have recently reported that signaling through Syk results in effective signal transduction of TLR-9 by induction of optimal expression of TRAF6, and that this signaling is important for the expression of various functions, such as antibody production, as well as for robust activation of B cells (14). These data suggest that the important role of Syk in B cells in the pathologic processes of RA is mediated, at least in part, through ACPA production.

These findings raise several important questions. How does Syk-related ACPA production affect the pathologic progression of RA? A high titer of ACPAs is an adverse prognostic factor for bone destruction (41), although the pathologic significance is still largely unknown. In a recent study, Amara et al (42) analyzed the immunoglobulin gene of IgG+ memory B cells collected from the synovial tissue of RA patients and found that patients who were positive for ACPAs had a gene sequence for an antibody that specifically binds to citrullinated antigen. In addition, Harre et al (43) re-

ported that ACPAs induce TNFα production by macrophages and indirectly induce the differentiation of osteoclasts. It has been demonstrated that the Syk inhibitor fostamatinib (R788) was ineffective in patients with active RA who did not respond to TNF inhibitors (44). Results of a recent study indicated that TNF inhibitors increase the levels of cytoplasmic Lyn, which phosphorylates Syk and plays a role in the initiation of the B cell receptor–mediated pathway (45). Our results (Figure 3A), however, showed 2 different subsets of TNF inhibitor–treated patients, one with an increase in pSyk levels and another with a decrease in pSyk levels after treatment with TNF inhibitors, despite improvement in disease activity at 24 weeks posttreatment. Further analysis is needed to explore this issue in more detail. Clarification of the relationship between Syk and TNFα and its role in RA pathologic processes may explain why Syk inhibitors are ineffective in patients with active RA who do not respond to TNF inhibitors.

In the treatment of RA, abatacept acts through a mechanism of action different from that of TNF inhibitors; it reduces T cell responses by limiting CD28-mediated signaling, which is required for T cell activation and differentiation. However, there is little or no information on the effect of abatacept on the pathologic development or progression of RA. The present findings showed that not only the phosphorylation of Syk but also the proportion of Tfh cells was significantly reduced by abatacept, while TNF inhibitors influenced neither Syk phosphorylation nor Tfh cell development. Platt and colleagues (46) demonstrated that treatment of mice with abatacept decreased the proportion of Tfh cells. Tfh cells are a critical T helper cell subset for the formation and function of B cells and play an important role in the pathogenesis of autoimmune diseases (17,18). Furthermore, B cells provide help for the survival of Tfh cells (47).

In a series of preliminary studies, the proportion of Tfh cells in RA patients was correlated significantly with the titers of various autoantibodies, such as RF and ACPAs, but was not correlated with the severity of disease activity or the levels of pSyk in B cells (results not shown). Previous studies showed that treatment with infliximab decreased the RF titer but did not change the ACPA level (48). In contrast, treatment with abatacept significantly reduced the levels of both RF and ACPAs (49). In the present study, treatment with TNF inhibitors did not reduce the proportion of Tfh cells, despite the fact that disease activity improved with the use of these drugs. However, the proportion of Tfh cells was significantly decreased by abatacept. Therefore, we argue that abatacept seems to selectively control Tfh cell activation, leading to the production of autoantibodies from pSyk-positive B cells. However, further studies are needed to determine whether this effect is selective for abatacept compared to other drugs such as MTX and tocilizumab.

Thus, the interaction between B cells and Tfh cells is required for autoantibody production. Our results suggest that abatacept inhibits Syk phosphorylation in B cells and inhibits the proliferation and differentiation of Tfh cells. Taken together, our findings highlight the importance of B cells in the pathogenesis of RA and describe the mode of action of abatacept, i.e., inhibition of B cell–T cell interactions. Further evaluation of Syk phosphorylation may help predict the response to abatacept therapy in patients with RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tanaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Iwata, Nakayamada, Fukuyo, Saito, Tanaka.

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Original article

Low complements and high titre of anti-Sm antibody as predictors of histopathologically proven silent lupus nephritis without abnormal urinalysis in patients with systemic lupus erythematosus

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Abstract

Objective. The aim of this study was to clarify the clinical characteristics and predictors of silent LN (SLN), a type of LN in SLE without abnormal urinalysis or renal impairment.

Methods. Of 182 patients who underwent renal biopsy, 48 did not present with abnormal urinalysis or renal impairment at the time of biopsy. The patients with LN (SLN group, $n=36$) and those without LN (non-LN group, $n=12$) were compared with respect to their baseline characteristics. Bivariate analysis comprised Fisher's exact test and the Mann–Whitney test, whereas multivariate analysis employed binomial logistic regression analysis.

Results. LN was histopathologically identified in 36 of 48 patients. According to the International Society of Nephrology/Renal Pathology Society classification, 72% of the SLN patients were classified as having class I/II, with a further 17% having class III/IV. Bivariate analyses indicated that platelet count, serum albumin, complement components (C3 and C4), complement haemolytic activity (CH50), anti-Sm antibody titre and anti-ribonucleoprotein antibody titre were significantly different between groups. Multivariate analysis indicated that CH50 and C3 titres were significantly lower in the SLN group, whereas anti-Sm antibody titre was significantly higher. The cut-off titre, calculated based on the receiver operating characteristic curve for CH50, was 33 U/ml, with a sensitivity and specificity of 89% and 83%, respectively. The cut-off titre for anti-Sm antibodies was 9 U/ml, with a sensitivity and specificity of 74% and 83%, respectively.

Conclusion. Low titres of CH50 and C3 and a high titre of anti-Sm antibody were identified as predictors of SLN.

Key words: systemic lupus erythematosus, silent lupus nephritis, complement, anti-Sm antibody.

CLINICAL

Introduction

SLE is a systemic autoimmune disease in which patients present with dysfunction of the CNS, haematopoietic organs, skin, kidneys and other organs. LN is one of the most serious manifestations of SLE, associated with poor prognosis and observed during the course of SLE in 30–60% of patients [1–3]. Furthermore, since 1976 there have been reports of silent LN (SLN) patients without abnormal urinalysis and renal impairment, who have been found to have LN through renal biopsy [4–10]. Therefore the actual prevalence of LN is inferred to be higher than reported. Previous reports classifying SLN according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification have indicated that class I/II LN—indicative of mild nephritis—accounts for

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~60–70% of cases, whereas class III/IV LN—presenting with proliferative GN and associated with poor prognosis—accounts for ~15–20% of cases [11, 12]. Although a renal biopsy is necessary for definitive diagnosis of LN, no clear criteria have yet been established for renal biopsy in SLE patients, in part because of the presence of SLN. Renal biopsies are also difficult to perform because of CNS involvement and complications such as haemorrhaging associated with needle puncture. Therefore predictors of LN in SLE patients who do not present with abnormal urinalysis results or renal impairment will help clinicians to identify cases in which renal biopsy should be performed, leading to early detection of nephropathy. Furthermore, even in cases in which a renal biopsy cannot be performed, the ability to infer the presence of SLN could help in determining a treatment strategy. Based on the above, the determination of predictors for SLN may lead to improved renal survival.

In our study we examined SLE patients who underwent renal biopsy at our hospital. Patients who did not present with abnormal urinalysis or renal impairment at the time of the biopsy were divided into two groups: those with and without histopathologically proven LN. Furthermore, the clinical characteristics of the two groups were compared to clarify the predictors of SLN.

