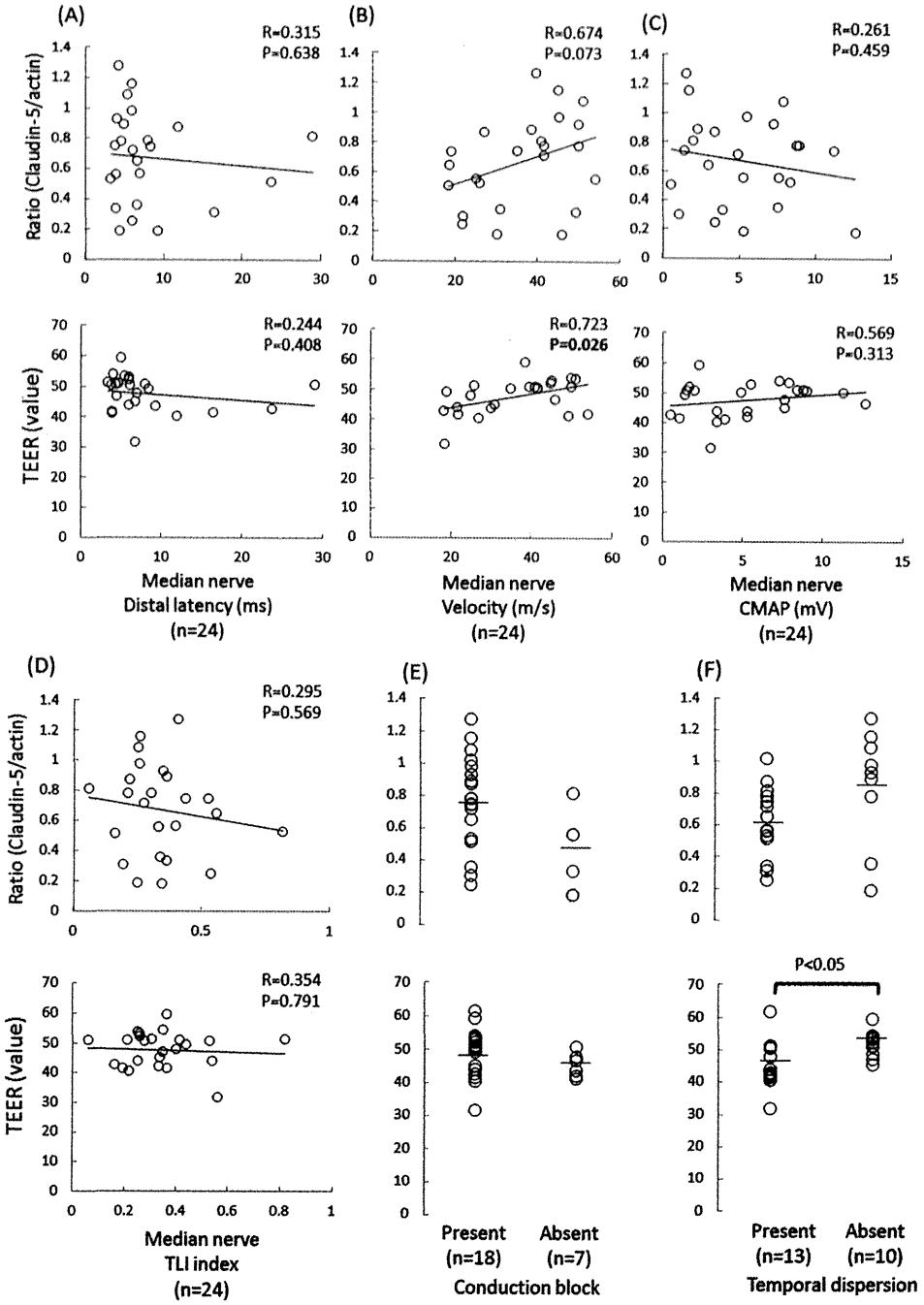

Figure 4. Correlation between the electrophysiological findings and BNB disruption in the patients with CIDP. Associations between the claudin-5 to actin protein ratios and the TEER values in the FH-BNBs following exposure to sera and the electrophysiological findings of the median nerve, including the distal nerve latency (A), conduction velocity (B), compound muscle action potential (CMAP) (C), terminal latency index (TLI index) (D) and presence of conduction block (E) or abnormal temporal dispersion (F) in the patients with CIDP. A lower TEER value was highly associated with slower motor nerve conduction and the presence of abnormal temporal dispersion.
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sera of patients with MADSAM or DADS. These results partly support the hypothesis suggested by the electrophysiological studies regarding the importance of BNB breakdown induced by humoral factors in t-CIDP sera.

We next examined the associations between the clinical, laboratory or electrophysiological findings and the degree of BNB damage following exposure to the sera obtained from the CIDP patients. Consequently, the severity of BNB damage after exposure to the sera significantly correlated with both a higher Hughes grade and lower MRC score, particularly in the iliopsoas muscle, which reflect the presence of clinical disability and proximal muscle weakness, respectively. Severe BNB breakdown was also found to be associated with a decrease in the speed of conduction in the median nerve in addition to abnormal temporal dispersion, thus indicating the presence of demyelination in the intermediate segments. Furthermore, this damage correlated with

an increased Q Alb value, which may reflect disruption of the BNB surrounding the nerve roots. Taken together, these findings suggest that the breakdown of the BNB induced by humoral factors in CIDP sera results in a wide range of symptoms of demyelination from the intermediate nerve trunk to the nerve root, and correlates with both clinical disability and proximal muscle weakness characteristics of t-CIDP. On the other hand, no associations were observed between impairment of the BNB and the duration of the disease or response to immunotherapy in the CIDP patients in our study. This finding suggests that BNB damage does not become more severe as the duration of disease increase, and that the extent of such damage cannot be used to predict the response to treatment.

