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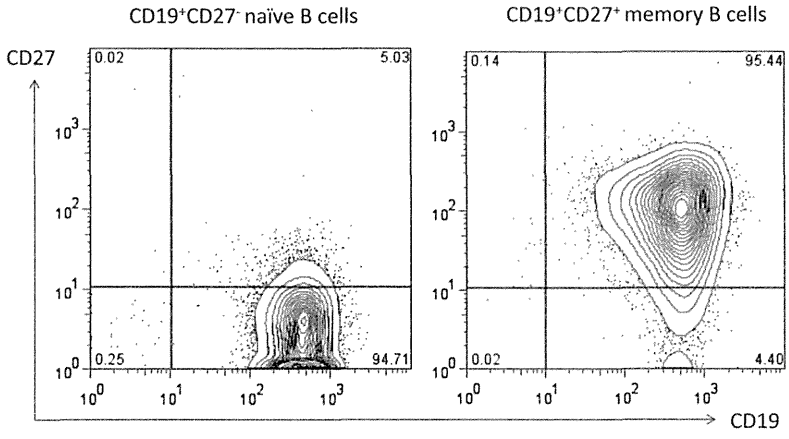


**FIG E1.** Phenotypic analysis of B-cell subsets in human peripheral blood. B cells were obtained by means of negative selection from PBMCs. $CD27^+$ memory B cells were then isolated by using positive selection from B cells with CD27 microbeads. The negative fraction of this isolation was assigned to $CD27^-$ naive B cells. The purity of naive and memory B cells was greater than 90% (*x-axis*, CD19; *y-axis*, CD27).

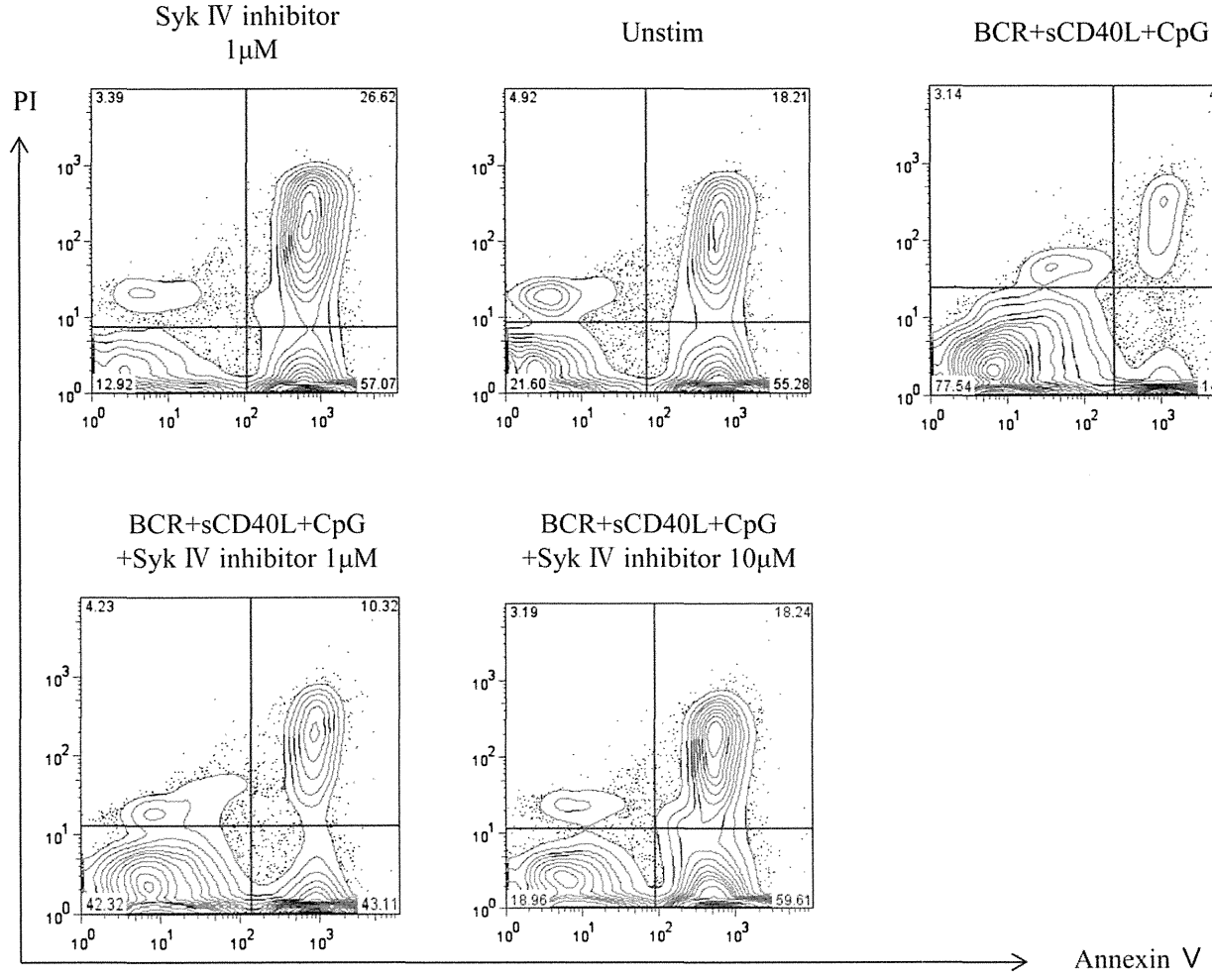


**FIG E2.** Syk provides survival signals for B cells after stimulation through all 3 receptors. B cells ($2 \times 10^5$ per well) were cultured in triplicate in 96-well plates with anti-Igλ and anti-Igk antibodies (1 μg/mL), soluble CD40 ligand (*sCD40L*; 2 μg/mL), and CpG-ODN 2006 (2.5 μg/mL) with or without Syk inhibitor IV for 72 hours. The percentage of apoptotic B cells was assessed by means of double-staining with FITC–Annexin V and PI (*x-axis*, PI; *y-axis*, Annexin V).

# B cell or T cell-dominant recurrence after rituximab therapy in patients with SLE

Systemic lupus erythematosus (SLE) is an autoimmune disease induced by autoreactive T cell activation and B cell autoantibody overproduction. The efficacy of rituximab in refractory SLE has been documented, although some patients show partial response only.[1–9] We report here two patients with SLE who showed T cell-dominant flare-up and two others who showed B cell-dependent flare-up, after long-term remission induced by rituximab administered at 375 mg/m$^2$ twice/week.

Rituximab rapidly depleted peripheral naive and memory B cells in patients with SLE. Patients with prolonged remission had persistent depletion of memory B cells for >2 years, whereas recovery of naive B cells occurred within 3–9 months. The expression levels of CD80 on B cells diminished rapidly and remained downregulated. Furthermore, CD69 and ICOS (inducible T-cell co-stimulator) expression levels on CD4+ T cells also decreased and remained at low levels.[10]

B cell-dominant recurrence occurred in two patients, who were concurrently positive for anti-ds-DNA antibodies and extractable nuclear antigen, with lupus nephritis (class II) before treatment (patients 2 and 3, table 1). Unlike patients with prolonged remission,[10] our patients had markedly high CD19+IgD memory B cells and overexpression of CD80 on CD19+ cells just before recurrence, with positive conversion of serum anti-ds-DNA antibodies and increased proteinuria (figure 1A and changes in Systemic Lupus Erythematosus Disease Activity Index and *British Isles Lupus Assessment Group Activity Index*, table 1). In contrast, no phenotypic changes were observed in T cells. Accordingly, the patients were re-treated with rituximab, which reduced CD19+IgD- B cells and anti-ds-DNA antibody to undetectable levels, and successfully controlled disease activity.

In contrast, a T cell-dominant recurrence was noted in two patients with negative anti-ds-DNA antibodies and extractable nuclear antigen. Patient 1 presented with fever, polyarthritis, lymphadenopathy and acute confusional state. Patient 4 had autoimmune haemolytic anaemia before treatment (table 1). In both patients, the signs and symptoms noted at initial presentation became evident again at recurrence after prolonged remission. Different from the previous two patients, there was neither an increase in memory B cells nor change in CD80 expression on B cells. Unlike patients with long-term remission [10], marked increases were noted in the number of CD4+CD45RO+ memory T cells and the expression levels of ICOS and CD69 on CD4+ T cells (figure 1B). Treatment commenced with intravenous cyclophosphamide pulse therapy and tacrolimus, which resulted in improvement of disease activity and peripheral CD4+ T cell abnormalities.