Materials and methods

Patients

We assessed 182 patients who underwent renal biopsy among the 449 hospitalized at the University of Occupational and Environmental Health between November 2002 and December 2012 and given a diagnosis of SLE based on the 1997 ACR classification criteria [13]. A renal biopsy was not performed for the following reasons: (i) patient consent could not be obtained; (ii) poor systemic condition, including complications such as CNS involvement and (iii) the attending physician judged the patient to be unsuitable for biopsy for reasons other than those mentioned above. For the 182 patients who underwent renal biopsy, abnormal urinalysis and renal impairment were defined based on the following three criteria: (i) <300 mg/day of proteinuria, (ii) no active urinary sediments and (iii) a glomerular filtration rate (GFR) $\geqslant$60 ml/min/1.73 m^2. According to the Kidney Disease Improving Global Outcomes 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease, the estimated GFR (eGFR) levels from serum creatinine <60 ml/min/1.73 m^2 should be reported as mildly to moderately decreased when reporting GFR [14]. Therefore we defined the silent range as GFR $\geqslant$60 ml/min/1.73 m^2 (GFR category G1–2) within which serum creatinine concentrations can be within the normal range [15]. We compared the baseline characteristics of the 48 patients who fulfilled these criteria with and without histopathologically proven LN. The study was conducted according to the principles of the Declaration of Helsinki. Our retrospective clinical observation was approved by the local ethics committee at the University of Occupational and Environmental Health.

Evaluation of clinical measurements

Before performing renal biopsy, the presence of all items in the 1997 ACR SLE classification criteria was determined and all of the following were measured: qualitative urinalysis, urinary sediment, quantitative determination of urinary protein and creatinine clearance (CCr) based on 24 h urine collection, complete blood count, serum albumin, serum creatinine, CRP, ESR, complement haemolytic activity (CH50), complement components (C3 and C4), IgG, anti-dsDNA antibodies, anti-Sm antibodies and anti-ribonucleoprotein (RNP) antibodies. GFR was measured by eGFR and 24 h CCr. The eGFR was calculated according to the described method using variables that included serum creatinine, age and sex [16]. SLE activity was assessed according to the SLEDAI and the BILAG index. CH50 was measured according to Mayer's method and C3, C4, and IgG were measured with immunonephelometry (Wako Pure Chemical Industries, Osaka, Japan). Anti-dsDNA antibodies (normal value $\leqslant$12 U/ml), anti-Sm antibodies (normal value <7 U/ml; values considered indeterminate: $\leqslant$7 to <30) and anti-RNP antibodies (normal value <15 U/ml) were measured with an enzyme immunoassay (Medical & Biological Laboratories, Nagoya, Japan). For all patients we confirmed that there was no significant change in laboratory results. Thus the values obtained from the serological evaluations at the time of the renal biopsy were considered representative of a stable disease state in all patients.

Evaluation of renal histopathology

Renal histopathology was classified based on the 2003 ISN/RPS classification [17]. Renal biopsy samples taken in 2003 or earlier were reclassified according to the 2003 ISN/RPS classification. Immunohistological diagnoses were determined using the direct immunofluorescence method. Patients who had no evidence of LN as determined by optical microscopy, immunofluorescence and electron microscopy were defined as not having LN.

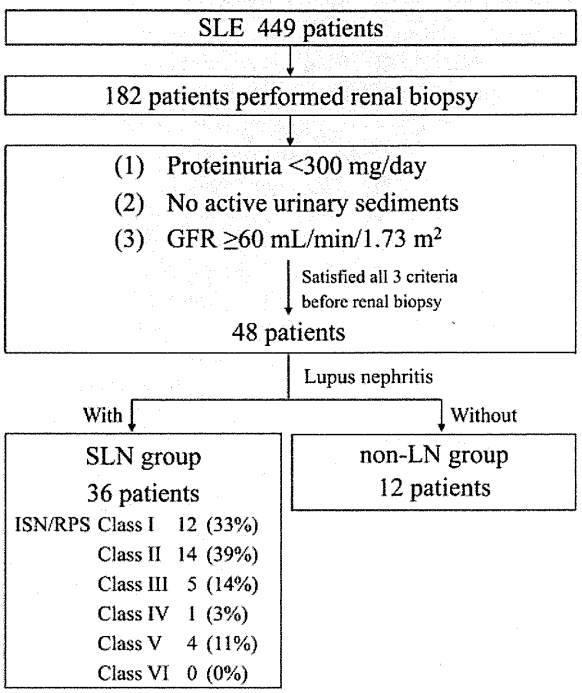
Statistical analysis

Values are expressed as mean (S.D.) or as number and percentage. The differences between patients with and without LN were examined for statistical significance using Fisher's exact test to compare frequencies and the Mann–Whitney *U* test to compare median values. Multivariate analysis was performed using binomial logistic regression analysis. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using JMP software, version 9 (SAS Institute, Cary, NC, USA).

Results

Of the 48 patients, 36 (75%) were classified as having histopathologically proven LN (SLN group). The remaining

FIG. 1 Patient disposition and ISN/RPS classification of the 48 SLE patients without abnormal urinalysis or renal impairment



SLN: silent LN; GFR: glomerular filtration rate; ISN/RPS: International Society of Nephrology/Renal Pathology Society.

12 (25%) patients had no observable immune deposits on immunofluorescence and no abnormal findings on optical or electron microscopy; these patients were classified as the non-LN group. In the SLN group, the frequencies of ISN/RPS class I–V nephritis were as follows: 12 (33%), 14 (39%), 5 (14%), 1 (3%) and 4 (11%), respectively (Fig. 1). No cases of class VI were observed. Moreover, characteristics such as age, sex, disease duration and prebiopsy treatment history were not significantly different between the groups. Furthermore, no significant differences were observed in the presence of hypertension and RP or in disease activity assessments (SLEDAI and BILAG) (Table 1).

A comparison of the 11 items in the 1997 ACR classification criteria showed that the SLN group had a significantly higher number of anti-Sm antibody-positive patients ($P = 0.02$), whereas no significant differences were observed for any of the remaining 10 items, including skin lesions, arthritis and neurological symptoms (Table 2). Prebiopsy blood tests showed that the SLN group had a significantly lower platelet count ($P = 0.03$) and significantly decreased serum albumin ($P = 0.048$) compared with the non-LN group. With respect to serum immunological tests, the SLN group exhibited markedly low titres of C3, C4 and CH50 (C3: $P < 0.001$; C4: $P < 0.001$; CH50: $P < 0.001$). With respect to antibody titres, the SLN group had markedly higher titres of anti-Sm and anti-RNP antibodies (anti-Sm: $P = 0.001$; anti-RNP: $P = 0.01$) compared with the non-LN group (Fig. 2). The lymphocyte count, inflammatory response, IgG and anti-dsDNA antibody titre were not significantly different between the groups.

Because there is a strong confounding relationship between CH50 and C3, a multivariate analysis based on the bivariate analysis results was performed with CH50 as the complement. In the SLN group, a low CH50 titre ($P < 0.001$) and a high anti-Sm antibody titre ($P = 0.02$) were considered independent factors (Table 3). Multivariate analysis after replacing CH50 with C3 indicated that C3 was also an independent factor ($P < 0.001$). To predict the presence of SLN, the cut-off titres for CH50, C3 and anti-Sm antibodies were calculated based on the receiver operating characteristic curve. The cut-off titre for CH50 was 33 U/ml, with a sensitivity of 89%, a specificity of 83% [odds ratio (OR) 40.0, $P < 0.001$], a positive predictive value (PPV) of 94% and a negative predictive value (NPV) of 71%. The cut-off titre for anti-Sm antibodies was 9 U/ml, with a sensitivity of 74%, a specificity of 83% (OR 14.4, $P = 0.001$), a PPV of 93% and an NPV of 53%. For the titre cut-offs of 33 U/ml for CH50 and 9 U/ml for anti-Sm antibodies, sensitivity was 66%, specificity was 100%, the PPV was 100% and the NPV was 50% (Table 4).