Katz et al. reported that patients with demyelinating sensory polyneuropathy and distal weakness can be classified as having DADS, in order to distinguish the phenotype from t-CIDP [8]. In addition, two-thirds of patients with DADS have IgM monoclonal gammopathy, and the disease is usually associated with anti-MAG antibodies [20,21]. DADS associated with positivity for anti-MAG antibodies, termed anti-MAG neuropathy, is separated from CIDP according to the 2010 EFNS/PNS guidelines [3,22]. In contrast, DADS without anti-MAG antibodies is often considered to be a variant of CIDP, and some reports have described differences in the response to immune treatment between DADS patients with and without anti-MAG antibodies [23,24]. In the present study, we assessed the effects of sera obtained from three patients with DADS without anti-MAG antibodies, and found that the level of BNB damage after exposure to the sera from these patients was milder than that observed following exposure to the sera of the t-CIDP patients. In addition, we demonstrated a prolonged distal latency and smaller terminal latency index, both of which suggest preferential demyelination in the distal nerve terminals, to be more frequent in the patients with DADS, although these findings did not correlate with the severity of BNB damage. These results suggest that the phenotypic discrepancies observed between t-CIDP and DADS may be due to differences in the location of BNB breakdown; namely, the "DADS phenotype" may be associated with primary involvement at the distal nerve terminal with a vulnerable BNB, as the humoral factors in DADS sera do not induce substantial BNB malfunction at the nerve trunk, compared to that observed in the setting of t-CIDP.

Based on the hypothesis suggested by the finding of electrophysiological studies, the conduction block in the nerve trunk noted in patients with MADSAM is thought to always be accompanied by focal breakdown of the BNB [10]. However, our present results suggest that this conduction block may have little relationship with the involvement of the BNB induced by humoral immunity, as the BNB damage observed after exposure to the sera obtained from the MADSAM patients was milder than that detected after exposure to the sera obtained from the t-CIDP and DADS patients and the presence of conduction block did not correlate with the severity of BNB damage after exposure to sera from any of the patients. Nevertheless, due to the *in vitro* nature of our experiments, we were unable to fully estimate the importance of the BNB breakdown induced by cellular immunity in the MADSAM patients because our data could not be used to elucidate the contribution of the sera to the passage of inflammatory cells across the BNB. It is possible that focal BNB breakdown at site(s) of conduction block is involved in the pathophysiology of MADSAM via the up-regulation of inflammatory cytokines and adhesion molecules. Therefore, further studies to clarify the association between BNB damage and cellular immunity in the setting of MADSAM are required.

The clinical and electrophysiological features of MADSAM and multifocal motor neuropathy (MMN) are very similar, although MADSAM can be distinguished from MMN by the presence of overt sensory involvement, infrequency of anti-GM1 IgM autoantibodies and responsiveness to steroid treatment [6,25]. In addition, the severity of BNB breakdown appear to differ between the two diseases. We previously reported that the sera derived from MMN patients decrease the claudin-5 protein level and the TEER values in the BNB by approximately 50% compared to that observed in healthy controls based on the same *in vitro* BNB model [18]. Comparing the finding of our previous and present studies, BNB damage is more severe in patients with MMN than in those with MADSAM, suggesting that humoral factors play a greater role in the onset of MMN than in that of MADSAM. This hypothesis implies that the pathological mechanism underlying the development of MMN are significantly different from those of MADSAM, although the two diseases share similar clinical features.

In conclusion, the present findings suggest that the severity of BNB breakdown differs depending on the clinical phenotype of CIDP, and may be associated with both the clinical disability and demyelination in the nerve trunk. Our data imply that measurements of the degree of the BNB breakdown would be useful diagnostic biomarkers for predicting both the clinical phenotype and course of CIDP. However, because this was retrospective, further prospective, large-scale studies are required to validate our findings. The accumulation of further data regarding the molecular mechanism(s) responsible for the BNB impairment observed in patients with CIDP may also lead to the development of improved or novel treatments for CIDP.

Ethic approval

The study was approved by the ethics committee of Yamaguchi University.

Provenance and peer review

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Author Contributions

Conceived and designed the experiments: FS TK. Performed the experiments: FS YS HN. Analyzed the data: FS YS MK TK. Contributed reagents/materials/analysis tools: SS MB SM SK. Contributed to the writing of the manuscript: FS SK TK.

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The Multicenter Study of a New Assay for Simultaneous Detection of Multiple Anti-Aminoacyl-tRNA Synthetases in Myositis and Interstitial Pneumonia