In conclusion, we experienced two patients with B cell-dominant recurrence and two patients with T cell-dominant one. SLE is known as a highly heterogeneous disease. Based on these results, rituximab-based B cell depletion therapy might expose the hidden B cell- or T cell-dependency during the SLE disease process. The phenotypic changes suggest that B cell- or T cell memory might be re-driven at recurrence after long-term remission. Thus, the phenotypic differences between B and

**Table 1** Characteristics of patients with systemic lupus erythematosus who showed a flare-up after remission by RTX

| Patient | Age/ sex | Disease duration (months) | Treatment prior to RTX | Initial major organ involvement | Anti-dsDNA antibody (IU/ml) | ENA | C3/ C4/ CH50 | ANAs | Latency to relapse (years) | SLEDAI (day 0→6 months→ at relapse) | BILAG (day 0→6 months→ at relapse) | Phenotype of lymphocytes at flare |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 29/F | 6 | CS, IVCY | Lymphadenopathy CNS | 5.4 (13.0) | | 114/ 18/31 | 320 | 2 | 9→0→17 | 12→0→21 | Persistently low memory B cells, high number of memory T cells and expression levels of ICOS |
| 2 | 32/F | 108 | CS, IVCY, CsA AZA, PE | Nephritis (IV) | 52.3 (18.9) | Ro, Sm, RNP | 90/ 15/39 | 640 | 3 | 16→0→20 | 17→0→9 | High number of memory B cells and expression level of CD80. Persistently low expression levels of CD69 and ICOS |
| 3 | 16/F | 30 | CS, IVCY MZ, CsA | Nephritis (IV) | 610.7 (159.6) | - Ro - | 54/8/ 25 | 320 | 1.5 | 13→0→10 | 23→0→13 | High number of memory B cells and expression levels of CD80. Persistently low expression levels of CD69 and ICOS |
| 4 | 19/F | 74 | CS, IVCY, PE AZA, TAC | AIHA | 7.9 (5.0) | | 54/ <5/9 | 80 | 1 | 4→0→2 | 4→0→13 | No recovery of B cells, high number of memory T cells and expression levels of CD69 |

AIHA, autoimmune haemolytic anaemia; ANAs, antinuclear antibodies; AZA, azathioprine; BILAG, British Isles Lupus Assessment Group Activity Index; CNS, central nervous system; CsA, cyclosporin; CS, prednisolone (or equivalent); ENA, extractable nuclear antigen; ICOS, inducible T-cell co-stimulator; IV, intravenous; IVCY, intravenous cyclophosphamide pulse therapy; MZ, mizoribine; PE, plasma exchange; RTX, rituximab; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; TAC, tacrolimus.

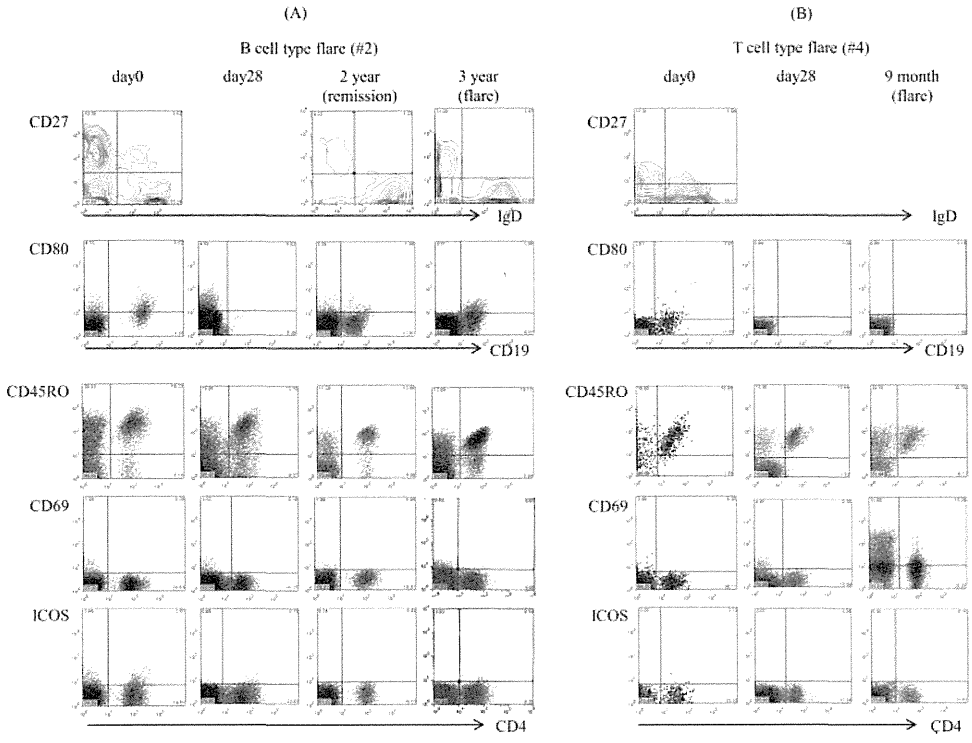


**Figure 1** (A) B and T cell surface antigen expression levels before and 28 days after treatment, and at the time relapse of systemic lupus erythematosus, 3 years after treatment, in patient 2 with B cell-dominant relapse. (1) Changes in CD19+$^{+}$ cell subsets (abscissa: IgD, ordinate: CD27), in the numbers of naive B cells (IgD$^{+}$CD27$^{-}$), memory B cells (IgD$^{-}$CD27$^{+}$ class switched memory B cells and IgD$^{-}$CD27$^{-}$ double negative memory B cells) and plasma cells (IgD$^{-}$CD27$^{high}$). (2) Changes in the expression level of CD80 (costimulatory molecule expressed on CD19$^{+}$ cells). (3) Changes in CD4$^{+}$ cell subsets (*abscissa*: CD4; *ordinate*: CD45RO; changes in numbers of naive T cells and memory T cells). (4) Changes in the expression levels of CD69 and ICOS, which are costimulatory molecules expressed on CD4+ cells. (B) B and T cell surface antigen expression levels before and 28 days after rituximab treatment, and at the time of relapse, 9 months after treatment, in patient 4 who showed T cell-dominant relapse.

T cells after rituximab therapy could partly explain the heterogeneity of SLE. The results also indicate that differential targeting therapies should be considered according to such heterogeneity. However, further analysis of a large sample of patients is needed.

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# Serum immune complex containing thrombospondin-1: a novel biomarker for early rheumatoid arthritis

The diagnosis of rheumatoid arthritis (RA) is based on classification criteria set by the 2010 RA classification criteria including serological assessment of rheumatoid factor (RF) and anticitrulline-containing protein/peptide (anti-CCP) antibody.[1 2] Anti-CCP antibody is specific (94–99%) for RA; however, 25% of patients with established RA and 40% of patients with early RA are negative for this marker.[3 4] Novel biomarkers, especially for early RA and/or for RA lacking RF and anti-CCP antibody markers (ie, seronegative RA) are therefore urgently required. Circulating immune complexes (CICs) present in the human

body are likely to contain many different antigens that may reflect underlying disease, so antigens incorporated into CICs are promising candidates for diagnostic biomarkers. We developed a novel proteomic strategy (immune complexome analysis) to identify and profile antigens in CICs and used this method to analyse CICs in patients with established RA and controls (healthy donors and patients with osteoarthritis).[5] CIC-associated thrombospondin-1 (TSP-1) was found in 81% and CIC-associated platelet factor 4 (PF4) in 52% of patients with established RA, but neither protein was found in CICs from any of the controls.[5] Both proteins are known as endogenous inhibitors of angiogenesis[6–8]; the formation of CICs may promote angiogenesis. We evaluated the diagnostic potential of CIC-associated TSP-1 and CIC-associated PF4 in patients with early RA divided into seropositive and seronegative groups.