Clinical course of patients with and without LN

The elevation of serum creatinine levels was not observed in any patients in either group within 5 years after renal biopsy. However, of the 32 patients in the SLN group who were observed for at least 1 year after renal biopsy, 6 (19%) showed the following abnormal urinalysis: only proteinuria (4 patients), only microscopic haematuria (1 patient) and both urinary findings (1 patient). The histopathological classification (ISN/RPS class) of these six patients at the time of SLN diagnosis was as follows: class I, one; class III, one; class IV, one; class V, three.

Discussion

Our study is the first to analyse predictors for SLN in SLE patients without abnormal urinalysis or renal impairment before renal biopsy. Surprisingly, SLN was observed in 75% of patients. Of the SLN patients, 72% were classified as ISN/RPS class I or II, whereas 17% were classified as class III or IV. Low titres of CH50 and C3 and a high titre of anti-Sm antibody were determined to be predictors of SLN. With respect to hypocomplementaemia, the cut-off titres for CH50 (33 U/ml; normal value 31.6–57.6) and C3 (65 mg/dl; normal value 65–135) were similar to the lower limits of their respective normal ranges. Hence it is highly likely that LN is already present in the early stage of low complement titres, even in the absence of abnormal urinalysis. In previous reports of SLN and hypocomplementaemia, Wakasugi *et al.* [12] reported that a low titre of C3 (cut-off value 55 mg/dl) was a predictor of ISN/RPS class III or IV SLN (sensitivity 85%, specificity 58%). In addition, Wada *et al.* [18] reported that patients with overt nephritis showed both persistent elevation of anti-dsDNA antibodies and persistent hypocomplementaemia for at least 24

Table 1 Baseline characteristics of the 48 patients and comparison between the SLN group and the non-LN group

	Total ($n = 48$)	SLN ($n = 36$)	Non-LN ($n = 12$)	*P*-value
Age at renal biopsy, mean (S.D.), years	37 (16)	37 (17)	36 (13)	0.79
Male/female, *n/n*	3/45	2/34	1/11	1.00
Disease duration, mean (S.D.), months	19 (33)	15 (19)	32 (57)	0.45
Patients received PSL, *n* (%)	15 (31)	11 (31)	4 (33)	1.00
Dosage of PSL, mean (S.D.), mg/day	25 (20)	28 (21)	17 (14)	0.43
Patients received immunosuppressive agent, *n* (%)	5 (10)	4 (11)	1 (8)	
MTX, *n*	1	1	0	
Mizoribine, *n*	1	1	0	
Cyclosporin A, *n*	1	1	0	
AZA, *n*	2	1	1	
Proteinuria, mean (S.D.), g/day	0.09 (0.07)	0.08 (0.06)	0.10 (0.08)	0.81
Serum creatinine, mean (S.D.), mg/dl	0.53 (0.11)	0.53 (0.12)	0.50 (0.07)	0.72
eGFR, mean (S.D.), ml /min/1.73 m^2	112 (26)	111 (29)	116 (19)	0.69
24 h CCr, mean (S.D.), ml /min	116 (34)	116 (36)	117 (29)	0.92
SLEDAI, mean (S.D.)	9.5 (4.4)	10.3 (4.6)	7.4 (3.1)	0.06
BILAG, mean (S.D.)	12 (7)	13 (8)	10 (6)	0.55
Hypertension, *n* (%)	3 (6)	3 (8)	0 (0)	0.56
RP, *n* (%)	10 (21)	4 (17)	4 (33)	0.40

P-value is estimated between the two groups. SLN: silent LN; PSL: prednisolone; eGFR: estimated glomerular filtration rate; CCr: creatinine clearance

Table 2 Comparison of each clinical manifestation in the 1997 ACR classification criteria between the SLN group and the non-LN group

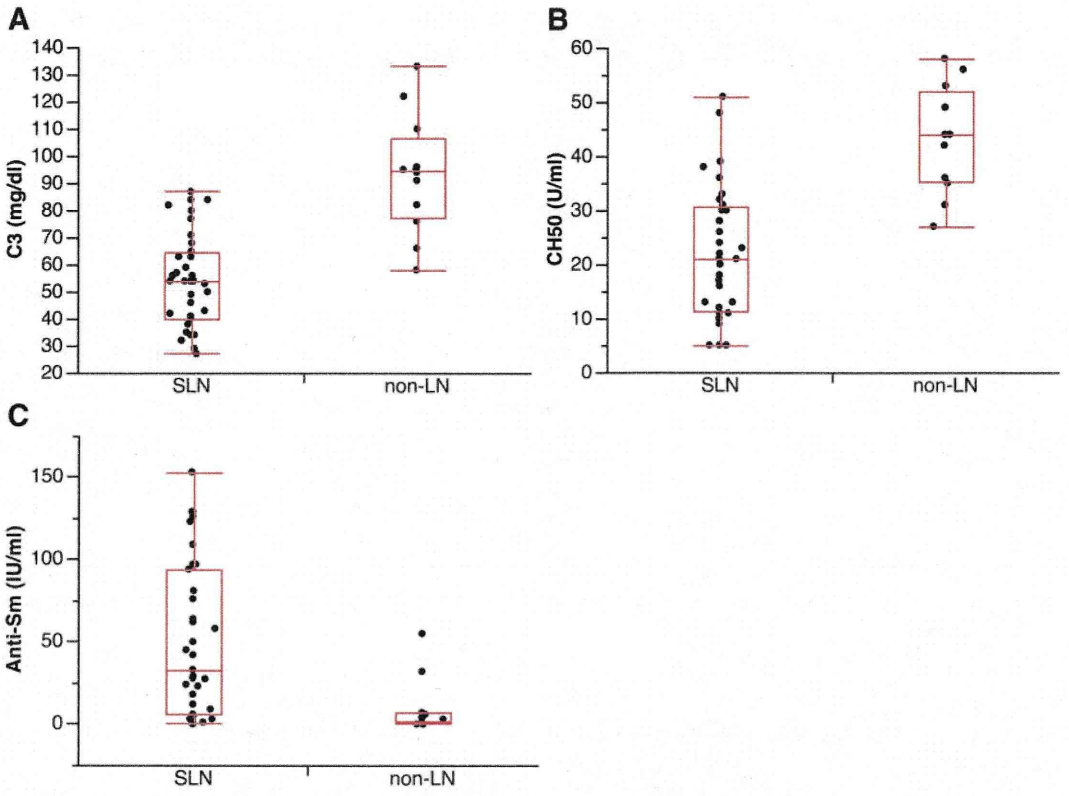
	Total ($n = 48$)	SLN ($n = 36$)	Non-LN ($n = 12$)	*P*-value
Malar rash	26 (54)	20 (56)	6 (50)	0.75
Discoid rash	10 (21)	7 (19)	3 (25)	0.69
Photosensitivity	21 (43)	13 (36)	8 (67)	0.10
Oral ulcers	12 (25)	10 (28)	2 (17)	0.70
Arthritis	34 (71)	28 (78)	6 (50)	0.14
Serositis	7 (15)	7 (19)	0 (0)	0.17
Renal disorder	0 (0)	0 (0)	0 (0)	
Neurological disorder[a]	21 (44)	16 (44)	5 (42)	1.00
Haematological disorder	43 (90)	33 (92)	10 (83)	0.59
Haemolytic anaemia	4 (8)	3 (8)	1 (8)	1.00
Leucopenia	22 (46)	19 (52)	3 (25)	0.18
Lymphopenia	42 (88)	33 (92)	9 (75)	0.16
Thrombocytopenia	6 (13)	5 (14)	1 (8)	1.00
Immunological disorder	44 (92)	35 (97)	9 (75)	0.09
Anti-DNA	39 (81)	32 (89)	7 (58)	0.09
Anti-Sm	27 (60)	24 (67)	3 (25)	0.02
aPL	14 (30)	11 (31)	3 (25)	1.00
ANA	47 (98)	36 (100)	11 (92)	0.25