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Abstract

Objective: Autoantibodies to aminoacyl-tRNA synthetases (ARSs) are useful in the diagnosis of idiopathic inflammatory myopathy (IIM) with interstitial pneumonia (IP). We developed an enzyme-linked immunosorbent assay (ELISA) system using a mixture of recombinant ARS antigens and tested its utility in a multicenter study. **Methods:** We prepared six recombinant ARSs: GST-Jo-1, His-PL-12, His-EJ and GST-KS expressed in *Escherichia coli*, and His-PL-7 and His-OJ expressed in Hi-5 cells. After confirming their antigenic activity, with the exception of His-OJ, we developed our ELISA system in which the five recombinant ARSs (without His-OJ) were mixed. Efficiency was confirmed using the sera from 526 Japanese patients with connective tissue disease (CTD) (IIM n = 250, systemic lupus erythematosus n = 91, systemic sclerosis n = 70, rheumatoid arthritis n = 75, Sjögren's syndrome n = 27 and other diseases n = 13), 168 with idiopathic interstitial pneumonia (IIP) and 30 healthy controls collected from eight institutes. IIPs were classified into two groups, idiopathic pulmonary fibrosis (IPF) (n = 38) and non-IPF (n = 130). Results were compared with those of RNA immunoprecipitation. **Results:** Sensitivity and specificity of the ELISA were 97.1% and 99.8%, respectively when compared with the RNA immunoprecipitation assay. Anti-ARS antibodies were detected in 30.8% of IIM, 2.5% of non-myositis CTD, and 10.7% of IIP (5.3% of IPF and 12.3% of non-IPF). Anti-ARS-positive non-IPF patients were younger and more frequently treated with glucocorticoids and/or immunosuppressants than anti-ARS-negative patients. **Conclusion:** A newly established ELISA detected anti-ARS antibodies as efficiently as RNA immunoprecipitation. This system will enable easier and wider use in the detection of anti-ARS antibodies in patients with IIM and IIP.

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Competing Interests: Murakami M. and Seto A. are the employees of Medical and Biological Laboratory Co., Ltd. (MBL). This study was performed under collaboration between the Aauthor 's' institutes and MBL. The all authors have declared that they have no other conflicts of interest.

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Introduction

A number of autoantibodies can be detected in sera from patients with idiopathic inflammatory myopathy (IIM), some of which are specific to IIM (known as myositis-specific autoantibodies: MSAs). Detection of these autoantibodies is closely associated with IIM clinical manifestations [1,2].

Among MSAs, autoantibodies against aminoacyl-tRNA synthetases (ARSs) are the most frequently detected in adult IIM patients. To date, eight anti-ARS antibodies have been described. Anti-Jo-1 (histidyl-tRNA synthetase) [3,4] is the most common, occurring in approximately 20% of IIM patients [2,5]. Anti-PL-7 (threonyl) [6], anti-PL-12 (alanyl) [7,8], and anti-EJ (glycyl) [9] occur in ~3–4%, and anti-OJ (isoleucyl) [10] and anti-KS (asparaginyl) [11] occur in < 2% of IIM patients. Anti-tyrosyl- and anti-phenylalanyl-tRNA synthetases were also reported in one case each [12,13]. Patients with anti-ARSs show a spectrum of common clinical manifestations known as anti-synthetase syndrome (ASS), including myositis, interstitial pneumonia (IP), non-erosive arthritis, fever, Raynaud's phenomenon, and mechanic's

hands. Of note, the prevalence of IP in anti-ARS-positive patients is as high as 75–95% and IP sometimes precedes myositis [1,14,15]. Yoshifuji *et al.* reported that anti-ARS-positive patients with IP respond better to initial corticosteroid therapy but suffer from a significantly higher recurrence than anti-ARS-negative patients [1]. Therefore, anti-ARS antibodies are useful not only in diagnosing IIM but also in predicting late-onset myopathy in IP-proceeding patients and the clinical course of IP in myositis.

Currently, anti-ARS antibodies are detected using an enzyme-linked immunosorbent assay (ELISA), immunodiffusion or immunoprecipitation, but all of the antibodies are not routinely detected except for anti-Jo-1. To detect anti-ARS antibodies more readily, we established an ELISA system using a mixture of five recombinant ARS antigens: Jo-1, PL-7, PL-12, EJ, and KS. Our intention was to detect these autoantibodies simultaneously as "multiple anti-ARS antibodies". This ELISA system that we developed could be used to detect not only anti-ARS-positive myositis patients but also anti-ARS-positive idiopathic interstitial pneumonia (IIP) patients.

Materials and Methods

Patients

Serum samples were obtained from 694 Japanese adult patients with connective tissue disease (CTD) and IIP who had been followed at eight University Hospitals in Japan and 30 healthy volunteers. Patient diagnoses included IIM (n = 250), systemic lupus erythematosus (SLE) (n = 91), systemic sclerosis (SSc) (n = 70), rheumatoid arthritis (RA) (n = 75), SS (n = 27), other diseases (n = 13), and IIP (n = 168). The diagnoses of IIM, SSc, SLE, and RA were made on the basis of corresponding criteria proposed by Bohan and Peter [16] or the American College of Rheumatology [17,18,19]. IIP was defined as IP of unknown cause in which a patient did not fulfill classification criteria for any specific CTD or vasculitis, or whose lung disease was potentially caused by a drug or occupational-environmental exposure [20]. Patients with IIP were classified into two groups; an idiopathic pulmonary fibrosis (IPF) (n = 38; 12 by histological diagnosis) group and a non-IPF (n = 130; according to the typical radiographic patterns of chest high-resolution computed tomography) group.

All patients and healthy volunteers gave their written informed consent to participate in this study prior to sample collection that was performed in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (Approval number: E544) and also by institutional review boards of all participating centers (Table S1).

Immunoprecipitation

The presence of anti-ARS antibodies was determined by RNA immunoprecipitation (RNA-IP) as previously described [21]. The immunoprecipitated RNA was resolved using urea-polyacrylamide gel electrophoresis and visualized using silver staining. Each anti-ARS antibody was identified according to its mobility and tRNA pattern compared with standard serum.

