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PAPER

Serum milk fat globule epidermal growth factor 8 elevation may subdivide systemic lupus erythematosus into two pathophysiologically distinct subsets

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Objective: Impaired clearance of apoptotic cells is a potential trigger of systemic lupus erythematosus (SLE). Milk fat globule epidermal growth factor 8 (MFG-E8) plays an important role in the clearance of dying cells. Previously, we reported serum MFG-E8 was elevated in some SLE patients. Here we further investigated the prevalence of MFG-E8 in active SLE and other autoimmune diseases and also tried to clarify the characteristics of MFG-E8-positive and -negative SLE. **Methods:** Serum MFG-E8 was measured in 40 active non-treated SLE patients, 104 disease controls and 104 healthy controls by ELISA. Clinical characteristics and serum cytokine profiles were compared between MFG-E8-positive and MFG-E8-negative SLE patients. **Results:** Prevalence of MFG-E8 was significantly higher in SLE patients (40%) than in various controls ($p < 0.05$). MFG-E8 level became negative after treatment, and increased again upon relapse. When compared, MFG-E8-positive SLE patients showed higher immune complex ($p = 0.021$) and lower complement ($p = 0.004$ for CH50). In contrast, MFG-E8-negative SLE patients tended to show higher CRP ($p = 0.094$). There was a positive correlation between MFG-E8 level and immune complex level ($r_s = 0.49$, $p = 0.049$). TNF-α ($p = 0.019$), IFN-γ ($p = 0.031$) and IL-10 ($p = 0.013$) were significantly higher in MFG-E8-positive SLE. **Conclusion:** MFG-E8-positive SLE and -negative SLE may have different clinical features, the one with stronger immunological response and the other with stronger inflammatory response, and those two groups may be two distinct subtypes of SLE driven by different mechanisms. Further, MFG-E8 could be used as a biomarker for diagnosis and monitoring of disease activity in certain SLE patients. *Lupus* (2014) **23,** 386–394.

Key words: Systemic lupus erythematosus; milk fat globule epidermal growth factor 8; subset; apoptosis

Introduction

Systemic lupus erythematosus (SLE) is a multi-organ inflammatory disease characterized by autoantibody production and immune complex deposition. The pathogenesis of SLE is not fully understood and is believed to be multifactorial. Impaired clearance of apoptotic cells is one of the potential triggers of SLE.[1]

In healthy people, dying cells are promptly cleared by phagocytes and rarely observed in vivo.[2] This rapid process is essential to avoid inflammation and maintain self-tolerance. If apoptotic cells are not removed efficiently, they may undergo secondary necrosis and release toxic or immunogenic intracellular materials, which may trigger autoimmunity.[3] In fact, decreased phagocytic activity and accumulation of apoptotic cells have been reported in some SLE patients.[4–8]

Milk fat globule epidermal growth factor 8 (MFG-E8) is a glycoprotein that plays an important role in the clearance of dying cells. It has an epidermal growth factor (EGF)-like domain that

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binds to the integrin on phagocytes at the N-terminus, and C1 and C2 domains that bind to phosphatidylserine on apoptotic cells at the C-terminus. In the process of apoptosis, dying cells expose internal phosphatidylserine on their surface as an "eat me" signal. MFG-E8 is secreted by activated macrophages or immature dendritic cells,[9,10] and acts as a bridging molecule between apoptotic cells and phagocytes, which promotes engulfment of apoptotic cells.[9] It is reported that there is an optimal concentration of MFG-E8 to promote phagocytosis. The activity of phagocytosis increases dose-dependently at low concentration, but above the optimal concentration, the effect changes toward inhibition. This can be explained by the hypothesis that excess MFG-E8 saturates the binding sites on apoptotic cells and phagocytes, and thus cannot work as a bridging molecule.[11] In this way, an excess as well as a deficiency of MFG-E8 causes impairment of apoptotic cell clearance and may induce autoimmune disease. This is supported by the finding that both MFG-E8–/– mice and mice injected with a large amount of MFG-E8 showed autoantibody production.[12,13] Previously, we reported that serum MFG-E8 was elevated in 17 of 172 adult SLE patients and 16 of 72 childhood SLE patients.[11] However, their clinical characteristics have not been assessed yet and the sera were from a mixture of active and inactive SLE patients. In this study, we further investigated the precise prevalence of MFG-E8 in active SLE and other autoimmune diseases and also tried to clarify the clinical and immunological characteristics of MFG-E8-positive and -negative SLE.