Data are given as number (%). *P*-value is estimated to allow comparisons between the two groups. [a]Neurological disorder includes only abnormality in cerebrospinal fluid (pleocytosis, elevation of IL-6 and IgG index) or neuroimaging. SLN: silent LN.

months before the onset of overt nephritis. Our study and these reports indicate that low titres of CH50 and C3 are clear predictors of SLN and the severity of SLN increases with lower complement titres and a longer duration of hypocomplementaemia. These findings can be explained by the primary pathology in LN, which is immune-complex nephritis. However, our study reveals the noteworthy fact that SLN was observed when complement components were present at the lower limit of their normal clinical range. Therefore low titres of CH50 and C3 are considered extremely useful predictors of SLN.

In this study we have shown for the first time that patients with SLN have a significantly higher titre of anti-Sm antibodies than those without LN. Anti-Sm antibodies are

FIG. 2 Comparison of titres of C3, CH50 and anti-Sm antibodies between the SLN group and the non-LN group



(**A**) C3, (**B**) CH50 and (**C**) anti-Sm antibodies. Each dot represents a patient. The bottom and top of the boxes are the first and third quartiles and the bands inside the boxes are the median values. The ends of the whiskers represent the minimum and maximum values.

TABLE 3 Comparison of laboratory results between the SLN group and the non-LN group

	SLN ($n = 36$)	Non-LN ($n = 12$)	*P*-value (bivariate)	*P*-value (multivariate)
WBCs, /μl	4578 (2178)	5741 (2008)	0.09	
Lymphocytes, /μl	866 (334)	1300 (721)	0.08	
Platelets, $\times 10^4$/μl	20.2 (7.7)	24 (5.1)	0.03	0.20
Serum albumin, g/dl	3.5 (0.5)	3.9 (0.5)	0.048	0.17
CRP, mg/dl	1.39 (4.7)	1.0 (1.3)	0.49	
ESR, mm/h	49 (32)	48 (28)	0.95	
C3, mg/dl	53 (17)	93 (22)	<0.001	<0.001
C4, mg/dl	8.3 (4.3)	16 (6.7)	<0.001	
CH50, U/ml	22 (12)	44 (9.9)	<0.001	<0.001
IgG, mg/dl	2273 (752)	2035 (1137)	0.21	
Anti-dsDNA, IU/ml	120 (132)	126 (176)	0.17	
Anti-Sm, IU/ml	50 (47)	9.3 (17)	0.001	0.02
Anti-RNP, IU/ml	79 (65)	21 (21)	0.01	

Data are given as mean (S.D.). *P*-value is estimated to allow comparisons between the two groups. SLN: silent LN.

antibodies against non-histone nuclear proteins; the corresponding antigens are small nuclear RNPs related to mRNA splicing (U1, U2, U4/U6 and U5RNP) [19, 20]. These antibodies are observed in 5–30% of SLE patients and they are included as a serum immunology criterion in the classification scheme because of their high specificity [21, 22]. The clinical significance of anti-Sm antibodies is that their presence in SLE patients is reportedly

Table 4 Predictors of SLN

Predictive factors	Sensitivity, %	Specificity, %	PPV, %	NPV, %	OR (95% CI)	*P*-value
CH50 <33 U/ml	89	83	94	71	40.0 (6.3, 251.8)	<0.001
C3 <65 mg/dl	78	92	97	58	38.5 (4.3, 344.9)	<0.001
Anti-Sm >9 U/ml	74	83	93	53	14.4 (2.6, 78.8)	0.001
CH50 <33 U/ml and anti-Sm >9 U/ml	66	100	100	50	—	<0.001

SLN: silent LN; PPV: positive predictive value; NPV: negative predictive value; OR: odds ratio.

associated with CNS involvement. Furthermore, many reports have stated that anti-Sm antibodies, along with anti-dsDNA antibodies, are expressed at a high rate in SLE patients with LN [23–27]. Although no studies have investigated the relationship between SLN and anti-Sm antibodies, anti-Sm antibodies were found to be associated with late-onset renal disease [28]. One report presented a comparison of patients who developed LN >5 years after SLE diagnosis with those who developed LN within 5 years, but observed no significant differences in the frequency of anti-Sm or anti-dsDNA antibody expression [29]. Some reports have indicated that anti-C1q antibodies are frequently expressed in patients with SLN and the anti-C1q antibody–C1q immune complex is involved in the early stage of LN onset [11, 30]; however, we did not investigate this association in our study.

The predictors of SLN may be used as criteria for deciding whether a renal biopsy should be performed for the early detection of LN. Complement activity and anti-Sm antibodies can be confirmed with an extremely simple blood test, thereby allowing the inference of LN onset from regular blood tests performed on an outpatient basis. Furthermore, LN can also be predicted in SLE patients when a renal biopsy is difficult, such as in elderly patients, when consent is not provided, in patients with CNS lesions or bleeding and in patients with circulatory or respiratory impairment. Based on the above, the predictors of SLN are considered useful for deciding the timing of renal biopsy and determining the treatment strategy.

In the long-term observations of SLN, renal survival and prognosis are reported to be more favourable than in cases of overt LN. Gonzalez-Crespo *et al.* [31] reported that the renal survival rate at 51 months is 98%, whereas the survival rate for patients with diffuse proliferative GN is 90%. However, in this study, even after the administration of medium-to-high doses of corticosteroids to more than half of the patients, abnormal urinalysis results were observed in 22 of the 211 (10.4%) patients during a mean observation period of 51 months, with 5 patients eventually developing end-stage renal failure. Exacerbated histopathological findings were also observed in 8 of the 47 patients who underwent repeat biopsy. Furthermore, another study reported abnormal urinalysis results and/or renal impairment in 25.8% of SLN patients at a mean duration of 58 months after renal biopsy with prednisolone administration of 40–60 mg/day [18]. Therefore it is necessary to analyse renal survival in SLN for >5 years and establish the basis for this therapy.

Our study has several limitations. First, it is a retrospective observational study. Second, renal biopsies were performed for only 182 of the 449 patients with SLE, thereby resulting in patient selection bias. Third, ~30% of the 48 patients examined were taking corticosteroids and/or immunosuppressants at the time of their renal biopsy, which may have concealed the clinical findings resulting from LN. Fourth, microalbuminuria was not examined in our study. The reasons for this were that our study was conducted in the clinical practice setting and that quantitative measurement of urine albumin excretion in non-diabetic patients was not covered by Japanese medical insurance. In some reports of SLN, clinical renal involvement was defined as >300–500 mg/day of proteinuria [11, 12, 31]. For these reasons we defined clinical renal involvement as overt proteinuria diagnosed in patients with ⩾1+ on urine dipstick testing and with ⩾300 mg/day of proteinuria. Fifth, our study did not include the anti-C1q antibody and urinary biomarkers such as the TNF-like weak inducer of apoptosis (TWEAK), monocyte chemoattractant protein 1 (MCP-1), neutrophil gelatinase-associated lipocalin (NGAL), CD4 T cell and CD8 T cell [32–36]. Despite our study's limitations, we believe that it has value in the development of LN diagnostics, as the above-mentioned limitations are often difficult to avoid in clinical research involving relatively rare pathologies such as LN.