Construction of expression plasmids for ARS-encoding cDNAs

For the expression and purification of recombinant proteins, full-length cDNAs of PL-12, EJ, PL-7, Jo-1, KS, and OJ (GenBank accession Numbers: D32050, U09587, NM_152295, AY995220, and BC001687, respectively) were first amplified using RT-PCR with HeLa total mRNA as a template. CDNAs for PL-12 and EJ were inserted into pET30a(+) (Novagen, Madison, WI, USA) and expressed as C-terminal His-tagged proteins. CDNAs for Jo-1 and KS were subcloned into pGEX4T-1 and pGEX6P-1 (GE Healthcare UK Ltd, Buckinghamshire, England), respectively, and expressed as N-terminal GST fusion proteins. CDNAs for PL-7 and OJ were engineered with a cMyc-epitope tag and His-tag sequence at their 3′ ends, and inserted into the pFastBacDual vector for baculovirus expression (Invitrogen, Carlsbad, CA, USA). Correct construction of plasmids was confirmed using DNA sequencing.

Expression and purification of recombinant ARSs

Expression and purification of His-tagged recombinant proteins: PL-12 and EJ were expressed in *Escherichia coli* BL-21(DE3) codon plus RIL bacteria (Stratagene, La Jolla, CA, USA). Competent cells were transformed with the vectors and the cells were incubated on Luria-Bertani (LB) agar plates containing 50 μg/mL kanamycin for 15 h at 37°C. A single colony was cultured in LB liquid medium containing kanamycin at 37°C. Addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to the medium was used to induce expression of recombinant PL-12 and EJ proteins. After a 2-h incubation, cells were harvested using centrifugation and resuspended in ice-cold phosphate buffered saline (PBS) at pH 7.5. The cells were sonicated and soluble cell lysates containing the His-tagged recombinant proteins were separated using centrifugation.

PL-7 and OJ were expressed in baculovirus-infected Hi-5 cells. Each of the expression vectors was transfected into SF-9 cells using Cellfectin (Invitrogen), and the baculovirus stock was prepared from the transfectant culture supernatant. Hi-5 cells infected with baculovirus were incubated for 72 h at 26°C and were harvested using centrifugation, and soluble cell lysates containing recombinant proteins were prepared as described above.

Soluble His-tagged recombinant ARSs were purified using immobilized metal ion affinity chromatography. Cell extracts were applied to TALON® Metal Affinity Resin columns (Clontech, Palo Alto, CA, USA), and the columns were washed with PBS containing 10 mM imidazole. Purified PL-12, EJ, PL-7, and OJ were eluted with PBS containing 50 mM imidazole.

Expression and purification of recombinant GST-ARS fusion proteins: Jo-1 and KS were also expressed in *E. coli* BL-21(DE3) codon plus RIL bacteria in the presence of ampicillin. Transformation, cultivation, induction, and extraction of soluble cell proteins were performed as described for PL-12 and EJ proteins. Soluble GST-Jo-1 and GST-KS fusion proteins were purified on Glutathione Sepharose 4B columns (GE Healthcare UK Ltd.) and eluted with Tris-HCl (pH 8.0) containing 15 mM GSH.

Immunoblotting of recombinant antigens

Purified recombinant ARS antigens were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane as described by Towbin *et al.* [22] with minor modifications. After blocking with 5% skimmed milk, the membrane was incubated for 60 min with serum diluted 1:100 and then incubated for 60 min with a 1:1000 dilution of goat anti-human IgG conjugated to peroxidase (Code No. 208, MBL, Nagoya, Japan). Immunoreactive bands were detected using the Western Blot Detection System WEST-one (iNtRON Biotechnology, Gyeonggi-do, Korea).

ELISA

For detection of each ARS autoantibody, purified recombinant ARSs were individually coated on 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY, USA). PL-12, EJ, PL-7, and

Jo-1 were diluted in PBS to a final concentration of 2.5 μg/mL, and KS to 5.0 μg/mL. Each diluent was added at 100 μL/well and incubated overnight at 4°C. The plates were washed twice with PBS, and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose overnight at 4°C. Sera from patients and normal healthy donors were diluted 1:100 in PBS containing 0.15% Tween 20 (PBS-T), 1% casein enzymatic hydrolysate, and 0.2 mg/mL *E. coli* extract, and 100 μL was applied to each well. After incubation for 60 min at room temperature (RT), the wells were washed four times with PBS-T. Goat anti-human IgG conjugated to peroxidase (Code No. 208, MBL) was diluted 1:7000 in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxyphenylacetic acid (peroxidase stabilizer), and 100 μL was added to each well. After incubation for 60 min at RT, the wells were washed four times with PBS-T, and 3,3',5,5'-tetramethylbenzidine substrate was then added. After a 30-min incubation at RT, the reaction was stopped by adding 100 μL of 0.25 N sulfuric acid and absorbance was read at 450 nm (A_{450}).

For simultaneous detection of five ARS autoantibodies, purified recombinant ARSs were diluted and mixed together in PBS and coated on plates. The final concentrations of PL-12, EJ, PL-7, Jo-1, and KS were 1.25 μg/mL, 0.63 μg/mL, 1.25 μg/mL, 0.63 μg/mL, and 2.5 μg/mL, respectively. The total protein concentration of the mixture was 6.25 μg/mL. ELISA plate preparation and assays were performed as described above. Conversion from A_{450} to a unit value (U/mL) was calculated using the following formula:

$$Unit\ Value\,(U/mL) = \frac{A_{450}<Sample> - A_{450}<Blank>}{A_{450}<Positive> - A_{450}<Blank>} \times 100$$

A_{450}<Positive> is the absorbance for an anti-Jo-1-positive patient serum that corresponds to a 100 U/mL value. A_{450}<Blank> is the background absorbance of buffer that does not contain serum. A_{450}<Sample> is the absorbance of a tested serum. The cutoff point was defined at 25 U/mL based on the analysis of the receiver operating characteristic curve in this multicenter study.