Patients and methods

Patients

We retrospectively studied 40 new-onset active SLE patients who were admitted to Kyoto University Hospital from March 2002 to April 2010. There was no overlap between patients in this study and those in our previous report.[11] All patients were diagnosed with SLE according to the American College of Rheumatology revised criteria.[14] No patients were pregnant or lactating. No patients had active infection. All sera ($n = 40$) were collected before treatment. As for MFG-E8-positive patients ($n = 16$), sera were also collected after treatment for re-evaluation. Sera from patients with other connective tissue diseases including rheumatoid arthritis (RA) ($n = 24$), polymyositis/dermatomyositis (PM/DM) ($n = 24$), systemic sclerosis (SSc) ($n = 20$), Sjögren's syndrome (SS) ($n = 20$) and vasculitis syndrome ($n = 16$), and healthy individuals ($n = 104$) were used as controls. All sera were kept at –20°C until analysis. This study was approved by the ethics committee of our institution, and the principles of the Helsinki Declaration were followed throughout the study. Informed consent was obtained from all participants.

Data collection

Demographic, clinical and serological information was obtained from medical records. Clinical manifestation was defined according to the American College of Rheumatology revised criteria.[14] Renal pathology was classified according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS).[15] Anti-double-stranded-DNA antibody (anti-dsDNA antibody) was detected by radioimmunoassay. Immune complex (IC) was detected by C1q solid-phase assay. Disease activity of SLE was evaluated by SLE Disease Activity Index (SLEDAI).[16] Remission of SLE was defined as absence of clinical manifestation and stabilized immunological activity. Relapse of SLE was defined as recurrence of disease activity that required intensification of treatment.

Measurement of serum MFG-E8

Serum MFG-E8 levels were measured by indirect sandwich enzyme-linked immunosorbent assay (ELISA) as previously described.[11] Briefly, the ELISA plate was coated with 1 µg/well of mouse anti-hMFG-E8 mAb (clone 1-H3) in 50 mM sodium bicarbonate buffer (pH 9.6), followed by blocking with Reagent Diluent Concentrate 2 (R&D Systems, Minneapolis, MN, USA). Fifty µl/well of serum samples diluted 1:10 in phosphate-buffered saline (PBS) containing 1% Triton-X 100 was added in duplicate and incubated for five hours at room temperature. The wells were then washed four times with PBS-Tween (T). In order to detect MFG-E8, 50 µl/well of 1500 × diluted biotinylated hamster mAb (clone 2-8E4A) was added, and incubated for one hour at room temperature. The wells were washed four times again, and incubated with 50 µl/well of 1500 × diluted alkaline phosphatase-conjugated streptavidin (Dako, Glostrup, Denmark) for 30 minutes at room temperature. After four additional washes, the color reaction was developed by Bluephos substrate (KPL, Gaithersburg, MD, USA). The absorbance values were determined at 620 nm. Recombinant human MFG-E8 diluted with 10% healthy donor serum was used to prepare

the standard curve. The cut-off value was set at 7.5 ng/ml so that the positivity in the healthy control samples became <5%.

Measurement of cytokine

Serum cytokine levels (interferon (IFN)-α, IFN-γ, interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-17A, soluble CD40 ligand (sCD-40L), tumor necrosis factor (TNF)-α, IL-21) were detected by multiplex bead array technology using Procarta® Cytokine Profiling Kits (Panomics, CA, USA). Serum IL-18 and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels were measured by ELISA using Human IL-18 ELISA Kit (MBL Co., Ltd., Nagoya, Japan) and Human GM-CSF ELISA Kit (Thermo Scientific, Wilmington, DE, USA).

Statistical analysis

Statistical analysis was performed using SPSS (Chicago, IL, USA). Mann-Whitney *U* test and the chi-square test were used for group comparisons. Spearman's correlation test was used to analyze correlations. Association with disease activity was analyzed using the Wilcoxon signed-rank test. A value of $p < 0.05$ was considered statistically significant. Since this study aimed at exploring the differences between MFG-E8-positive and -negative populations, we did not apply correction of multiple testing.

Results

Prevalence of serum MFG-E8 in various collagen vascular diseases

We measured MFG-E8 concentration in the sera of 40 active SLE patients before treatment as well as other collagen vascular diseases in active or nontreated conditions. We found that 16 patients (40%) out of 40 nontreated SLE patients were positive for MFG-E8, whereas the positive rates of MFG-E8 in other collagen vascular diseases were much lower than that in SLE (Figure 1). The specificity of serum MFG-E8 for SLE was 94.2% when various collagen vascular diseases were used as controls.