Conclusion

Diagnosing SLN is extremely difficult because of the lack of clinical symptoms and examination findings. However, some cases of SLN involve proliferative GN such as ISN/RPS class III or IV, with renal dysfunction progressing over the long term in some cases. Our study findings suggest that LN is present at a high rate in SLE patients with low titres of CH50 and C3 and a high titre of anti-Sm antibodies, including in those without abnormal urinalysis or renal impairment. Given the above, the possibility of the presence of LN is considered extremely high when the CH50 concentration is <33 U/ml and anti-Sm antibodies are present at >9 U/ml. In such cases, a renal biopsy is strongly recommended. The use of these indicators allows the early detection and treatment of LN, which is expected

to lead to improvements in renal survival and prognosis for SLE patients.

Rheumatology key messages

- LN was histopathologically identified in 75% of SLE patients without abnormal urinalysis or renal impairment.
- Low complements and high titre of anti-Sm antibody were identified as predictors of silent LN.

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BRIEF COMMUNICATION

Distinguishing the cerebrospinal fluid cytokine profile in neuropsychiatric systemic lupus erythematosus from other autoimmune neurological diseases

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Abstract
Neuropsychiatric systemic lupus erythematosus (NPSLE) is a serious complication in SLE. Although the mechanism of NPSLE remains unclear, cytokines and chemokines are considered to be involved in their pathogenesis. Here we used Bio-Plex Pro assays to examine 27 types of cytokines and chemokines in the cerebrospinal fluid (CSF) of 32 NPSLE patients. We used the CSF of 20 patients with multiple sclerosis (MS) and 22 patients with neuromyelitis optica (NMO) as a disease control group. Fourteen of 27 cytokines/chemokines were significantly higher in the NPSLE patients compared to the MS/NMO patients. We could identify six "minimum predictive markers" by using a weighted-voting algorithm that could distinguish NPSLE from MS and NMO: interleukin (IL)-17, IL-2, interferon (IFN)-γ, IL-5, basic fibroblast growth factor (FGF)-basic and IL-15. The determination of various types of CSF cytokine profiles may contribute to the diagnosis of NPSLE and may help elucidate the mechanisms underlying this disease.

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1. Introduction

Neuropsychiatric systemic lupus erythematosus (NPSLE) syndromes involve both the central and peripheral nervous systems. Despite advances in the understanding of the immunopathogenic and clinical aspects of SLE, NPSLE remains a diagnostic and therapeutic challenge [1]. Cytokines and chemokines are considered biomarkers and therapeutic targets in NPSLE. Of note, abnormalities in cerebrospinal fluid (CSF) have been reported in patients with NPSLE. Increased levels of proinflammatory cytokines and chemokines have been reported in the CSF of patients with NPSLE, including cytokines such as interleukin (IL)-6, IL-8, IL-10, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), C-C motif ligand (CCL)2/monocyte chemoattractant protein-1 (MCP-1) and C-X-C motif ligand (CXCL) 10/inducible protein-10 (IP-10) [2,3]. Among these, CSF IL-6 is major cytokine for the diagnosis of NPSLE. IL-6 is a proinflammatory cytokine secreted by immune cells and activated astrocytes, with a wide variety of functions. The sensitivity and specificity of the diagnosis of lupus psychosis were 87.5% and 92.3%, respectively, at the cut-off value of 4.3 pg/mL [4].

Multiple sclerosis (MS) and neuromyelitis optica (NMO) are chronic autoimmune inflammatory diseases affecting the central nervous system (CNS). Disruption of the blood–brain barrier (BBB) is a known mechanism of the disease process in these two CNS diseases. MS and NMO are also considered T cell-mediated autoimmune diseases, and both the Th1/Th2 balance and Th17 cells play an important role in the pathogenesis [5]. Elevated CSF IL-6 and IL-8 levels in NMO patients have also been reported [6]. CSF IL-6 and IL-8 levels are significantly higher in patients with NMO than in patients with MS [6]. Similar cytokines/chemokines have been evaluated in NPSLE, MS and NMO but the therapeutic strategy and management are quite different among these three diseases.

In this study we evaluated multiple cytokines, chemokines and growth factors in NPSLE compared to MS and NMO as disease controls. We found a specific combination of cytokines, chemokines and growth factors in NPSLE that can be distinguished from the profiles of the other two diseases. This analysis might help clarify the mechanism of NPSLE caused by inflammation and may provide an important resource for pharmaceutical developments.

2. Methods

2.1. Study design and patients

We studied 32 patients who were admitted to Nagasaki University Hospital in a 7-year period from 2006 through 2013 and fulfilled at least four of the 11 revised criteria of the American College of Rheumatology (ACR) for the classification of SLE [7]; they were all diagnosed with NPSLE by rheumatologists and psychiatrists. Neuropsychiatric manifestations showing psychiatric symptoms such as mood disorder, anxiety disorder, psychosis, acute confusional state, or cognitive dysfunction were evaluated by a psychiatrist and classified according to the ACR nomenclature and case definitions for NPSLE [8].

All information about clinical symptoms and laboratory data were reviewed retrospectively using the patients' medical records. The patients' age, gender, clinical events, results of serum laboratory tests, the CSF analysis, brain magnetic resonance imaging (MRI), and single photon emission computed tomography (SPECT) and their diagnoses and treatment were all analyzed.

As disease controls, we used samples from 20 relapsing remitting MS (RRMS) patients, samples from 22 NMO patients, 11 normal pressure hydrocephalus (NPH) patients, and 16 viral meningitis (VM) patients from the Department of Clinical Neuroscience and Neurology, Nagasaki University Hospital. For the diagnosis of NMO, we defined NMO spectrum disorder (NMOSD) based on the revised NMO criteria [9]. All of the NMO patients were positive for anti-AQP4 antibodies in sera. CSF of NPH patients were used for non-autoimmune, non-inflammatory neurological controls and VM patients were used for positive controls. The protocol was approved by the Institutional Review Board of the Nagasaki University Hospital.

2.2. Multiplex cytokine bead assay

We performed a multiplex cytokine bead assay using undiluted CSF supernatants and the Bio-Plex Pro Human Cytokine Group I 27-Plex Panel analyzed with a Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. CSF samples were centrifuged within 30 min at 1500 rpm at 4 °C for 5 min, and the liquid phase of the CSF was stored at −80 °C until use. The levels of 27 cytokines/chemokines and growth factors in the liquid phase of the CSF, namely, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8/IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, basic fibroblast growth factor (FGF)-basic, CCL11/eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-γ, IP-10, CCL2/MCP-1, CCL3/macrophage inflammatory protein (MIP)-1α, CCL4/MIP-1β, platelet-derived growth factor (PDGF)-BB, CCL5/regulated on activation, normal T cell expressed and secreted (RANTES), TNF-α, and vascular endothelial growth factor (VEGF) were measured as described [10,11].