Statistical analysis

Statistical analyses were performed using StatView version 5.0 software. Clinical information of anti-ARS-negative and positive non-IPF patients was compared using the two-sample t-test or the Fisher's exact test.

Results

Autoantigen preparation

We first prepared six recombinant His-tagged ARS antigens, which were all expressed in *E. coli*. Immunoblot analysis showed that four of them, Jo-1, PL-12, EJ, and KS, were identified by their corresponding autoantibodies as well as by using an ELISA, whereas PL-7 and OJ reacted weakly with their corresponding autoantibodies (data not shown). Because we hypothesized that poor antigenic activity of recombinant PL-7 and OJ was due to a lack of posttranslational modification or proper structural folding, we prepared both fusion proteins expressed in eukaryotic Hi-5 cells using the baculovirus system. We confirmed antigenic activity of the new recombinant PL-7 using an ELISA (Fig.ô 1a) but the activity was lost when examined using immunoblotting (Fig.ô 1c). Recombinant PL-7, denatured using urea or SDS, had weaker antigenic activity than non-denatured PL-7, showing that the 3-dimensional protein structure played an important role in the reaction between the threonyl-tRNA synthetase and the anti-PL-7 antibody (Fig.ô 1a). Because of this antigenic characteristic of PL-7, we decided to prepare other recombinant ARSs, without denaturing reagents, as soluble polypeptides in PBS. Because His-Jo-1 and His-KS were insoluble, they were expressed as GST-recombinant proteins. ELISA revealed that the five newly prepared ARS antigens, His-PL-12, His-EJ, GST-Jo-1, GST-KS, and His-PL-7, displayed suitable antigenic reactivity. Immunoblotting also showed that four of the five ARS antigens, except for His-PL-7, had sufficient antigenic activity (Fig.ô 1b and c).

The recombinant OJ expressed in Hi-5 cells had weak antigenic activity, as confirmed using both immunoblotting and an ELISA (data not shown), suggesting that it is difficult to prepare a recombinant OJ as a single polypeptide that retains antigenic activity.

Establishing an ELISA system for simultaneous detection of five ARS antibodies

To detect multiple ARS antibodies simultaneously, we developed an ELISA system using a mixture of the five recombinant ARSs except for OJ. We tested a variety of antigen mixtures to estimate the most appropriate ratio and concentration to use, and we found that anti-ARS-positive sera showed reactivity with all five different ARSs with the highest sensitivity and specificity occurring at antigen concentrations of 0.63, 1.25, 1.25, 0.63, and 2.5 μg/mL (6.25 μg/mL in total) for histidyl-, threonyl-, alanyl-, glycyl-, and asparaginyl-tRNA synthetases, respectively. To assess potential cross-reactivity, we compared the absorbance values (A_{450}) obtained using an ELISA on every single recombinant ARS with those obtained with the new ELISA using the ARS mixture. When tested using a single-peptide-ELISA, each of the five anti-ARS antibodies showed reactivity with only its corresponding autoantigen. Samples positive for anti-PL-7, PL-12, or KS antibodies showed higher A_{450} values with the new mixed-peptide-ELISA than with the single-peptide ELISA, whereas the samples positive for anti-Jo-1 or EJ antibodies showed no significant difference in A_{450} values obtained with the two ELISAs. Such differences in A_{450} values may be due to different peptide-coating efficiencies because the total peptide concentration was higher in the mixed-peptide-ELISA than in the single-peptide ELISA (data not shown).

Clinical significance of anti-ARS ELISA in CTD

To confirm the efficiency of this newly established ELISA, we screened a total of 694 serum samples from patients with various CTDs and IIP, and 30 healthy controls. The results were compared between the ELISA and the RNA-IP assay (Fig.ô 2). A total of 102 samples were positive for anti-ARS antibodies using the ELISA and all of them, except for one, were identified to have any anti-ARS, other than anti-OJ, using the RNA-IP assay (Tableô 1). The sensitivity and specificity of the new ELISA in the detection of anti-ARS antibodies (including anti-OJ) compared with the RNA-IP technique were 97.1% and 99.8%, respectively (Tableô 1). Anti-ARS antibodies were detected in 30.8% (77/250) of IIM and 2.5% (7/276) of other CTDs (Tableô 2). None of the healthy controls were positive (Fig.ô 2). In IIM, 30.8% (33/107) of polymyositis (PM), 35.5% (33/93) of dermatomyositis (DM), 13.0% (3/23) of amyopathic DM, and 33.3% (1/3) of overlap myositis were positive for anti-ARS antibodies (Tableô 3). Among the 95 anti-ARS-positive IIM patients, 85 (89.4%) had IP, 54 (56.8%) arthralgia/arthritis, 24 (25.3%) had mechanic's hand, 37 (38.9%) had high fever, and 31 (32.6%) had Raynaud's phenomenon, which were consistent with previous reports [15]. The prevalence of these ASS symptoms was significantly higher in the anti-ARS-positive patients than in the negative patients (data