Characteristics of MFG-E8-positive SLE

The full list of MFG-E8 concentration and clinical characteristics of SLE at the onset of disease are shown in Table 1. Comparisons of serological manifestations between MFG-E8-positive and

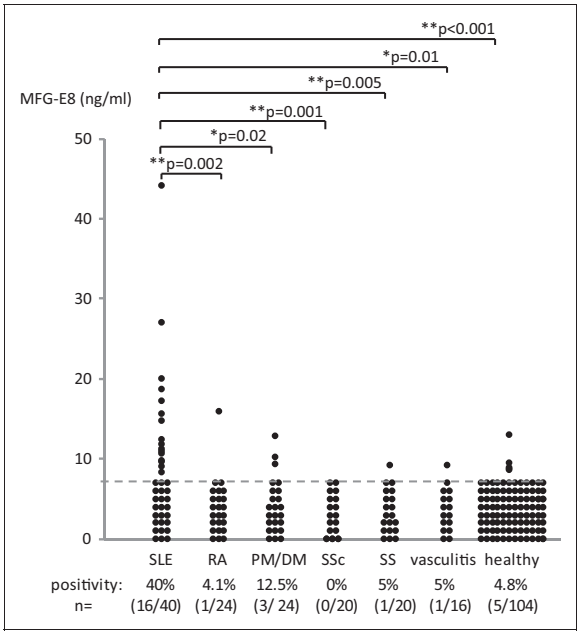

Figure 1 Prevalence of serum MFG-E8 concentration in SLE patients and various controls. The serum MFG-E8 concentrations were measured in various collagen vascular diseases of active state including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), polymyositis/dermatomyositis (PM/DM), systemic sclerosis (SSc), Sjögren's syndrome (SS), vasculitis syndrome as well as in healthy controls. The positivity of MFG-E8 in each disease is shown under the graph. The dotted line indicates the cut-off value. $*p < 0.05$, $**p < 0.01$. MFG-E8: Milk fat globule epidermal growth factor 8.

MFG-E8-negative SLE patients are shown in Table 2. MFG-E8-positive patients showed significantly higher levels of IC ($p = 0.021$) and lower levels of C3 ($p = 0.016$), C4 ($p = 0.008$) and total hemolytic complement activity (CH50) ($p = 0.004$) than MFG-E8-negative patients. Anti-dsDNA antibody titer also tended to be higher ($p = 0.116$) in MFG-E8-positive patients, although no significant differences were found in antinuclear antibodies (ANA) titer. In contrast, C-reactive protein (CRP) level tended to be higher in MFG-E8-negative patients ($p = 0.094$). Therefore, it seems that MFG-E8-positive SLE patients show stronger immunological responses, such as high IC and low complement, and MFG-E8-negative SLE patients show more of an inflammatory response, such as high CRP. However, clinically, organ involvement profile, SLEDAI and treatment needed to achieve remission and relapse rate were not significantly different between the groups (Supplementary Table 1). Next, we analyzed the association between MFG-E8 level and some disease activity markers. As shown in Figure 2, there was a positive correlation between MFG-E8 level