Serum samples were collected from 25 disease-modifying antirheumatic drug (DMARD)-naïve seropositive patients with early RA (mean±SD age 52.8±18.4 years; 21 women; disease duration 0.25–12 months; CRP 0.01–8.55 mg/dl) and 15 seronegative patients with early RA (mean±SD age 60.5±17.9 years; 8 women; disease duration 1–6 months; CRP 0.02–14.4 mg/dl) at Nagasaki University Hospital. All the seropositive patients were positive for RF and 20 were positive for anti-CCP antibody, while all the seronegative patients were negative for both RF and anti-CCP antibody. The diagnosis of RA was made by the 2010 RA classification criteria as well as administration of DMARDs within the first 12 months.[1 2] Serum samples from 16 patients with Sjögren's syndrome (SS) (mean±SD age 60.9±13.0 years) and 14 patients with systemic lupus erythematosus (SLE) (mean±SD age 42.6±12.4 years) who fulfilled the international criteria for the diagnosis of SS[9] and SLE[10] and 11 healthy donors (mean±SD age 49.5±10.3 years) were used as controls. CICs purified by magnetic beads with immobilised protein G were reduced and alkylated, followed by tryptic digestion. The peptide mixture (1 μl) was subjected to nano-liquid chromatography/electrospray ionization/tandem mass spectrometry. More details of the analytical method can be found in our earlier report.[5]

As shown in table 1, CIC-associated TSP-1 was found only in patients with early RA and was not found in disease controls (patients with SS or SLE) or healthy donors (100% specific). Twenty-two (55%) of the total of 40 patients with early RA (56% (14/25) of the seropositive patients and 53% (8/15) of the seronegative patients) had CIC-associated TSP-1. PF4-containing CICs were found in only three patients (8%) with early RA compared with 52% of the patients with established RA.[5] These PF4-containing CICs may therefore promote disease progression.

In conclusion, we have shown that CIC-associated TSP-1 has high potential as a novel biomarker for diagnosing early and/or seronegative RA. Further analyses using a large number of patients are warranted to determine the clinical benefit of using this novel biomarker.

**Table 1** Number of patients with early RA carrying CIC-associated TSP-1 or CIC-associated PF4

| | Early RA patients (n=40) | | | | |
|---|---|---|---|---|---|
| | Seropositive (n=25) | Seronegative (n=15) | SS patients (n=16) | SLE patients (n=14) | Healthy donors (n=11) |
| TSP-1 | 14 | 8 | 0 | 0 | 0 |
| PF4 | 3 | 0 | 0 | 0 | 0 |

CIC, circulating immune complex; PF, platelet factor; RA, rheumatoid arthritis; SS, Sjögren's syndrome; SLE, systemic lupus erythematosus; TSP, thrombospondin.

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Original article

# The diagnostic utility of anti-melanoma differentiation-associated gene 5 antibody testing for predicting the prognosis of Japanese patients with DM

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## Abstract

**Objective.** Interstitial lung disease (ILD), especially rapidly progressive ILD (RPILD), is a major poor prognostic factor in patients with DM. We investigated the association of anti-melanoma differentiation-associated gene 5 (MDA5) antibody (Ab) with clinical characteristics and mortality in Japanese patients with DM.

**Methods.** Seventy-nine DM patients, comprising 58 classic DM and 21 clinically amyopathic DM (CADM) patients, were enrolled. Serum Abs were screened by immunoprecipitation assays, and an immunosorbent assay (ELISA) was used for MDA5. The relationships of clinical characteristics and mortality with each Ab were investigated.

**Results.** Anti-MDA5 Ab was detected in 17 patients. Anti-clinically amyopathic DM 140 kDa polypeptide Abs (anti-CADM-140 Abs) were found in 16 of the 17 anti-MDA5 $Ab^+$ patients. Skin ulcers, palmar papules, CADM, RPILD and mediastinal emphysema were widely distributed in anti-MDA5 $Ab^+$ patients. Mortality at 6 months as well as 5 years was also significantly higher in anti-MDA5 $Ab^+$ patients than in anti-MDA5 $Ab^-$ patients. In a multivariable Cox regression analysis, mortality was independently associated with anti-MDA5 Ab (relative hazard 6.33; 95% CI 1.43, 28.0). All of the deaths in anti-MDA5 $Ab^+$ patients were attributed to respiratory failure of RPILD; however, RPILD did not worsen in any of the anti-MDA5 $Ab^+$ patients who survived the first 6 months.

**Conclusion.** The presence of anti-MDA5 Ab identifies the characteristic skin, musculoskeletal, pulmonary and prognostic features in patients with DM. In addition, anti-MDA5 Ab seems to predict a group of patients with CADM-complicated fatal RPILD.

**Key words:** anti-MDA5 Ab, CADM, RPILD.

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## Introduction

A number of autoantibodies can be detected in the sera of patients with DM, some of which are specific to DM and are known as myositis-specific autoantibodies (MSAs). Moreover, these autoantibodies are closely associated with clinical manifestations of DM, such as symptoms, complications, reactivity to therapy and prognosis [1].

In recent years, the autoantibodies found in patients with inflammatory myopathies have been mainly classified into several types by immunoprecipitation assays: anti-aminoacyl-tRNA synthetase antibodies (anti-ARS Abs), Abs to the signal recognition particle (anti-SRP Abs), anti-Mi2 Abs, PM/Scl-100 Abs and PM/Scl-75 polypeptides Abs (anti-PM-Scl Abs), anti-clinically amyopathic DM 140 kDa polypeptide Abs (anti-CADM-140 Abs), anti-155/140 kDa polypeptide Abs (anti-p155/140 Abs) and autoantibodies to a 142 kDa protein (anti-MJ Abs). These autoantibodies are strongly associated with the clinical presentation [2–6]. In this regard, we have reported a high frequency of rapidly progressive interstitial lung disease (RPILD) and clinically amyopathic DM (CADM) associated with anti-CADM-140 Abs [7, 8]. Recently RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) was identified as a major autoantigen in patients with CADM, which is targeted by anti-CADM-140 Abs [9, 10].

Gono *et al.* [11] have also recently reported that anti-MDA5 Ab predicts a fatal outcome in patients with DM combined with RPILD; however, the long-term prognosis and other clinical characteristics of anti-MDA5 Ab$^+$ DM patients remain to be elucidated. In the present study we have tried to investigate the clinical value of anti-MDA5 Ab for DM patients in a single cohort.

## Patients, materials and methods

### Patients

Sera samples were obtained from 79 patients with DM who were undergoing medical treatment at the Graduate School of Biomedical Sciences, Nagasaki University, from September 1999 to August 2010, and were stored at −20°C until use. Most of the sera samples were obtained at the first visit so the interval from initiation of therapy was minimal. We collected the data from all of the DM patients examined in our department. Twenty-one patients did not fulfill Bohan and Peter's criteria [12, 13] but fulfilled Sontheimer's criteria (CADM) [14, 15] because of the absence of clinical skeletal muscle symptoms and the presence of persistent clinical DM skin features. Clinical manifestations, laboratory data, radiographic data and the presence of internal malignancies were extracted from medical records and verified by T.K., N.I. and K.F. The patients were diagnosed with ILD according to the results of chest X-ray and high-resolution chest CT, reported by Japanese board-certified radiologists. All of the subjects underwent routine examination of internal malignancies and chest radiography. A subset of patients with RPILD was defined as those presenting with progressive dyspnoea and progressive hypoxaemia, and a worsening of interstitial change on chest radiography within 1 month from the onset of respiratory symptoms, as described previously [2]. A signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University, was obtained from each patient.

### Immunoprecipitation and ELISA

MSAs, including anti-CADM-140 Abs, anti-ARS Abs and anti-155/140 Abs, were detected by immunoprecipitation assays using extracts of leukaemia cell line K562, as described previously [3]. Interpretation of the results of immunoprecipitation was undertaken without knowledge of patients' clinical status. An ELISA system using recombinant MDA5 as an antigen source was performed as described previously [10]. All samples were examined in duplicate, and the Ab units were calculated from the optical density at 450 nm, using a standard curve obtained from serial concentrations of a serum sample containing a high titre of anti-CADM-140 Abs. The cut-off level was set at 8.0 U, based on 10 S.D. above the mean value obtained from 32 healthy control sera. Interpretation of the results of ELISA was undertaken without knowledge of the clinical status of the patients and the results of immunoprecipitation assays.