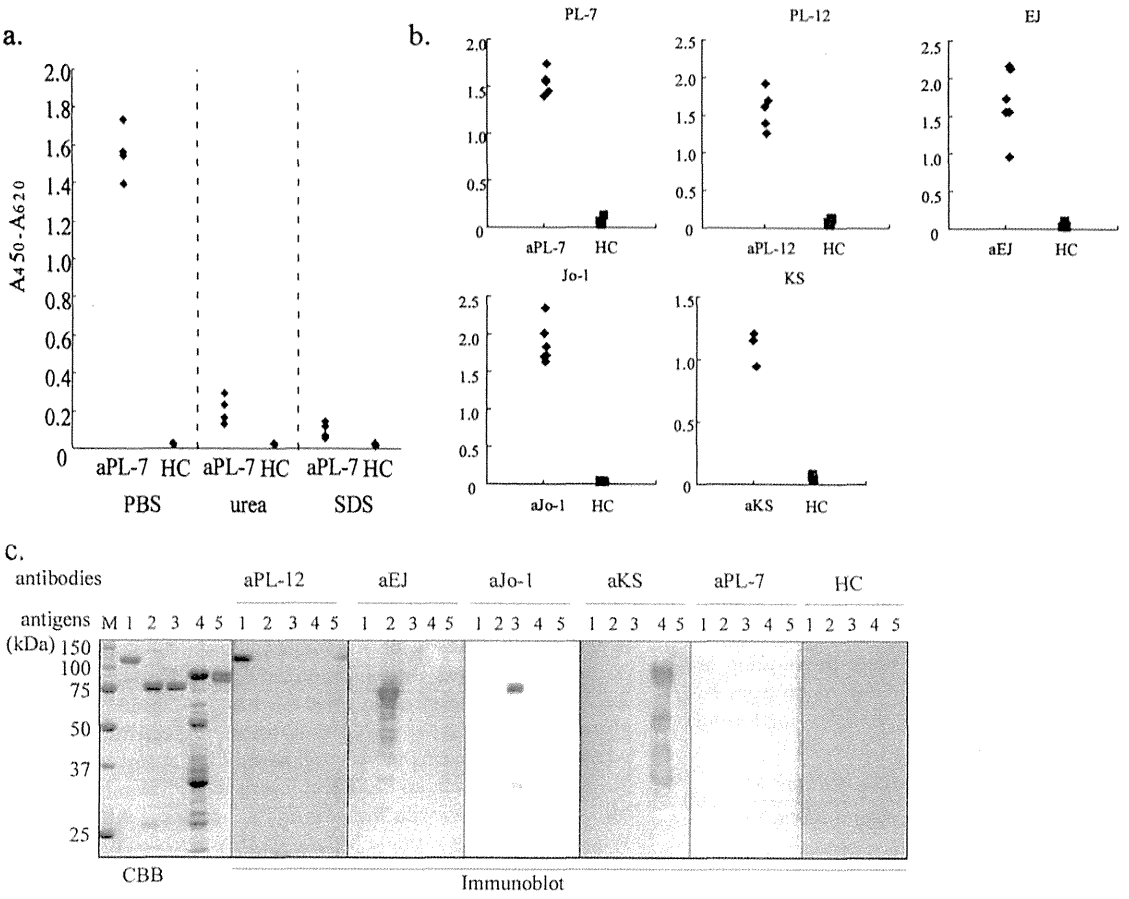

Figure 1. Antigenic activity of recombinant autoantigens a. Antigenic activity of PL-7 in various conditions. Left, purified recombinant PL-7 was eluted and diluted in PBS and coated on ELISA plates. Middle and Right, purified recombinant PL-7 was eluted in PBS and diluted in 8M urea and 2 × SDS sample buffer, respectively, and then coated onto ELISA plates. b. Five recombinant ARS antigens (His-PL-12, His-EJ, GST-Jo-1, GST-KS, and His-PL-7) were prepared as soluble polypeptides in PBS and their antigenic activity was tested in an ELISA using sera from five patients each containing corresponding autoantibodies (only GST-KS was tested using sera from three patients). Six healthy controls were used in each ELISA. c. Purified recombinant ARS antigens were electrophoresed on SDS-PAGE and transferred to a PVDF membrane followed by immunoblot analysis. CBB; Coomassie Brilliant Blue staining of gels, M; molecular weight marker, HC; healthy control, Lane 1; His-PL-12, Lane 2; His-EJ, Lane 3; GST-Jo-1, Lane 4; GST-KS and Lane 5; His-PL-7.
doi:10.1371/journal.pone.0085062.g001

not shown). There were seven anti-ARS-positive patients with other CTDs; two SSc patients were positive for anti-PL-12, two SLE patients were positive for anti-KS or anti-PL-12, and three RA patients were positive for anti-KS, anti-OJ or anti-PL-12.

Table 1 Comparison of the results between the new ELISA system and RNA-IP.

		RNA-IP		
		+	-	
anti-ARS ELISA	+	101*	1*	102
	-	0* (3)†	622* (619)†	622
	total	101* (104)†	623* (620)†	724

*The results detecting the five anti-ARS antibodies (anti-Jo-1, PL-12, EJ, KS, and PL-7) are described (sensitivity: 100%, specificity: 99.8%).
†Numbers in parenthesis are the results detecting all anti-ARS antibodies (including anti-OJ) (sensitivity: 97.1%, specificity: 99.8%).
doi:10.1371/journal.pone.0085062.t001

Clinical significance of anti-ARS ELISA in IIP

Anti-ARS antibodies were positive in 10.7% (18/168) of IIP patients. Only two patients (5.6%) with IPF were positive for anti-ARS; conversely, 16 patients (12.1%) with non-IPF were positive for anti-ARS antibodies (Tableô 2). To investigate whether the anti-ARS-positive IIP were clinically distinct from anti-ARS-negative IIP patients, we compared clinical backgrounds and treatments between anti-ARS-positive and negative non-IPF patients (Tableô 4). The anti-ARS-positive patients were significantly younger and a higher proportion was female ($p<0.01$), and they were treated more frequently with glucocorticoids (GC) or the combination of GC and immunosuppressants ($p<0.05$ and $p<0.01$, respectively).