Table 1 Serum MFG-E8 level and characteristics of SLE patients

				Serological marker							*Organ involvement*								*treatment*[c]	
	Patient	*Age /sex*	*MFG-E8 (ng/ml)*	*C3 (mg/dl)*	*C4 (mg/dl)*	*CH50 (U/ml)*	*IC (μg/ml)*	*ANA*	*Anti-dsDNA Ab (U/ml)*	*CRP (mg/dl)*	*Rash*	*Arthritis*	*Cytopenia*[a]	*Serositis*	*Nephritis*[b] *(class)*	*CNS*	*SLEDAI*	*PSL (mg/day)*	*IS*
	1	28/F	44.2	56.1	7.0	23.5	30.4	1:1280	65	0.1	–	+	+	–	+	–	8	50	
	2	24/F	27.1	17.4	<2.0	<7.0	>50.0	1:320	1243	0.0	–	+	+	–	+	+	27	50	
	3	23/F	20.0	49.4	<2.0	9.6	21.7	1:160	95	0.0	–	+	–	–	–	–	6	44	
	4	27/F	18.7	15.7	<2.0	<7.0	11.4	1:2560	1274	1.2	+	+	+	–	–	–	14	50	
	5	25/F	17.2	38.2	3.3	15.4	9.8	1:640	83	0.0	–	–	+	–	+ (IV+V)	–	19	50	
	6	27/F	15.7	13.5	<2.0	<7.0	>50.0	1:320	228	0.0	+	–	+	–	+ (III+IV)	–	25	50	
MFG-E8	7	43/F	14.8	48.4	<2.0	11.5	6.3	1:320	129	0.0	+	–	+	–	+	–	24	55	MZB
positive	8	17/F	12.5	42.8	3.2	9.5	15.1	N/D	51	0.1	+	+	+	–	–	–	16	20	
($n = 16$)	9	36/F	11.8	34.3	<2.0	<7.0	2.7	1:2560	35	1.4	+	–	+	+	+	–	23	60	IVCY
	10	25/F	11.2	103.4	27.2	41.9	<1.5	1:1280	3	9.9	+	+	+	–	–	–	7	30	
	11	30/F	10.9	28.5	<2.0	<7.0	>50.0	1:1280	168	0.1	+	+	–	–	–	–	10	10	
	12	28/F	10.7	34.4	<2.0	7.1	23.4	1:640	230	0.0	–	+	+	–	–	+	13	45	IVCY
	13	32/F	9.8	51.3	9.8	23.7	1.8	1:160	17	0.0	+	+	+	–	–	–	9	20	
	14	21/M	9.7	86.7	26.8	44.3	3.6	1:2560	4	1.1	+	-	+	+	–	–	7	55	
	15	16/F	9.0	26.1	2.5	12.1	8.2	1:640	170	0.0	+	+	+	–	–	–	11	20	
	16	21/F	8.3	19.4	3.1	<7.0	9.1	1:320	1100	0.2	+	+	+	–	+	–	19	55	
	17	23/F	<6.0	38.2	16.6	34.2	<1.5	1:1280	17	0.0	+	–	+	–	–	–	19	25	
	18	15/F	<6.0	50.5	6.6	17.6	<1.5	N/D	36	0.0	+	–	+	–	–	+	13	55	
	19	24/F	<6.0	55.2	8.4	22.1	3.2	1:640	27	0.1	+	–	–	–	–	+	14	30	
	20	17/F	<6.0	45.1	4.1	15.0	11.7	1:2560	172	1.3	+	+	–	–	–	–	17	35	
	21	41/F	<6.0	71.4	13.8	32.8	10.4	1:80	8	0.0	–	+	–	–	+ (IV+V)	–	13	60	IVCY
	22	66/M	<6.0	81.5	29.0	45.6	<1.5	1:1280	15	10.8	–	–	+	+	+	–	12	60	
	23	19/F	<6.0	23.4	<2.0	<7.0	31.2	1:1280	184	0.6	+	+	+	–	–	–	10	30	
	24	48/F	<6.0	55.4	9.0	25.9	5.3	1:80	26	0.0	+	–	+	–	+ (III)	–	20	60	
	25	44/M	<6.0	140.3	31.1	53.3	<1.5	1:320	2	9.2	–	+	+	–	+	–	22	40	
	26	15/F	<6.0	119.7	19.2	45.4	1.9	1:320	14	3.8	+	+	+	–	+	–	10	60	TAC
MFG-E8	27	28/F	<6.0	68.2	16.1	38.8	3.3	1:2560	21	1.6	–	+	–	–	–	–	10	20	
negative	28	29/F	<6.0	38.7	2.4	15.2	26.5	1:1280	108	1.9	–	+	–	+	+	–	12	40	
(n=24)	29	16/F	<6.0	61.7	19.1	32.0	2.2	1:640	59	0.3	+	–	+	–	–	–	18	25	
	30	21/F	<6.0	28.7	2.0	<7.0	9.4	1:1280	400	0.1	+	+	+	–	+ (II)	–	5	20	
	31	22/F	<6.0	38.5	4.1	12.8	14.2	N/D	254	0.0	-	-	+	+	+ (IV)	–	21	60	MZB
	32	18/F	<6.0	25.4	2.4	8.9	7.0	1:640	243	0.0	+	+	+	–	+ (IV)	–	19	55	TAC
	33	20/F	<6.0	32.1	<2.0	7.2	16.1	1:1280	47	0.1	+	+	+	–	–	–	9	20	TAC
	34	42/F	<6.0	50.6	15.1	30.6	<1.5	1:1280	12	2.7	+	+	+	+	–	–	6	55	IVCY
	35	26/M	<6.0	66.9	15.9	37.5	3.2	1:1280	12	0.2	+	–	+	–	+ (V)	–	8	70	TAC
	36	35/F	<6.0	114.8	25.3	45.2	<1.5	1:640	54	5.6	–	+	+	–	–	–	3	15	
	37	33/F	<6.0	51.5	8.6	31.3	5.9	1:1280	60	0.4	–	+	+	–	–	–	10	15	

(continued)

Table 1 Continued

Patient	*Age /sex*	*MFG-E8 (ng/ml)*	*Serological marker*					*Organ involvement*							*treatment*[c]	
			C3 (mg/dl)	*C4 (mg/dl)*	*CH50 (U/ml)*	*IC (μg/ml)*	*ANA*	*Anti-dsDNA Ab (U/ml)*	*CRP (mg/dl)*	*Rash*	*Arthritis*	*Cytopenia*[a]	*Serositis*	*Nephritis*[b] *(class)*	*CNS*	*SLEDAI*	*PSL (mg/day)*	*IS*
38	28/F	<6.0	85.1	14.7	38.7	6.6	1:320	27	0.0	+	+	+	–	–	–	6	30	
39	40/F	<6.0	67.7	9.6	34.1	N/D	1:320	560	3.8	–	+	+	–	–	–	9	25	
40	37/M	<6.0	65.1	7.3	33.3	1.8	1:640	3	0.4	+	+	+	–	–	–	6	20	