The cytokine/chemokine/growth factor concentrations were calculated based on the respective standard curve for each cytokine/chemokine/growth factor concentration of the standards assayed in the same manner as the CSF samples. The detection limit for each molecule was determined by the recovery of the corresponding standard, and the lowest values with more than 70% recovery were set as the lower detection limits. All samples were analyzed in duplicate.

2.3. Construction of diagnostic systems

Next, we selected which of the 27 cytokines/chemokines/growth factors were useful markers for distinguishing NPSLE from MS and NMO. The weighted-voting (WV) algorithm was used as described [12,13]. The ranking of the 27 cytokines/chemokines for this algorithm was based on the signal-to-noise ratio (SNR). For each marker set of 27

cytokines/chemokines/growth factors based on the absolute value of the SNR, we calculated the sensitivity, specificity, accuracy, positive predictive value, negative predictive value, and Matthews correlation coefficient (MCC). The MCC is used as a measure of the quality of binary (two-class) classifications. We defined the "distinction set" as a set that had a minimum number of markers with higher MCCs.

In the WV algorithm, each marker belonging to a predictor set is assigned a vote value, and prediction is based on the relative vote value in NPSLE and in MS and NMO. The vote value of marker 'a,' v_a, and the weight of marker 'a,' w_a, are calculated by the following two formulas:

$$v_a = w_a \times \left| x_a - \frac{\overline{X}_{na} + \overline{X}_{oa}}{2} \right|$$

$$w_a = \frac{\overline{X}_{na} - \overline{X}_{oa}}{S_{na} + S_{oa}}.$$

Here, X_a is the expression level of each marker a in a CSF sample. $\overline{X}_{na}$ is the mean expression level of each marker 'a' in NPSLE in the learning set. $\overline{X}_{oa}$ represents the mean expression level in the other diseases (i.e., MS and NMO). S_{na} is the standard deviation in NPSLE, and S_{oa} represents the standard deviation in the others. The SNR used for ranking the markers is the absolute value of W_a; the diagnostic markers were selected in descending order of SNR. We selected 'i' pieces of markers, in the order of the absolute number of SNR, and we defined the prediction strength (PS) value of the i pieces of markers using the following formula:

$$\text{Prediction strength value} = \frac{\sum_{n=1}^{i} v_a}{\sum_{n=1}^{i} |v_a|}.$$

In the case of missing values, the vote of the gene is assigned as 0. PS is defined in which positive and negative PS indicates the prediction of others and NPSLE, respectively.

2.4. Statistical analysis

We used a *t*-test and the nonparametric Wilcoxon rank sum test for the inter-group comparisons of multiple variables. The Spearman rank correlation coefficient was used for the analyses of correlations between cytokines, chemokines, and growth factors in the patients with NPSLE, MS and NMO. All of the statistical analyses were performed using JMP® Pro10 (SAS Institute, Cary, NC, USA) and R Statistical Software (Foundation for Statistical Computing, Vienna, Austria). The significance level was set at $p < 0.05$.

3. Results

3.1. Demographic and disease-related variables

Supplementary Table 1 shows the demographic and disease-related characteristics of the 32 NPSLE patients at examination. Thirty-one of the 32 patients (96.9%) were females. The median age at the onset of NPSLE was 34.9 yrs, ranging from 15 to 50 yrs. The median duration from SLE onset to first neuropsychiatric event was 8.7 yrs. The median SELENA-SLEDAI score at the disease onset of NPSLE was 13.3. The median levels of anti-ds-DNA antibodies (U/mL), C3 (mg/dL) and C4 (mg/dL) were 20.2, 86.3 and 19.4, respectively. The median level of CSF IgG index was 0.61. Seven (21.9%) of the NPSLE patients also fulfilled the criteria for the antiphospholipid syndrome. Eight (25.0%) of the patients had anti-ribosomal P antibodies, and 17 (53.1%) patients had abnormal MRI findings.

A total of 39 neuropsychiatric manifestations were observed in the 32 NPSLE patients. Nine of the 19 ACR Ad Hoc Committee classifications of NPSLE manifestations [2] were identified in this study (Suppl. Table 2). Nine of the 32 (28.1%) patients presented more than one NPSLE manifestation. Most of the NPSLE manifestations (97.4%) were in the CNS. The most frequent manifestation was headache (35.9%), followed by psychosis (25.6%) and mood disorder (12.8%).

3.2. Comparison of cytokine/chemokine/growth factor levels

We analyzed 32 NPSLE patients, 20 RRMS patients, samples from 22 patients with NMO, 11 NPH patients and 16 VM patients. The representative results are shown in Supplementary Fig. 1. Although some of the cytokine and chemokine levels of the VM patients were extremely high compared to the other groups, we did not observe any significant differences among the NPSLE, MS and NMO groups. We therefore referred to a previous report [14] and compared the results among only the present NPSLE, MS and NMO groups. Since there was no significant difference between the MS and NMO groups (Fig. 1), we speculated that it is problematic to distinguish MS and NMO, and we thus compared NPSLE and the "others" (i.e., the combined group of MS and NMO patients).

Fourteen of the 27 cytokines/chemokines/growth factors were significantly higher in the NPSLE group compared to the others (Table 1). CSF IL-6 and IL-8 levels, which had been reported to be increased in NPSLE and NMO, were not significantly different between the present NPSLE group and the others. Only the CSF level of IL-1β was lower in the NPSLE group compared to the others.

3.3. Ranking of the cytokines/chemokines/growth factors for diagnosing NPSLE

Our ranking of the cytokines/chemokines/growth factors for diagnosing NPSLE determined by the weighted-voting algorithm showed that the combination of IL-17, IL-2, IFN-γ, IL-5, FGF-basic and IL-15 had the highest MCC (88.99%). According to this method, the accuracy of discriminating a responder from a non-responder was 94.59% (Table 2). The WV values (v_a) of each of these six markers and the PS value were obtained by the formulas shown in Supplementary Table 3; X_a is the expression level of each marker 'a' in a CSF sample.

4. Discussion

NPSLE is still diagnosed based on a combination of clinical observations, laboratory tests and imaging techniques,