### Statistical analysis

Fisher's exact probability test and the Mann–Whitney U-test were used to compare the differences. We also examined the cumulative survival rates from the first visit to the hospital with DM-related symptoms up to 5 years by the multivariate Cox proportional hazard model adjusted for patient age at symptoms onset, gender, with or without CSs and with or without immunosuppresants. A $P < 0.05$ was considered significant.

## Results

### Clinical characteristics of anti-MDA5 Ab$^+$ patients

Table 1 summarizes the 17 DM patients with anti-MDA5 Ab and the 62 DM patients without anti-MDA5 Ab. There were 21 patients with CADM in the present study and we have found that anti-MDA5 Ab is detected in 14 of 21 patients. In this group, 11 of 14 (79%) patients had complicated RPILD and 7 (50%) patients died. Our present data confirm the recent publications regarding the characteristics of anti-MDA5 Ab$^+$ patients, including the CADM, RPILD, low CK, high ferritin and high mortality found in these patients [11]. Since anti-MDA5 Ab is mostly attributed to anti-CADM-140 Abs, a high prevalence of palmar papules and mediastinal emphysema, which has been reported as typical of anti-CADM-140 Abs$^+$ DM patients by our group [7], was also preferentially found in anti-MDA5 Ab$^+$ patients. The present finding that skin ulcers are highly prevalent in anti-MDA5 Ab$^+$ patients is new, however. Muscle biopsy or lung biopsy was not performed. Skin biopsies were taken from eight patients positive for anti-MDA5 Abs, and six patients were

**Table 1** Comparison of clinical manifestations between patients with anti-MDA5 Ab and patients without anti-MDA5 Ab

| Variable | Anti-MDA5 Ab | | |
|---|---|---|---|
| | Positive ($n=17$) | Negative ($n=62$) | *P*-value |
| Age at onset, years | 55.5 (13.0) | 55.3 (15.0) | 0.27 |
| Female, *n* (%) | 15 (88) | 37 (60) | 0.056 |
| Skeletal muscle and skin features | | | |
| Muscle weakness, *n* (%) | 4 (24) | 38 (62) | **0.005** |
| Gottron's sign, *n* (%) | 13 (76) | 32 (52) | 0.07 |
| Ulcer region, *n* (%) | 10 (59) | 7 (12) | **0.00007** |
| Heliotrope rash, *n* (%) | 8 (47) | 23 (39) | 0.56 |
| Palmar papules, *n* (%) | 11 (65) | 13 (22) | **0.0014** |
| Periungual erythema, *n* (%) | 10 (59) | 24 (41) | 0.2 |
| Clinical diagnosis | | | |
| CADM, *n* (%) | 14 (82) | 7 (11) | **$4.2 \times 10^{-9}$** |
| Pulmonary involvement and malignancy | | | |
| ILD, *n* (%) | 16 (94) | 37 (61) | **0.008** |
| RPILD, *n* (%) | 12 (71) | 4 (7) | **$9.8 \times 10^{-9}$** |
| Mediastinal emphysema, *n* (%) | 6 (35) | 1 (2) | **$2.1 \times 10^{-5}$** |
| Malignancies, *n* (%) | 0 (0) | 6 (10) | 0.17 |
| Laboratory data | | | |
| CPK, IU/l | 173 (53–468) | 905 (107–1607) | **0.00024** |
| KL-6, U/ml | 1361 (825–1903) | 1040 (345–1510) | 0.36 |
| Ferritin, ng/ml | 1365 (894–1751) | 180 (90–244) | **0.016** |
| Therapy | | | |
| Maximum PSL, mg/day | 40 (35–50) | 40 (22.5–50) | 0.99 |
| Immunosuppressant, *n* (%) | 16 (94) | 29 (47) | 0.17 |
| Outcome | | | |
| Death, *n* (%) | 7 (41) | 3 (5) | **$6.6 \times 10^{-6}$** |
| MSA profile | | | |
| Anti-140 Ab positive, *n* (%) | 16 (94) | 0 (0) | **$3.76 \times 10^{-15}$** |
| Anti-155/140 Ab positive, *n* (%) | 0 (0) | 7 (11) | 0.35 |
| Anti-ARS Ab positive, *n* (%) | 0 (0) | 30 (48) | **0.002** |
| Autoantibody negative | 1 (6) | 25 (40) | **0.005** |
| Anti-MDA5 Ab titre | 230 (22–448) | 1.3 (1.1–1.9) | **$1.62 \times 10^{-10}$** |

Ages are presented as mean (S.D.) values, while laboratory markers are medians (interquartile range). *P*-values were established using Fisher's exact test or the Mann–Whitney U-test. Bold indicates significant values. CPK: creatinine phosphokinase; PSL: prednisolone.

diagnosed pathologically with dermatitis consistent with DM. One patient revealed only mild mucin deposition, and another revealed only hyperpigmentation. A potential limitation of the present study is the fact that biopsies were taken from only a small number of patients. EMG was performed in one anti-MDA5 $Ab^+$ patient, revealing myogenic conversion consistent with myositis. Only one patient was found to have preceding ILDs among anti-MDA5 $Ab^+$ patients. Skin manifestations preceded ILDs in the other patients. We showed the typical images about mediastinal emphysema, palmar pustule and regional ulcers in anti-MDA5 $Ab^+$ patients with CADM (Fig. 1). In the frequency of cancer, anti-MDA5 $Ab^+$ patients have no malignancy (0/17), whereas 6 of 62 (10%) patients in anti-MDA5 $Ab^-$ group were complicated malignancies. Anti-155/140 Abs were found in all six patients with cancer. We confirmed the profile of autoantibodies regarding the presence or absence of anti-MDA 5 Ab: namely, all DM patients positive for anti-ARS Abs, anti-155/140 Abs and other types of autoantibodies were among the anti-MDA5 $Ab^-$ group. There was no overlap between anti-MDA5 Ab and any other types of autoantibodies. Immunoprecipitation of anti-CADM-140 Abs from patients with anti-MDA5 Ab is shown in Fig. 2.

### Survival rate of anti-MDA5$^+$ patients

Ten (12%) patients died within 5 years from the first treatment. The cumulative 6-month survival rates were 57.4 and 98.4% for DM with anti-MDA5 Ab and those without anti-MDA5 Ab, respectively (Fig. 3). The survival rates from the first visit to our hospital after adjusting for age, gender, with or without CSs and with or without immunosuppresants were significantly different between each subset ($P=0.0151$). The first visit to our hospital was almost identical to the diagnosis of each patient. The presence of anti-MDA5 Ab was independently associated with mortality (relative hazard 6.33; 95% CI 1.43, 28.0) in a multivariable Cox regression model that included patient age at onset, gender, with or without CSs and with or without immunosuppresants. We have tried to compare

FIG. 1 Typical clinical manifestations of patients with anti-MDA5 Ab. The palmar pustules (**A**) were mainly located near the MCP and PIP joints (arrows) and multiple ulcer regions (**B**) were also observed. Chest CT scan (**C**) shows mediastinal emphysema in the middle of the chest cavity (arrows).