[a]White blood cells<4000/μl, lymphocytes<1500/μl, hemolytic anemia, or platelets<100,000/μl. [b]Persistent proteinurea>0.5 g/day or >3+ when quantification was not performed, or existence of cellular casts. Histology of ISN/RPS is shown in parentheses. [c]Maximum dose of prednisolone and immunosuppressant usage to induce remission.
SLE: systemic lupus erythematosus; N/D: no data available; F: female; M: male; MFG-E8: milk fat globule epidermal growth factor 8 (normal range: <6 ng/ml); C3: complement component 3 (normal range: 70.5-125.6 mg/dl);
C4: complement component 4 (normal range: 10.6–33.0 mg/dl), CH50: total hemolytic complement activity (normal range: 28–51 U/l); IC: immune complex (normal range: <3.0 μg/ml);
ANA: antinuclear antibody (normal range: <1:40); anti-dsDNA Ab: anti-double-stranded-DNA antibody (<6 U/ml); CRP: C-reactive protein (normal range: <0.2 mg/dl);
CNS: central nervous system involvement; SLEDAI: SLE Disease Activity Index; PSL: prednisolone; IS: immunosuppressant; MZB: mizoribine; IVCY: intravenous cyclophosphamide; TAC: tacrolimus.

Table 2 Comparison of serological manifestations between MFG-E8-positive and -negative SLE patients

Serological manifestations	*MFG-E8 positive (n = 16)*	*MFG-E8 negative (n = 24)*	*values*
C3, median (range)	36.3 (13.5–103.4)	55.3 (23.4–140.3)	0.016[a]
C4, median (range)	2.3 (<2.0–27.2)	9.3 (<2.0–31.1)	0.008[b]
CH50, median (range)	9.6 (<7.0–44.3)	31.7 (<7.0–53.3)	0.004[b]
IC, median (range)	10.6 (<1.5–>50)	3.3 (<1.5–31.2)	0.021[a]
ANA, median (range)	1:640 (1:160–1:2560)	1:960 (1:80–1:2560)	0.750
anti-dsDNA Ab, median (range)	112 (3–1274)	42 (2–560)	0.116
CRP, median (range)	0.05 (0.0–9.9)	0.35 (0.0–10.8)	0.094

MFG-E8: milk fat globule epidermal growth factor 8; SLE: systemic lupus erythematosus; CH50: total hemolytic complement activity; IC: immune complex; ANA: antinuclear antibodies; anti-dsDNA Ab: anti-double-stranded DNA antibodies; CRP: C reactive protein. [a]$p < 0.05$, [b]$p < 0.01$.

and IC level ($r_s = 0.49$, $p = 0.049$). However, MFG-E8 level did not significantly correlate with other parameters, such as C3, C4, CH50, anti-dsDNA antibody, CRP (Figure 2) and SLEDAI (Figure 3(a)).

MFG-E8 level correlates with disease activity of SLE

We wondered whether MFG-E8 level correlates with disease activity along with the treatment. All of the 40 patients had active disease before treatment and were treated with prednisolone (PSL) with or without immunosuppressant. All patients achieved remission, and relapse was observed in 13 patients.

After treatment (median follow-up period was 41.5 months (ranging from 10 to 105 months)), MFG-E8 level significantly decreased in all MFG-E8-positive patients ($p = 0.001$) (Figure 3(b)). Typical clinical course and the MFG-E8 level of patient No. 1 are shown in Figure 3(c). Clinical manifestations of SLE improved soon after initiation of corticosteroid treatment. MFG-E8 level gradually decreased in parallel with anti-dsDNA antibody titer and IC level, which was accompanied by gradual increase of CH50.

In cases of disease relapse, three of four patients showed high MFG-E8 level again. As an example, the clinical course and the MFG-E8 level of patient No. 3 are shown in Figure 3(d). We found that MFG-E8 decreased once along with the clinical improvement and increased again upon relapse. When this patient finally achieved remission, MFG-E8 decreased again along with the clinical improvement (not shown). The level of