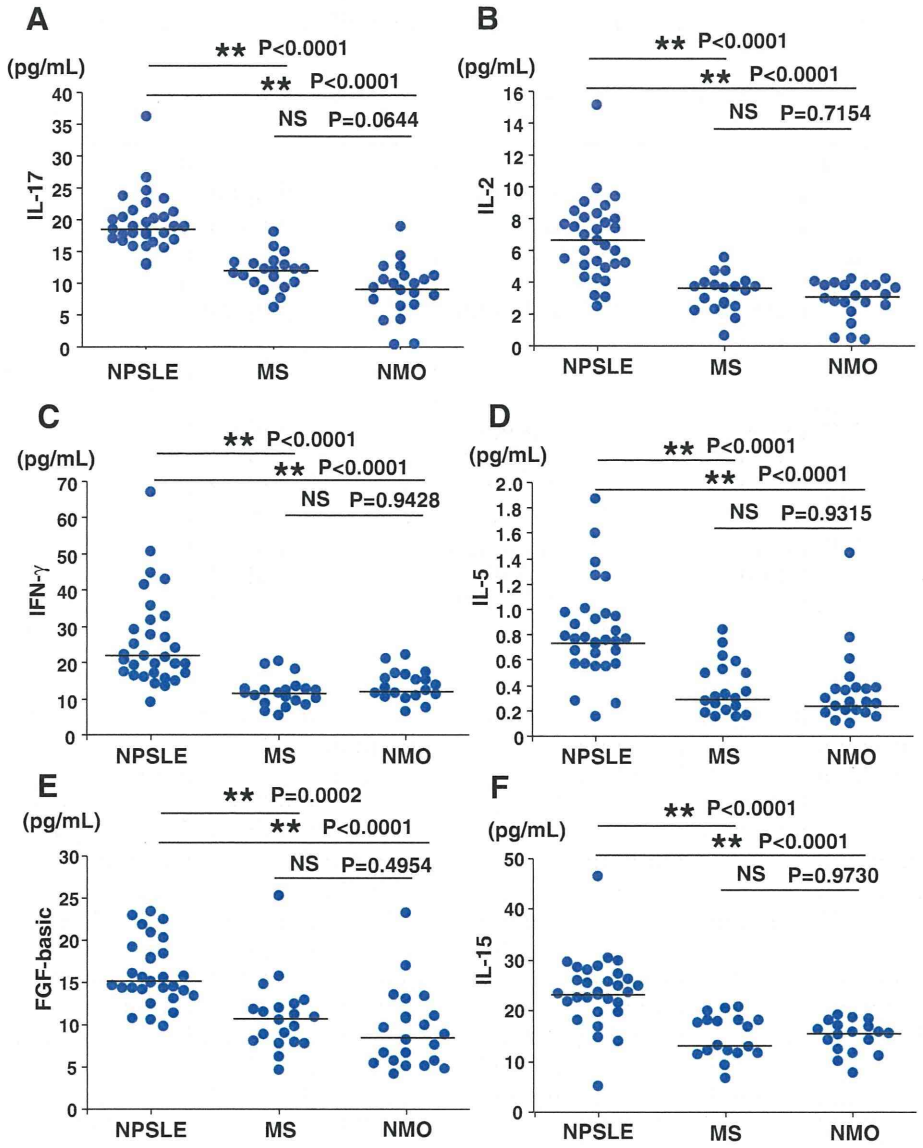

Figure 1

because there are no specific markers for NPSLE. Multiple cytokines and chemokines have been implicated in the pathophysiology of NPSLE. Previous studies obtained evidence of the intrathecal production of IL-6 [3,4] and other cytokines including IL-8 [3,15], IL-10 [16], TNF-α [16] and IFN-γ [17] in patients with NPSLE. Cytokines associated with NPSLE are produced by neuronal and glial cells, probably in response to autoantibodies within the intrathecal space.

MS and NMO, chronic autoimmune inflammatory diseases affecting the CNS, are sometimes difficult to distinguish from NPSLE. An overlap diagnosis often referred to as lupoid sclerosis has been described [18]. In SLE, MS and NMO, the BBB is pivotal. There is evidence implicating BBB damage as an important component in the development of NPSLE, MS and NMO, occurring through damage to the barrier's integrity by environmental triggers such as cytokines, chemokines and growth factors.

For example, the stimulation of human brain microvessel endothelial cells (HBMECs) with cytokines such as IL-1β, IL-8, TNF-α, and IFN-γ is known to induce increased permeability of the monolayers [19,20]. Additionally, CSF from NPSLE patients with IL-6 and IL-8 was correlated with MMP-9 levels, and the latter is associated with degradation of the BBB extracellular matrix [21]. In both an in vitro investigation and in $CCR2^{-/-}$ mice, CCL2 signaling appeared to play a role in BBB disruption [22,23].

We found that 14 of the 27 CSF cytokines/chemokines/growth factors used in the present study were significantly higher in the NPSLE group compared to the others (Table 1). In addition, we measured the serum markers of NPSLE, MS and NMO at nearly the same time that the lumbar puncture was performed. We found that serum IL-15 and IFN-γ were significantly increased in the non-NPSLE patients compared to the NPSLE patients (Suppl. Table 4). It is interesting that these results were the inverse of the CSF cytokines' results, and these results might indicate that BBB disruption is much stronger in NPSLE compared to non-NPSLE.

Table 1 Comparison of each marker in cerebrospinal fluid among NPSLE, MS and NMO. *P*-value of each disease vs. the other two is shown.

Marker	NPSLE vs. others	MS vs. others	NMO vs. others	Up/down of NPSLE vs. others
IL-17	7.1981E-13	0.005200983	6.06716E-08	Up
IL-2	1.79273E-09	0.000183297	3.05064E-07	Up
FGF-basic	3.17974E-08	0.048298753	0.000240424	Up
IL-5	1.73358E-07	0.001280693	0.000544469	Up
IL-15	2.32436E-07	0.003814834	0.000574384	Up
IL-9	3.0018E-07	0.000287076	0.041266563	Up
IFN-γ	1.15444E-06	1.34983E-05	0.000209175	Up
IL-12(p70)	2.06811E-06	0.016465613	0.001083612	Up
IL-10	1.28056E-05	0.001591964	0.00011084	Up
IL-7	2.57896E-05	0.01531653	0.004787909	Up
GM-CSF	0.000174931	0.822364295	4.22879E-05	Up
IL-13	0.000194842	0.023994687	0.022546951	Up
TNF-α	0.000984547	0.002751192	0.012722463	Up
Eotaxin	0.002694469	0.005361471	0.00337151	Up
IL-8	0.923993657	0.003915445	0.106276224	Up
IL-6	0.974937316	0.014641205	0.153928475	Up

CSF IL-6 is a major cytokine for the diagnosis of NPSLE because of its sensitivity and specificity in certain conditions [4]. Elevated CSF IL-6 levels in NPSLE and NMO patients have been reported [3,6]. However, our present findings showed that there was no significant difference in the CSF IL-6 levels among the NPSLE, MS and NMO patients (data not shown). We speculate that CSF IL-6 is involved to some extent in the pathogenic mechanism of all of these autoimmune neurological diseases, and thus CSF IL-6 was not a marker that could be used to distinguish them.

Our study shows for the first time identified predictive markers in the CSF of NPSLE patients compared to MS and NMO patients. Among them, IL-17, IL-2, IL-5, FGF-basic and IL-15 have not been well documented in NPSLE. There has been an increasing focus on the role of Th17, which might be a promising therapeutic target for SLE. CD3 + CD4(−)CD8(−) (double-negative) T cells are an important source of IL-17 in SLE. IL-17 produced by double-negative and CD4 T cells participates in the pathogenesis of SLE [24]. IL-17 promotes the production of IL-6 [25] and other inflammatory mediators by endothelial cells, fibroblasts, macrophages and astrocytes [26].

IL-2, a cytokine with multifaceted effects, is important for immune cell activation and peripheral tolerance. The therapeutic efficacy of IL-2 in SLE has been suggested [27,28]. In another study, no association with IL-2 was found in NPSLE patients who had neurological symptoms without evidence of a neurological disease [29]. However, our patients' backgrounds are different from those of the patients in that study; we compared NPSLE patients with those with other autoimmune neurological disorders. The meaning of elevated IL-2 in NPSLE requires further investigation.

As with IL-2, IL-15 can expand T-cell populations, and IL-15-deficient mice have greatly reduced numbers of lymphocytes [30]. Moreover, IL-15 can promote excessive antibody production in B cells [31]. Increased levels of serum IL-15 have also been observed in patients with SLE, and the IL-2R are correlated with markers that are potential targets of IL-15 [32].

Table 2 Ranking of the cytokines/chemokines for the diagnosis of NPSLE determined by a weighted-voting algorithm with the highest Matthews correlation coefficient.