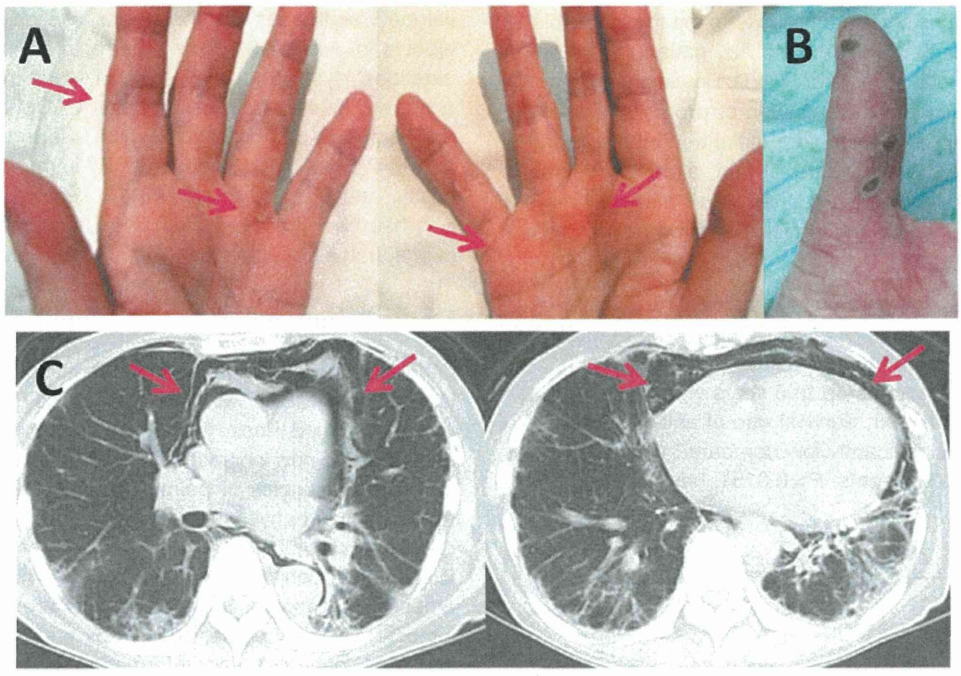
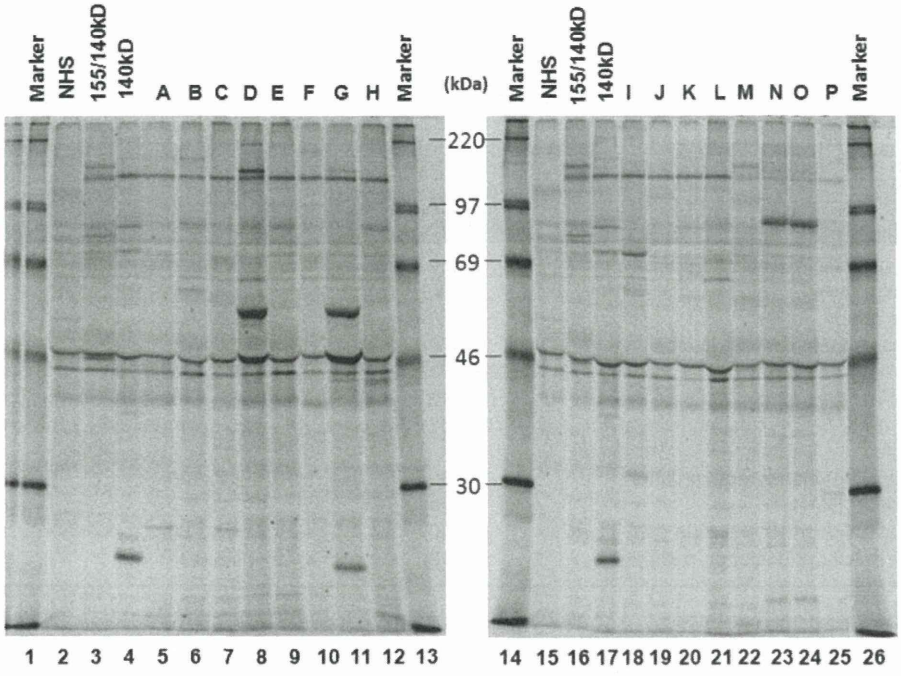
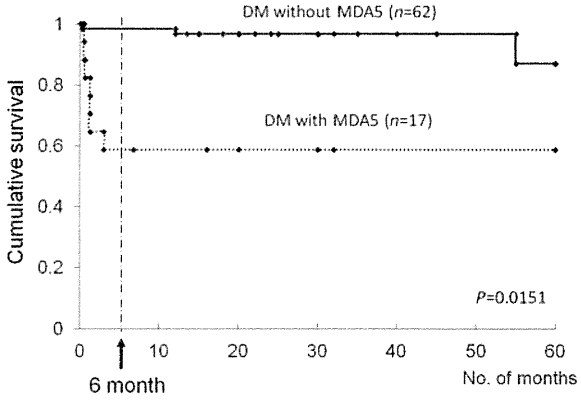



FIG. 2 Immunoprecipitation with anti-CADM-140 Ab from the 35S-labelled K562 cell extract. Lanes 5–12 and 18–25 show the results with anti-CADM-140-positive sera from DM patients with anti-MDA5 Ab$^+$ (A–P). The results of the prototype sera of anti-155/140 Abs and anti-CADM-140 Abs are also shown (lanes 3, 16 and 4, 17, respectively). One sera of an anti-MDA5 Ab$^+$ patient immunoprecipitated not anti-CADM-140 Abs, but anti-U1-RNP Ab, which was deleted from Fig. 2.



the variables within anti-MDA5 Ab$^+$ DM patients who were alive or dead and found that the regime of therapy was not different between two groups although the $PaO_2/FiO_2$ and serum CPK levels were higher in the former. The value of anti-MDA5 Ab is significantly lower in the former (Table 2). All the deaths in the anti-MDA5 Ab$^+$ patients were attributed to respiratory failure of RPILD. However, importantly, there was no acute exacerbation or progressive worsening of ILD by CT images after initial treatments in any of the anti-MDA5 Ab$^+$ patients. In fact, all of the deaths of anti-MDA5 Ab$^+$ patients occurred within the first 6 months (Fig. 3). In addition, no patient required home oxygen therapy after discharge among anti-MDA5 Ab$^+$ patients who were alive during the first 6 months. We showed a short case presentation describing a patient with CADM positive for anti-MDA5 Ab. A 60-year-old female developed erythemas on the upper eyelids, fingers and elbows in July 2005. Three months later she developed exertional dyspnoea. A CT scan revealed interstitial lung shadow (Fig. 4A). We measured anti-CADM-140 Ab levels and anti-MDA5 Ab levels, which were both positive (anti-140 kDa Abs were detected by immunoprecipitation assay, and the titre of anti-MDA5 Abs was 544.109 U). She has been treated at our outpatient department and is in a stable condition (Fig. 4B).

**Fig. 3** The adjusted cumulative survival rates in the presence or absence of anti-MDA5 Ab. The cumulative survival rates from the first visit to the hospital with DM-related symptoms up to 5 years were examined as described in the text. Survival rate of anti-MDA5 Ab$^+$ patients was significantly low compared with that of anti-MDA5 Ab$^-$ patients. $P = 0.0151$, between the two groups.




## Discussion

Other Japanese groups recently identified the characteristics of anti-MDA5 Ab$^+$ DM patients [11]. Our present data confirmed their findings. Additionally, we have shown some new characteristics of these patients, such as high frequencies of palmar papules, skin ulcers and mediastinal emphysema, as well as no overlapping of other types of autoantibodies. These data may help physicians to recognize features of anti-MDA5 Ab$^+$ patients among DM patients. Since physicians are urged to start intense immunosuppressive therapy early for anti-MDA5 Ab$^+$ DM patients, this information may be clinically indispensable.