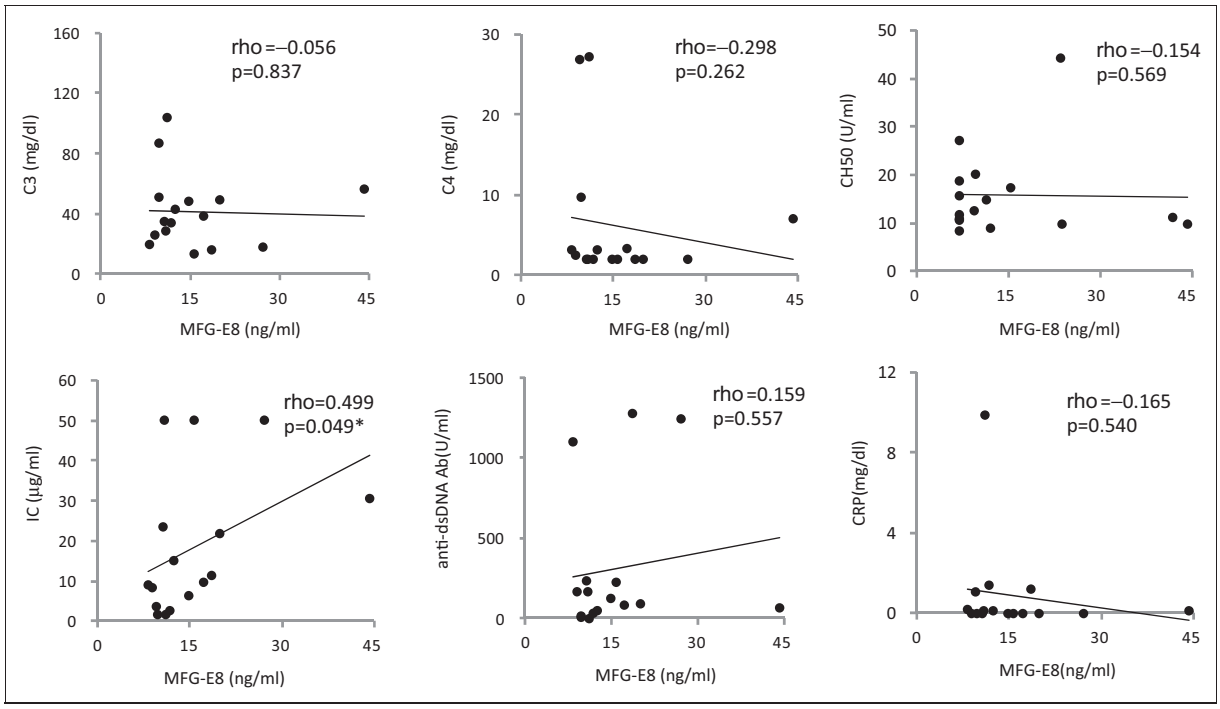

Figure 2 Correlation between serum MFG-E8 level and serological markers. The serum MFG-E8 concentration and either the level of C3, C4, CH50, immune complex (IC), anti-dsDNA antibody or CRP at the disease onset are plotted. Regression line, correlation coefficient and p value are shown in each graph. *$p < 0.05$.
MFG-E8: milk fat globule epidermal growth factor 8; CH50: total hemolytic complement activity; anti-dsDNA; anti-double-stranded DNA; CRP: C-reactive protein.

anti-dsDNA antibody, IC and CH50 also reflected the disease activity.

Comparison of serum cytokine profile between MFG-E8-positive and -negative SLE patients

Since it seems that MFG-E8 may classify SLE into immunological reaction-dominant type and inflammatory reaction-dominant type, we measured various cytokines in the sera before treatment to determine whether specific proinflammatory cytokines are more dominant in the MFG-E8-negative subset. Representative cytokine profiles are shown in Figure 4 and other cytokines are shown in Supplementary Figure 1. Contrary to our hypothesis, most proinflammatory cytokines as well as anti-inflammatory cytokines tended to be higher in MFG-E8-positive patients. In particular, TNF-α ($p = 0.019$), IFN-γ ($p = 0.031$) and IL-10 ($p = 0.013$) were significantly higher in MFG-E8-positive patients. There were no statistically significant differences in other cytokines, such as IFN-α, IL-1β, IL-6, IL-17A, sCD-40L, IL-21, IL-18 and GM-CSF. No cytokine levels correlated with MFG-E8 titer (data not shown).

Discussion

We revealed that serum MFG-E8 was elevated in 40% of active SLE patients, which was more frequent than in our previous report.[11] This is probably due to the different proportion of active disease because serum MFG-E8 decreases after treatment as shown in this report. We also revealed that MFG-E8-positive SLE and -negative SLE have different clinical features: MFG-E8-positive SLE showed significantly stronger immunological response, such as high IC concentration and low complement, whereas MFG-E8-negative SLE tended to show more of an inflammatory response, such as high CRP level. This is quite interesting because such different characteristics may reflect different pathological mechanisms.

We also found that MFG-E8 level correlated with disease activity. Serum MFG-E8 significantly decreased after remission and increased upon relapse. When we followed two patients, MFG-E8 level seemed to move in parallel with other conventional parameters (anti-dsDNA antibody,