Discussion

Among MSAs/myositis-associated autoantibodies (MAAs), anti-ARSs are the most frequently detected (28–37% [1,23,24]) in adult IIM patients, and anti-ARS-positive patients develop common characteristic symptoms known as ASS. Not only IIM but also apparent IIP patients can be positive for anti-ARS antibodies because IP often precedes myositis [1,14,20,25]. Both myopathy

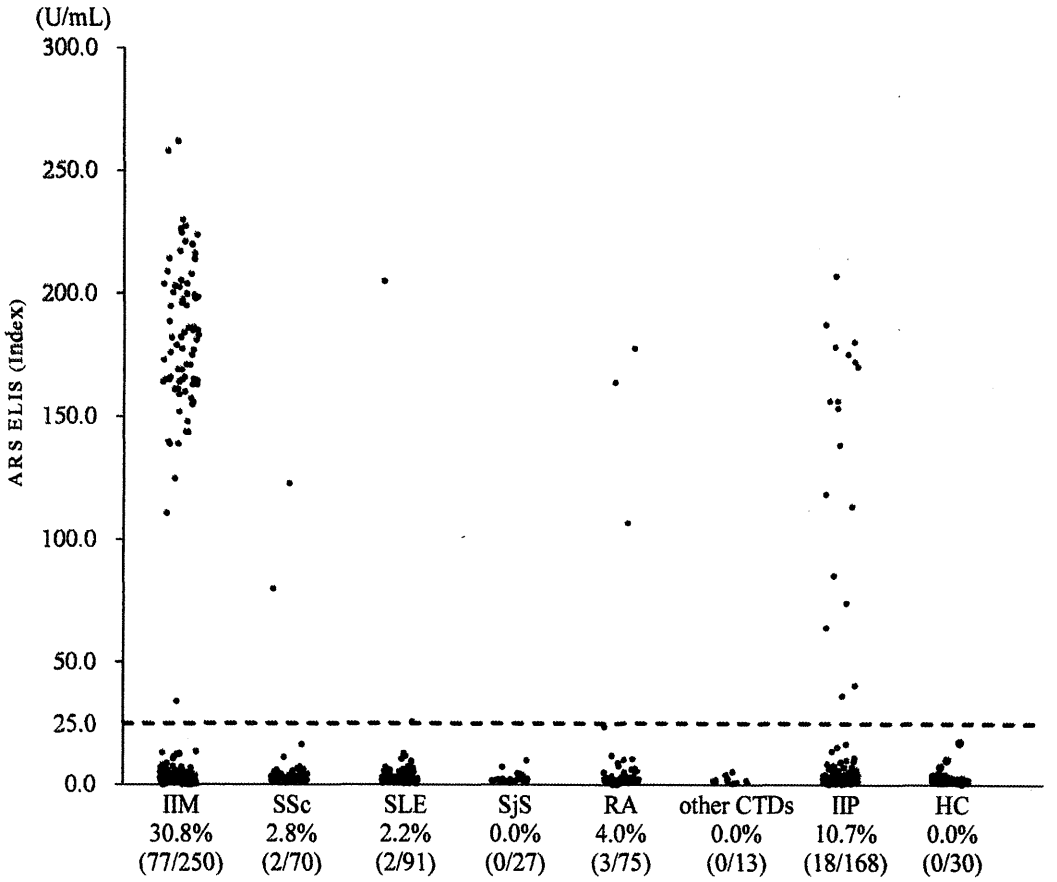

Figure 2. Confirmation of the efficiency of the ELISA system. Using the ELISA system, ARS antibodies were measured in 694 serum samples from patients with various CTDs and IIP, and 30 serum samples from healthy controls. The cutoff value (25 U/mL) is indicated by a horizontal dotted line.
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and IP anti-ARS-positive patients showed a better response to initial GC therapy but it can exacerbate the condition more often in anti-ARS-positive than in anti-ARS-negative patients [1,26]. Therefore, anti-ARS antibodies are useful not only in diagnosis, predicting the clinical course and therapy decisions in IIM, but also in classifying IP patients and predicting late-onset myopathy in IP-preceding patients.

An immunoprecipitation assay has been used to detect each anti-ARS antibody but to date, it can only be performed in a limited number of laboratories. To detect them more easily and routinely, we aimed to establish an ELISA system using the six recombinant ARS antigens to simultaneously detect anti-Jo-1, PL-7, PL-12, EJ, OJ, and KS antibodies. We did not include anti-tyrosyl or phenylalanyl synthetase because they have been reported only in one case each. However, some differences in clinical manifestations and prognoses among patients expressing different ARS antibodies, especially between anti-Jo-1 and non-anti-Jo-1 patients, have been observed [14,15]. However, different treatments for patients expressing different anti-ARSs have not been established. Currently, we treat anti-ARS-positive patients

Table 2 The frequency of each anti-ARS antibody in IIM, other CTD and IIP.