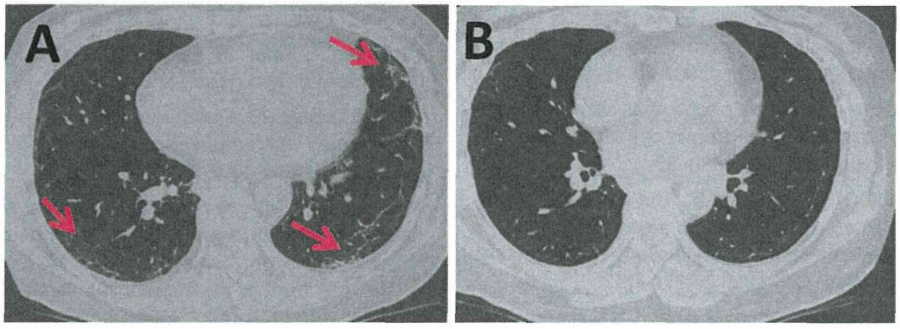
Although the prognosis of anti-MDA5 Ab$^+$ patients was worse than that of anti-MDA5 Ab$^-$ patients, none of the surviving anti-MDA5 Ab$^+$ patients experienced acute exacerbation or progressive worsening of ILD after the initial treatment. This is quite different from anti-MDA5 Ab$^-$ patients, since ILD recurred in several of these patients and death ensued during long-term follow-up (Fig. 3). One of the characteristics of anti-MDA5 Ab$^+$ patients is hyperferritinaemia [11, 16]. There are many

**Table 2** Comparison of clinical parameters between alive and dead anti-MDA5 Ab$^+$ patients

| Variable | Anti-MDA5 Ab positive ($n = 17$) | | |
|---|---|---|---|
| | Alive ($n = 10$) | Dead ($n = 7$) | *P*-value |
| Age at onset, years | 52 (42–58.5) | 59 (53–70) | 0.051 |
| Female, *n* (%) | 9 (90) | 6 (86) | 1.00 |
| Ulcer region, *n* (%) | 5 (50) | 5 (71) | 0.70 |
| Palmar papules, *n* (%) | 7 (70) | 5 (71) | 1.00 |
| CPK, IU/l | 208 (90.3–864) | 169 (33.5–359) | **0.014** |
| Anti-MDA5 Ab titre | 168 (16.3–436) | 230 (76.0–478) | **0.032** |
| $PaO_2/FiO_2$ before treatment, mmHg | 395 (370–462) | 203 (114–240) | **0.027** |
| Therapy | | | |
| Steroid pulse therapy, *n* (%) | 5 (50) | 7 (100) | 0.09 |
| CYC, *n* (%) | 4 (40) | 4 (57) | 0.84 |
| Oral calcinurin inhibitor, *n* (%) | 6 (60) | 7 (100) | 0.18 |
| I.V. calcinurin inhibitor, *n* (%) | 1 (10) | 3 (43) | 0.32 |

Ages are presented as mean (S.D.) values, while laboratory markers are medians (interquartile range). *P*-values were established using Fisher's exact test or the Mann–Whitney U-test. Bold indicates significant values. $PaO_2$: partial pressure of arterial oxygen; $FiO_2$: fractional inspired oxygen concentration.

FIG. 4 A chest CT scan before and after treatment. A reticular shadow was revealed in the lower lung field (**A**) and it improved 4 years after disease onset (**B**). Arrows indicate the region that improved with treatment.




reports evaluating hyperferritinaemia in patients with autoimmune diseases [17]. The highest ferritin levels in autoimmune disorders are typically seen in patients with macrophage activation syndrome (MAS), often associated with adult-onset Still's disease (AOSD) [18]. It is well known that many viruses produce double-stranded (ds) RNA that can be recognized by two major arms of the innate immune system: the toll-like receptors (TLRs) and the Rig-I-like receptors (RLRs). MDA5 is a member of the RLR family that recognizes dsRNA within the cytosolic compartment and induces the production of inflammatory cytokines and cell surface molecules involved in the anti-viral response [19]. Considering that MAS could be induced by various infectious agents [20], and given the critical role of MDA5 in innate immune defence against viruses, one hypothesis is that the production of anti-MDA5 Ab is an epiphenomenon during virus infection that is associated with the onset of CADM and RPILD; namely, infection of the skin and lung epithelium by certain viruses. In general, innate immune responses do not recur; therefore we have not found exacerbation of ILD during the follow-up periods of anti-MDA5 $Ab^+$ DM patients.

Most patients with ILD-complicated DM appear to be well controlled by CSs and immunosuppressants [21]. In contrast, patients with RPILD observed in DM were resistant to a variety of treatments [22, 23]. We have introduced CSs, cyclophosphamide and calcineurin inhibitor to anti-MDA5 $Ab^+$ patients with RPILD. We could not find any significant difference in therapy between alive and dead patients. $PaO_2/FiO_2$, serum CPK level and the value of anti-MDA5 Ab before treatment were prognostic factors. We showed the significance of the duration of preceding symptoms in patients positive for anti-MDA5 Abs. Although we do not have any definitive evidence, shorter duration of preceding symptoms to treatment could lead to better outcomes (supplementary Table 1, available as supplementary data at *Rheumatology* Online). Thus it is recommended that anti-MDA5 $Ab^+$ patients who have typical CADM with signs of ILD be treated promptly with the combination of CSs, cyclophosphamide and calcineurin inhibitor.

In conclusion, the measurement of anti-MDA5 Ab by ELISA enables us to predict the prognosis of patients with CADM-complicated fatal RPILD. The characteristics of anti-MDA5 $Ab^+$ DM patients could be explained by the nature of MDA5 in innate immune responses to viruses. A multicentre, prospective study is warranted to confirm our results.

**Rheumatology key messages**

- Anti-MDA5 Ab is associated with characteristic pulmonary and skin involvement in patients with DM.
- Anti-MDA5 Ab predicts patients with CADM complicated by RPILD.

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*Disclosure statement*: The authors have declared no conflicts of interest.

## Supplementary data

Supplementary data are available at *Rheumatology* Online.

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特集　キナーゼ阻害によるリウマチ性疾患の治療 —現在と未来—

# CaMKⅣ

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Key Words　全身性エリテマトーデス　CaMKⅣ　KN-93　Th17

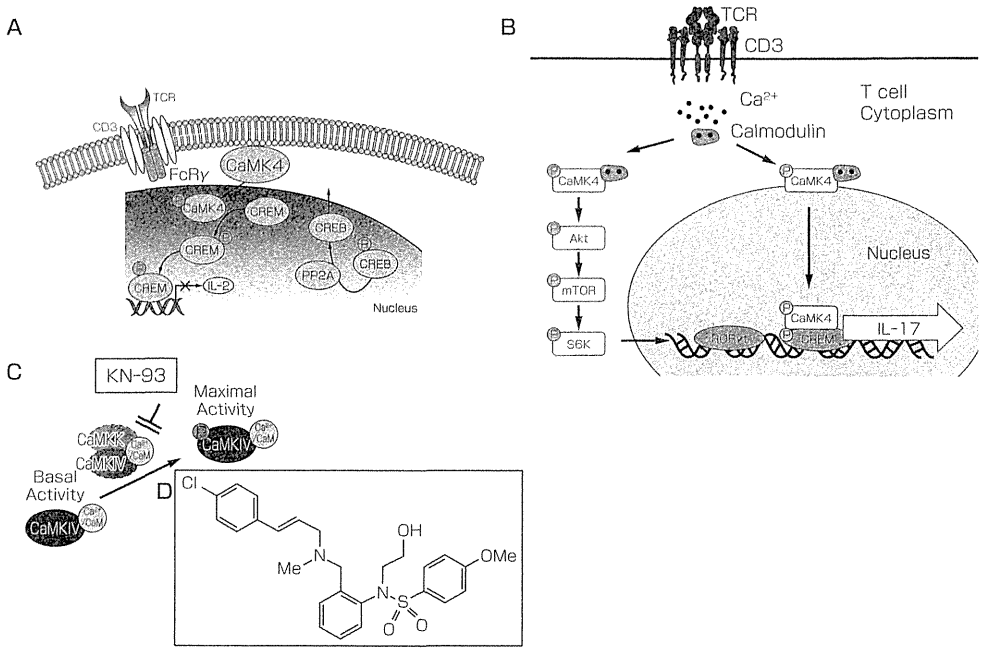
$Ca^{2+}$/calmodulin-dependent protein kinase Ⅳ（CaMKⅣ）はリンパ球，血球系前駆細胞，神経細胞，胸腺細胞，骨芽細胞などに広く発現しており，シグナル伝達物質として細胞の核内に移行し，さまざまな転写因子活性を有する．CaMKⅣ阻害薬であるKN-93はT細胞におけるIL-2やIL-17発現をコントロールし，全身性エリテマトーデス（SLE）などの自己免疫疾患の病態を制御する可能性がある．またT細胞だけではなく，抗原提示細胞や単球・マクロファージに類似した機能をもつ腎メサンギウム細胞，破骨細胞にも関与し，炎症や増殖を抑制することによって，さまざまな病態における治療的側面を担う可能性がある．