Marker	TP	FN	TN	FP	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value	MCC
IL-17	29	3	38	4	90.63%	90.48%	90.54%	87.88%	92.68%	80.83%
IL-2	28	4	39	3	87.50%	92.86%	90.54%	90.32%	90.70%	80.69%
IFN-γ	28	4	41	1	87.50%	97.62%	93.24%	96.55%	91.11%	86.38%
IL-5	28	4	41	1	87.50%	97.62%	93.24%	96.55%	91.11%	86.38%
FGF-basic	28	4	40	2	87.50%	95.24%	91.89%	93.33%	90.91%	83.49%
IL-15	30	2	40	2	93.75%	95.24%	94.59%	93.75%	95.24%	88.99%
IL-8	7	25	39	3	21.88%	92.86%	62.16%	70.00%	60.94%	21.35%
IL-6	7	25	39	3	21.88%	92.86%	62.16%	70.00%	60.94%	21.35%

TP: true positives (predicted positive, actual positive); FN: false negatives (predicted negative, actual positive); TN: true negatives (predicted negative, actual negative); FP: false positives (predicted positive, actual negative). MCC: Matthews correlation coefficient.

The possible role of IL-5 in the pathogenesis of lupus has not been extensively evaluated. Several studies suggested that abnormally high levels of IL-5 may have a role in the abnormal expansion of auto-reactive B-1 cells and the subsequent suppression of autoimmune disease [33]. Additionally, elevated IL-5 mRNA has been reported in cutaneous lupus erythematosus [34]. The authors of those studies suggested that IL-5, produced by the Th2 subset of CD4+ T cells may contribute to the development of autoimmunity in lupus. However, the potential relevance of these results to NPSLE is unclear.

FGF-basic is a potent angiogenic factor whose activity is involved in endothelial cell and fibroblast survival, proliferation, migration, and tube formation, together with VEGF [35]. An increased FGF-basic level was reported in lupus and tended to be correlated with disease activity [36].

There has been no thorough comparison of the CSF of NPSLE, MS and NMO patients, to the best of our knowledge. Here, we found that NPSLE patients showed significantly higher levels of cytokines, chemokines and growth factors compared to MS and NMO patients. Among these factors, IL-17, IL-15, IL-9, IL-5, IL-12 p70, IL-7, FGF-basic, eotaxin, IL-13, GM-CSF and VEGF had not been observed in the CSF of NPSLE patients. In the present examination, we were able to detect cytokines, chemokines and growth factors with the Bio-plex assay, even if they were present in very small amounts. Since almost all of the above markers had already been shown elsewhere to be increased in MS or NMO, it would be difficult to distinguish MS or NMO from NPSLE using the above markers. We therefore devised a numerical prediction scoring system that clearly separated the NPSLE patients from the other patients. We identified six "minimum predictive markers"—IL-17, IL-2, IFN-γ, IL-5, FGF-basic and IL-15—by a weighted-voting algorithm that showed the highest MCC and predicted the test set with 94.59% accuracy.

These predictive markers seem not to be correlated with each other; however they certainly involve Th1, Th2 and Th17 cells. This may indicate that these six markers contribute more strongly to induce both pro-inflammatory and antibody-mediated neuronal and glial cells' destruction in NPSLE compared to MS and NMO, and we suspect that they are essential for the pathogenesis of NPSLE.

Focusing on other markers is also important. We found that the correlations of pairs of cytokines/chemokines/growth factors showed different patterns in NPSLE, MS and NMO. The correlation coefficients of the cytokines/chemokines were the most significant: PDGF-BB, MIP-1a, MIP-1b and RANTES in NPSLE, IL-1b and IL-1ra in MS, and IL-12(p70) and VEGF in NMO (Suppl. Fig. 2). Examinations of each single marker may be meaningless, because each marker operates together with one or more other markers in a rather complex system. These results indicate that the downstream pathway of Th1/Th2/Th17-related mediators differs among NPSLE, MS and NMO.

Various markers are known to be involved in the pathology of NPSLE; however, no predictive markers for detecting NPSLE were reported prior to the present study. Here, we were able to identify six minimum predictive markers (IL-17, IL-2, IFN-γ, IL-5, FGF-basic and IL-15), by using a weighted-voting algorithm with the highest MCC that could predict NPSLE from other autoimmune neurological diseases with 94.59% accuracy. We examined the correlations of cytokines/chemokines/growth factors in NPSLE, MS and NMO, and we found that many cytokines/chemokines/growth factors were operating together in NPSLE compared to MS and NMO. The determination of various types of CSF cytokine profiles may contribute to the diagnosis of NPSLE and may help elucidate the mechanisms underlying this disease.

Conflict of interest

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2015.01.010.

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Direct Infection of Primary Salivary Gland Epithelial Cells by Human T Lymphotropic Virus Type I in Patients With Sjögren's Syndrome

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Objective. **To investigate whether human T lymphotropic virus type I (HTLV-I) directly infects salivary gland epithelial cells (SGECs) and induces the niche of the salivary glands in patients with Sjögren's syndrome (SS).**

Methods. **SGECs were cultured with the HTLV-I–producing CD4+ T cell line HCT-5 or with Jurkat cells. Antibody arrays, immunofluorescence analysis, and enzyme-linked immunosorbent assay (ELISA) were used to determine the profiles of inflammation-related molecules, and the profiles of apoptosis-related molecules were determined by antibody array and immunofluorescence analysis. The presence of HTLV-I–related molecules was assessed by immunofluorescence analysis and in situ polymerase chain reaction. Apoptosis of SGECs was evaluated by TUNEL staining.**

Results. **Among the SGECs, 7.8 ± 1.3% (mean ± SD) were positive for HTLV-I–related proteins after 96-hour coculture with HCT-5 cells. Nuclear NF-κB p65 was also detected in 10% of the SGECs. The presence of HTLV-I proviral DNA in SGECs after coculture with HCT-5 cells was detected by in situ polymerase chain reaction. After coculture of SGECs with HCT-5, the expression of cytokines and chemokines, including soluble intercellular adhesion molecule 1, RANTES, and interferon γ–induced protein 10 kd (IP-10/CXCL10) was increased in a time-dependent manner. The expression of proapoptotic molecules (e.g., cytochrome c and Fas) and antiapoptotic molecules (e.g., Bcl-2, Heme oxygenase 2, and Hsp27) was increased in the SGECs cocultured with HCT-5, showing that apoptosis of SGECs was not detected after coculture with HCT-5 or Jurkat cells.**

Conclusion. **HTLV-I is thought to infect SGECs and alter their cellular functions. These changes may induce the niche of SS and contribute to the development of SS in anti–HTLV-I antibody–positive individuals.**

Human T lymphotropic virus type I (HTLV-I) has been reported to be involved in the pathogenesis of primary Sjögren's syndrome (SS) in endemic areas, including Nagasaki City, Japan (1–3). The extremely high prevalence of SS among patients with HTLV-I–associated myelopathy (HAM) appears to confirm a strong relationship between HTLV-I infection and SS (4–6). A previous study by our group also revealed the clinical characteristics of anti–HTLV-I antibody–positive SS patients and showed that the labial salivary glands (LSGs) of such patients are not destructible compared with the LSGs of anti–HTLV-I antibody–negative patients with SS (7). In addition, the low prevalence of ectopic germinal centers (GCs) as well as the low expression of CXCL13 in infiltrating mononuclear cells in LSGs were shown to be immunohistologic characteristics of anti–HTLV-I antibody–positive patients with SS (8).

HTLV-I preferentially infects T cells, especially CD4+ T cells, and the observations described above indicate that the T cell lineage may primarily contribute

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