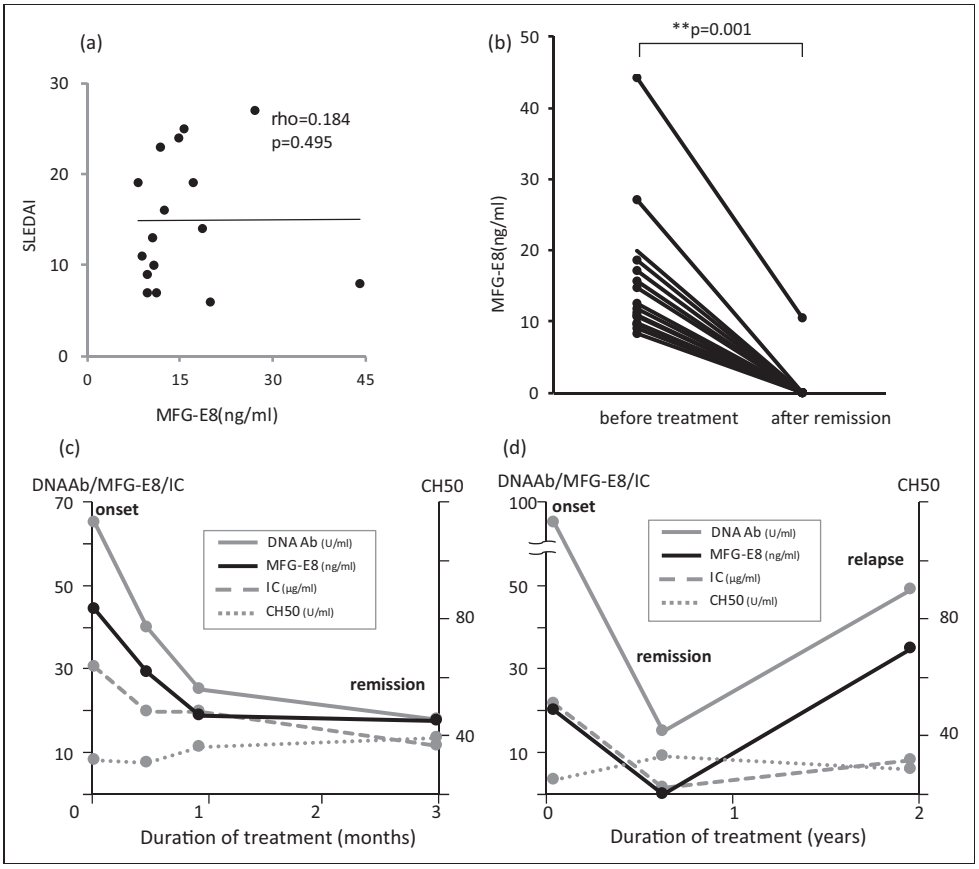

Figure 3 (a) Correlation between serum MFG-E8 level and SLEDAI in 40 active SLE patients before treatment. (b) Comparison of serum MFG-E8 level before and after treatment. $^{**}p < 0.01$. (c) Clinical course of patient No. 1: Clinical manifestation of SLE improved soon after initiation of prednisolone. MFG-E8 level gradually decreased in parallel with anti-dsDNA antibody titer and immune complex (IC) level, which was accompanied by gradual increase in CH50. DNA Ab: anti-dsDNA antibody. (d) Clinical course of patient No. 3. MFG-E8 decreased once along with the clinical improvement and increased again upon relapse. The titer of anti-dsDNA antibody, IC and CH50 also reflected the disease activity.
MFG-E8: milk fat globule epidermal growth factor 8; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; SLE: systemic lupus erythematosus; anti-dsDNA: anti-double-stranded DNA; CH50: total hemolytic complement activity.

IC and CH50). These findings indicate that serum MFG-E8 level is associated with immunological and disease activity in a subset of SLE patients, and may be useful as a disease activity marker in such cases.

The close association of MFG-E8 level with disease activity suggests the possible involvement of MFG-E8 in the pathogenesis of SLE. We have already shown that an excess level of MFG-E8 inhibited phagocytosis of apoptotic cells in an in vitro study and repeated injection of MFG-E8 caused autoantibody production in the mouse model.[11,13] Since serum MFG-E8 was usually negative in healthy controls, it is possible that MFG-E8-positive SLE patients have excessive amounts of MFG-E8 in circulation and/or tissues. In such cases, apoptotic cells may not be removed efficiently and subsequent leakage of autoantigen may trigger strong autoimmune reaction including autoantibody production, IC formation and complement consumption.

It is not clear why MFG-E8 is elevated in some SLE patients, but there are several possibilities. Previously, we reported a mutation of MFG-E8 in 2 out of 322 SLE patients, which caused aberrant splicing leading to the truncated form of MFG-E8. When the mutant MFG-E8 was injected into mice, it remained longer in the circulation and induced autoantibody production at a lower dose than the wild-type MFG-E8.[17] Another group showed that a single nucleotide polymorphism (SNP) in the coding sequence of MFG-E8 correlated with SLE.[18] Although we could not replicate the result in our cohort (data not shown), rare variants of MFG-E8 gene or polymorphisms in the regulatory region of MFG-E8 might be influencing