## はじめに

$Ca^{2+}$/calmodulin-dependent protein kinase Ⅳ（CaMKⅣ）は，リンパ球や脳などに多く存在しており，細胞内では核内に局在していることが知られている．CaMKⅣはcAMP response element modulator（CREM）などの転写因子をリン酸化することにより，カルシウム依存的に遺伝子の発現調節をおこなっている．最近のゲノムワイド関連研究（genome-wide association study：GWAS）ではリウマチ性疾患のなかでも全身性エリテマトーデス（systemic lupus erythematosus：SLE）はほかの多因子疾患と比較して，免疫系における機能を有する関連多型が多く見出されている[1]．SLEのT細胞の核内ではCaMKⅣの発現が増加しており，Interleukin（IL）-2を介したT細胞機能の異常を引き起こすことが報告されている．正常のT細胞のSLE患者血清で刺激をおこなうと，血清中の抗CD3抗体によりT細胞レセプター（T-Cell Receptor：TCR）を介してCaMKⅣが活性化され，核内に移行することが想定されている[2]．核内に移行したCaMKⅣはIL-2プロモーター領域の−180にあるCREのsiteでCREM$\alpha$をリン酸化しIL-2発現を低下させている（図❶A）．

一方，IL-17を産生するT helper（Th）17細胞は関節リウマチ，乾癬，SLEなどの自己免疫疾患の発症に関与することが知られている．そのなかでSLEではIL-17を産生するTh17細胞と制御性T細胞（Treg）のアンバランスが発症に関与していると考えられている．KogaらはCaMKⅣがCREM$\alpha$を介したIL-17産生をコントロールしながらTh17細胞の分化に関与することを報告し，Th17細胞におけるCaMKⅣ-CREM pathwayの役割を明らかにした[3]（図❶B）[4]．

またCaMKⅣはSLEのモデルマウスであるMRL/*lpr*マウスのT細胞でも発現が亢進している．われわれはCaMKⅣ阻害薬である低分子化合物KN-93（図❶C，D）




**図❶ CaMKIV（$Ca^{2+}$/calmodulin-dependent protein kinase IV）の細胞内シグナルにおける役割と CaMKIV 阻害薬 KN-93 の構造**

A，B：CaMKIV は $Ca^{2+}$/CaM 複合体の下流にある CaMKK を介しリン酸化を受け核内に移行する．つづいてリン酸化を受けた CREM は IL-2 を抑制，あるいは IL-17 産生を促進させる．また別の経路では AKT/mTOR signaling を活性化させ，Th17 細胞の分化に関与する（Koga T *et al*, 2014[3]/Crispin JC *et al*, 2010[4]より引用）．

C：KN-93 は CaM と競合的にはたらき，CaMKK による CaMKIV のリン酸化を阻害する（Racioppi L *et al*, 2008[7]より改変引用）．

D：KN-93 の化学式：$C_{26}H_{29}ClN_2O_4S$，分子量＝501.1（TOCRIS 社 http://www.tocris.com より引用）

を MRL/*lpr* マウスの腹腔内に投与することにより，腎炎進展抑制効果や疾患の活動性を調節することを報告した[5)6)]．これらのことから CaMKⅣをターゲットとしたキナーゼ阻害薬が SLE をはじめとする自己免疫疾患の治療薬として有用となりうることが示唆された．

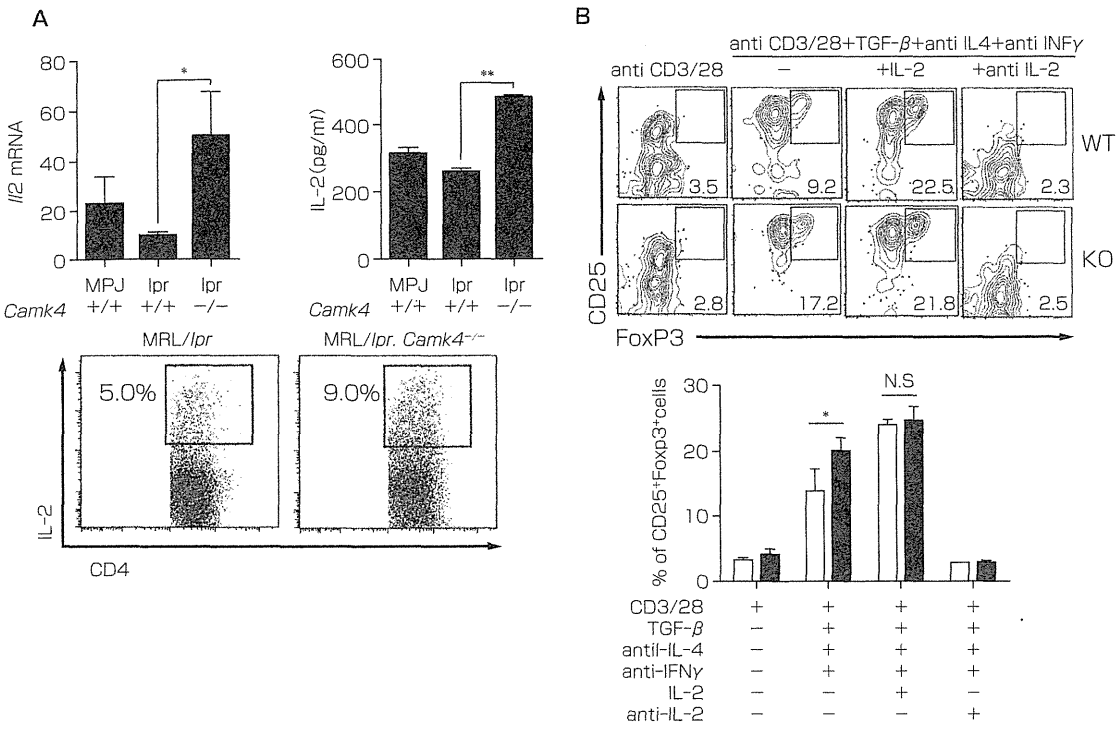
本稿では自己免疫疾患における CaMKⅣの役割および治療標的分子としての可能性について概説したい．

## 1．CaMKⅣ

$Ca^{2+}$シグナルは神経細胞，骨格筋細胞とともに免疫細胞でもセカンドメッセンジャーとして細胞増殖，分化，分泌，遊走などに重要な役割を担っている．カルモジュリン（calmodulin：CaM）は細胞内でカルシウムと結合するおもな蛋白で，$Ca^{2+}$/CaM 複合体を形成する．この複合体は $Ca^{2+}$/CaM 依存性セリン-スレオニンキナーゼ（CaMK）ファミリーである CaMKI，CaMKⅡ，CaMKⅣを活性化させる．そのなかで CaMKⅣは $Ca^{2+}$/CaM 複合体の下流にある CaMK キナーゼ（CaMKK）を介しリン酸化を受け核内に移行し，さまざまな転写因子に結合した結果，細胞の機能変化をもたらすものと考えられている．CaMKⅣはリンパ球や脳などに多く発現しており，血球系前駆細胞，神経細胞，胸腺細胞，骨芽細胞などで細胞の生存や分化に関与していることが報告されている[7)]．

免疫細胞では SLE 患者の T 細胞の核内で CaMKⅣの発現が亢進しており，IL-2 のプロモーター領域にある転写因子 CREM$\alpha$ のリン酸化を誘導し，IL-2 の発現抑制メカニズムが示された[2)]．さらに MRL/*lpr*.*Camkiv*$^{-/-}$マウスでは MRL/*lpr* マウスにくらべて IL-2 の発現が回復しており，Treg の発現を誘導した結果，SLE の病態改善効果をもたらしていることが示唆された[6)]（**図❷A，B**）．