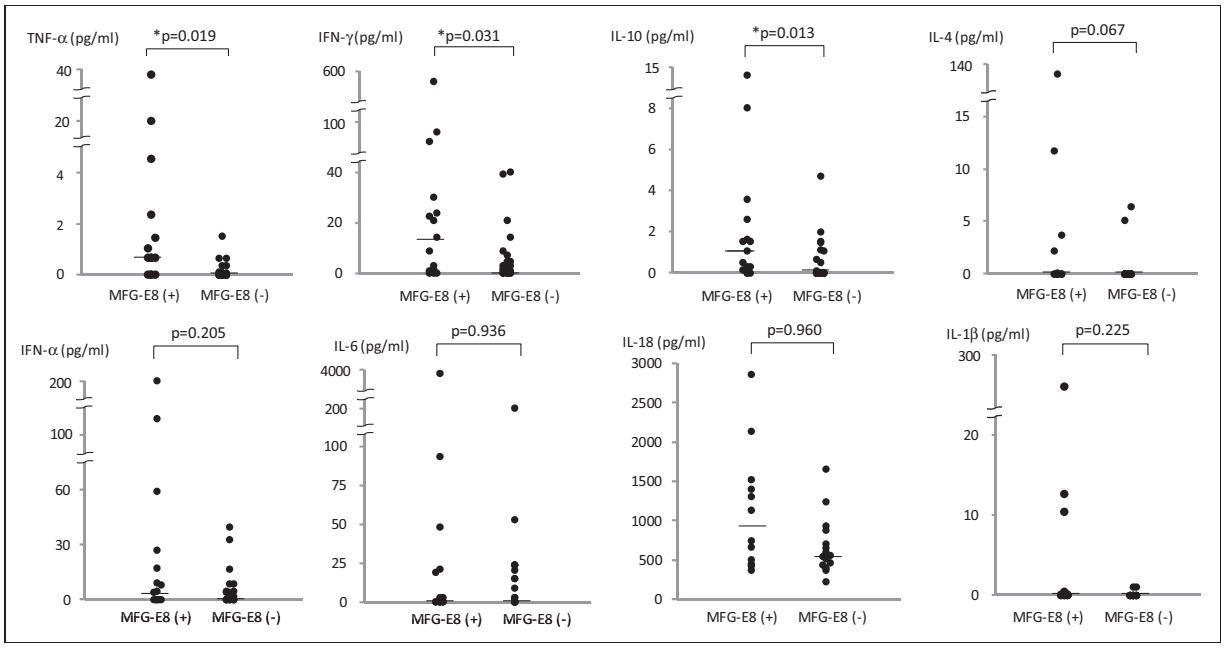

Figure 4 Comparison of serum cytokine level between MFG-E8-positive and -negative SLE patients. Various cytokine levels were measured in the sera of 16 MFG-E8-positive and 24 MFG-E8-negative SLE patients before treatment, and plotted in each graph. The horizontal lines indicate the median value. $*p < 0.05$.
MFG-E8: milk fat globule epidermal growth factor 8; SLE: systemic lupus erythematosus.

the MFG-E8 level. It is also possible that MFG-E8 elevation is a secondary effect of inflammation or immune reaction in SLE. Since we previously showed that a large amount of MFG-E8 was produced in thioglycollate-elicited peritoneal macrophages and GM-CSF and IL-18 induced MFG-E8 gene expression in macrophages, a certain type of inflammation may cause MFG-E8 production.[9,11] In this study, we could not find any differences in serum GM-CSF and IL-18 concentration between the two subsets; however, serum TNF-α, IL-10 and IFN-γ were significantly higher in MFG-E8-positive patients than in MFG-E8-negative patients. These results did not classify each subset into typical Th1, Th2 or Th17 phenotypes and may indicate overall activation of the immune system in MFG-E8-positive patients. Hormonal imbalance in SLE patients is another possible cause, because it is reported that prolactin up-regulated MFG-E8 expression in macrophages and enhanced engulfment of apoptotic cells.[19] The mechanism of MFG-E8 elevation may be multifactorial and further investigation is needed.

Although there appeared to be clear distinct characteristics in MFG-E8-positive and -negative SLE subsets, there were some exceptional cases. For example, patient No. 10 in the MFG-E8-positive subset showed high CRP and low immunological activity. She had a unique organ involvement, mastitis. Since MFG-E8 is expressed in the mammary gland, serum MFG-E8 elevation may be derived from mastitis in her case, although no MFG-E8 data are available for mastitis. On the other hand, some patients in the MFG-E8-negative subset, for example, No. 23 and No. 28, showed markedly high IC and quite low complement. These patients might have extremely low levels of MFG-E8, which could cause the similar phenotype to MFG-E8-positive patients, or they might have defects in other molecules responsible for apoptotic cell clearance, such as C1q, Mer and Tim4.[20–25] The defects in such molecules should be examined in the future.

In conclusion, MFG-E8-positive SLE and -negative SLE seem to have different clinical features, the one with stronger immunological response and the other with stronger inflammatory response, and these two groups may be two distinct subtypes of SLE driven by different mechanisms. Further investigation on the pathological mechanisms of MFG-E8 elevation and its clinical utility in SLE patients is needed.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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