免疫細胞以外においてもわれわれは MRL/*lpr* マウスにおける腎糸球体からメサンギウム細胞を単離し，



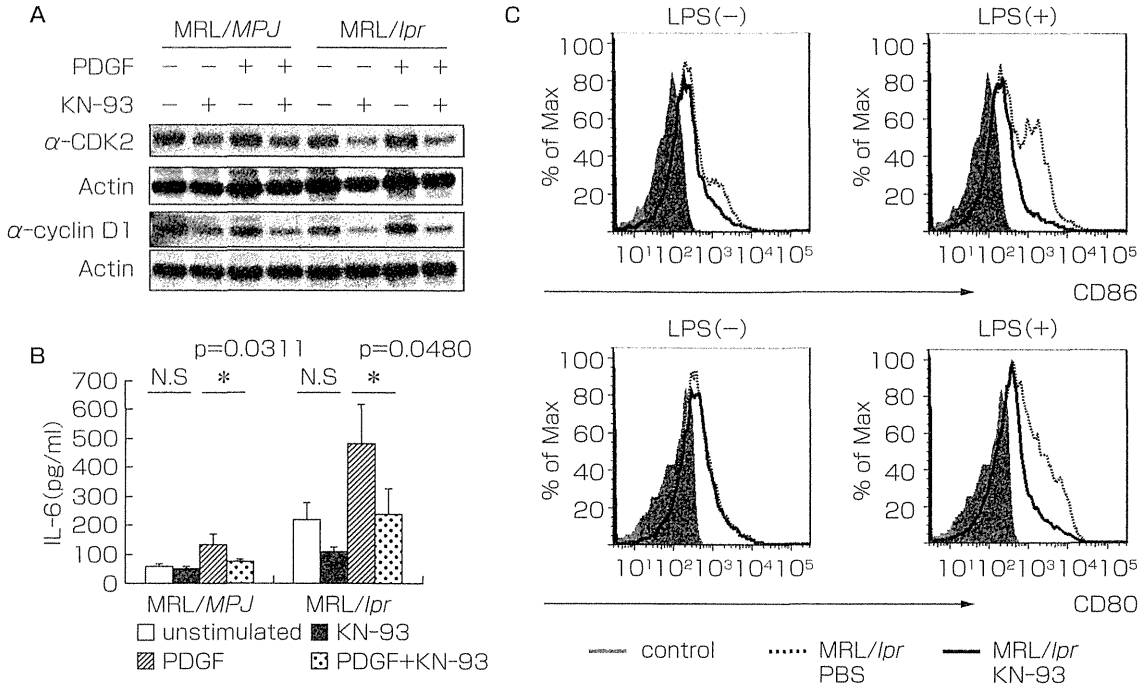

**図❷ CaMKIV は pCREM を介した IL-2 発現を制御する**
A：T 細胞由来の IL-2 発現は MRL/*lpr* マウス（WT）で抑制され，MRL/*lpr.camkiv*$^{-/-}$マウス（KO）でその発現が回復していた．B：MRL/*lpr.camkiv*$^{-/-}$マウスでは WT マウスにくらべ，IL-2 依存性に制御性 T 細胞（Treg）の発現を誘導した．（$^{*}p<0.05$，$^{**}p<0.01$）
（Koga T *et al*, 2012[6]より引用）

CaMKⅣを介した増殖能，サイトカイン産生能を検討した．その結果 MRL/*lpr* マウス由来のメサンギウム細胞では CaMKⅣ発現が亢進しており，MRL/*lpr.Camkiv*$^{-/-}$マウスおよび KN-93 投与下では，c-Jun の発現制御を介してメサンギウム細胞の増殖能や IL-6 産生が抑制されていることが明らかとなった．これらの結果より，ループス腎炎におけるメサンギウム細胞の CaMKⅣ発現亢進が腎炎進展にも関与している可能性が考えられた[8]（**図❸A，B**）．

Sato らは破骨細胞形成の Receptor activator of NF-κB ligand（RANKL）シグナルのトランスクリプトーム解析を進め，カルシウムを介する転写制御シグナルに CaMKⅣが重要であることを報告した．RANKL 刺激により CaMKⅣとその下流で活性化された phosphorylated cyclic AMP response element binding protein（pCREB）が破骨細胞分化の必須転写因子である c-Fos の誘導を介して分化を制御し，*Camkiv*$^{-/-}$マウスでは wild type と比較して破骨細胞数が減少しており，それに伴って骨量が増加していることが明らかとなった．さらに *in vivo* の炎症性骨破壊モデルや卵巣摘出骨粗鬆症モデルにおいても，CaMKⅣ阻害薬は治療効果があるとされ，今後の新たな治療標的分子となりうることが示唆された[9]．

## 2．CaMKⅣ-CREM pathway

SLE では IL-2 プロモーター領域の－180 部位にある CRE への CREMα と CREB（cAMP response element-binding）の相互的な結合により，T 細胞における IL-2 発現を制御している．CREM は alternative splicing により多くの isoform に分けられるが，CREMα は IL-2 産生を抑制するはたらきがある．一方，もう一つの転写因子である CREB はリン酸化を受けると IL-2 の転写活性を亢進させるはたらきがある．SLE の T 細胞では CaMK




**図❸ CaMKIV 阻害薬 KN-93 による腎メサンギウム細胞増殖および MRL/*lpr* マウス B 細胞活性化の抑制**
A，B：KN-93 は MRL/*lpr* マウスの腎メサンギウム細胞において細胞増殖に関与する CDK-2，cyclin D1 の蛋白発現を抑制し，IL-6 産生を低下させた．これらの効果は血小板由来成長因子（PDGF）刺激で有意な差がみられた．C：KN-93 は MRL/*lpr* マウスにおける脾臓由来の $CD19^+$B 細胞では LPS 刺激下で CD80 および CD86 の発現が低下していた．
（Ichinose K *et al*, 2011[5]/Ichinose K *et al*, 2011[8]より引用）

Ⅳによってリン酸化された CREMα と Protein phosphatase 2A（PP2A）により脱リン酸化された CREB により IL-2 発現が抑制される[4)10)]（**図❶A**）．

一方，SLE では IL-17 を産生する Th17 細胞と制御性 T 細胞のアンバランスが発症に関与していると考えられている．Koga らは CaMKⅣが CREMα を介した IL-17 産生を制御し，Th17 細胞における CaMKⅣ-CREM pathway の役割を明らかにした．また同時に CaMKⅣは AKT/mTOR signaling を活性化させ Th17 細胞の分化に関与することを報告した[3)]（**図❶B**）．

## 3．CAMKⅣ-CD86，CD80 制御

抗原提示細胞としての B 細胞，マクロファージ，樹状細胞は T 細胞とかかわり，co-stimulatory pathway である CD80，CD86 などを介して T 細胞の自己抗原に対する免疫寛容状態の破綻により，interferon-γ（IFN-γ）や tumor necrosis factor α（TNF-α）などの炎症性サイトカインを誘導する．

Illario らは単球由来の樹状細胞の表面に発現している CD83 や CD86 がリポ多糖（lipopolysaccharide：LPS）刺激下で KN-93 により抑制されていることを報告した．この toll-like receptor 4（TLR4）によって活性化されるシグナルは pCREB，Bcl-2，Bcl-xL を介して CaMKⅣによって制御されており，樹状細胞の cell survival に寄与していることを明らかにした[11)]．

われわれは MRL/*lpr* マウスに CaMKⅣ阻害薬である KN-93 を 8 週齢および 12 週齢から 16 週齢まで腹腔内投与し，蛋白尿，血清サイトカイン発現，抗 ds-DNA 抗体価，脾臓，リンパ節における炎症性サイトカイン，表面抗原の発現変化についての検討をおこなった．8 週齢および 12 週齢から KN-93 を投与されたマウス（KN-93 投与群）では，リン酸緩衝生理食塩水（PBS）投与群にくらべ蛋白尿抑制と腎炎進展抑制効果が認められた．抗 ds-DNA 抗体価，IFN-γ や TNF-α などの炎症性サイトカイン発現は 8 週齢から投与開始された KN-93 投与群

マウスで有意に低下していた．脾臓由来のCD19$^+$ B細胞ではLPS刺激下でCD80およびCD86がKN-93投与群で低下していた[5]（**図❸C**）．以上の結果から，KN-93は抗原提示細胞とT細胞の相互作用を制御した結果，細胞増殖，炎症や抗体産生能を抑制し，ループス腎炎の治療に寄与する可能性がある．

## おわりに

CaMKⅣはT細胞だけではなく，抗原提示細胞や単球・マクロファージに類似した機能をもつ腎メサンギウム細胞，破骨細胞にも関与し，炎症や増殖を抑制することによって，さまざまな病態における治療的側面を担うことが期待される．今後はヒトへの臨床的応用に向けて，投与方法や薬剤の安全性について更なる検討をおこなう必要がある．

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