		RNA-IP(%)					
	ARS ELISA	Jo-1	PL-7	PL-12	EJ	KS	OJ
IIM	30.8% (77/250)	13.6	13.2	2.0	6.0	0.0	0.8
other CTDs	2.5% (7/276)	0.0	0.0	1.4	0.0	0.7	0.4
IIP	10.7% (18/168)	3.6	2.4	0.6	1.2	2.4	0.0
IPF	5.3% (2/38)	0.0	0.0	2.6	0.0	2.6	0.0
non-IPF	12.3% (16/130)	4.6	3.1	0.0	1.5	2.3	0.0

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Table 3 The frequency of each anti-ARS antibody in subsets of IIM.

IIM classification	Total	Jo-1	PL-7	PL-12	EJ	KS	n (%)
I polymyositis	107	18	7	3	5	0	33 (30.8)
II dermatomyositis	93	13	10	1	9	0	33 (35.5)
III amyopathic dermatomyositis	23	0	2	0	1	0	3 (13.0)
IV malignancy-associated myositis	7	0	1	0	0	0	1 (14.3)
V juvenile myositis	1	0	0	0	0	0	0 (0)
VI overlap myositis	3	1	0	0	0	0	1 (33.3)
VII unclassified	6	2	3	1	0	0	6(37.5)

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Table 4 Comparison of clinical backgrounds between anti-ARS (+) and (−) non-IPF patients.

	non-IPF n = 130		
	anti-ARS		
	(−) n = 114	(+) n = 16	p-value
age at the onset of the disease (yr) mean	69.6±9.5	56.9±14.5	<0.01
female (n; (ratio%))	39(34.2)	12(75.0)	<0.01
chronic (n; (ratio%))	104(91.2)	13(81.3)	N.S
subacute + acute (n; (ratio%))	5(4.4)	1(6.3)	N.S
acute (n; (ratio%))	2(1.8)	1(6.3)	N.S
glucocorticoids(GC) (n;(%))	49(43)	11(68.8)	<0.05
GC + immunosuppressants(IS) (n;(%))	19(16.7)	8(50.0)	<0.01
only drugs other than IS (n;(%))	8(7.0)	2(12.5)	N.S
PaO_2 at rest (Torr) mean	75.9±14.9	86.5±37.4	N.S
SpO_2 at rest (%) mean	95.7±2.4	97.1±2.1	<0.05
SpO_2 after 6 min walk test (Torr) mean	88.6±5.5	86.9±6.0	N.S
%VC (%) mean	87.7±22.5	77.9±17.4	<0.05
%DLCO (%) mean	51.0±19.5	58.0±23.1	N.S
KL-6 (U/mL) mean	1132±949	1287±693	N.S
SP-D (ng/mL) mean	207±180	180±136	N.S

%VC: % vital capacity, %DLCO: % diffusing capacity of carbon monoxide.
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with expectation of a standard clinical course in which the disease can recur with tapering of GC and in which exacerbation of IP is associated with a poor prognosis [1,14]. Therefore, presently, we are focusing on determining whether a patient with IIM or IIP is anti-ARS positive or not for the first screening when we begin treatment. This is why we decided to use a mixture of ARS antigens and not just single antigens to detect 'multiple anti-ARS antibodies' simultaneously.

We first prepared recombinant ARSs in *E. coli*, but recombinant PL-7 and OJ did not react well with their corresponding autoantibodies either using immunoblotting or an ELISA. For PL-7, structural conformation was important for antigenic activity because the recombinant PL-7 showed good reactivity only when it was expressed in a eukaryotic Hi-5 cell and was not denatured prior to being measured in the ELISA. Conversely, when recombinant PL-7 was denatured with urea or SDS, it was weakly detected with the PL-7 antibody, although its antigenicity was not completely lost. Such antigenic characteristics have also been reported previously by others [27]. This suggests that the synthetase epitope recognized by the anti-PL-7 antibody is in its native tertiary conformation.

In contrast, recombinant OJ (isoleucyl-tRNA synthetase) was not well detected even when it was expressed in Hi-5 cells and analyzed under non-denaturing conditions. This may be due to the unique feature of this isoleucyl-tRNA synthetase, which is a component of the multi-enzyme complex containing nine ARSs with three nonenzymatic factors [28,29]. In screening tests, positivity of anti-OJ in patients' sera was determined by the pattern of immunoprecipitation using HeLa cell extracts as originally described by Targoff *et al.* [28]. But there is a possibility that some 'anti-OJ antibodies' may recognize other components of the multi-enzyme complex rather than isoleucyl-tRNA synthetase itself, or alternatively the structural conformation of the complex may be important for recognition by anti-OJ, as was previously suggested by Targoff *et al.* [10]. They examined 11 patient sera with anti-OJ for evidence of reaction with other components of the complex. Ten out of 11 sera significantly inhibited enzyme activity of isoleucyl-tRNA synthetase, but some of them also significantly inhibited other ARSs such as leucyl-, lysyl-, or arginyl-tRNA synthetases. Moreover, immunoblot analysis of anti-OJ revealed that the majority of the sera could not identify a shared band and only a few sera recognized isoleucyl-tRNA synthetase. These results suggest that most 'anti-OJ sera' may react with multiple synthetases of the multi-enzyme complex or react with conformational epitopes of the complex. For this reason, we considered that it would be difficult to prepare the immunoreactive OJ antigen as a single molecule; therefore, we developed an ELISA system using the other five recombinant ARSs. This may not significantly affect the sensitivity of the ELISA because the prevalence of anti-OJ antibodies in patients is very low among the six anti-ARS antibodies.

The efficiency of this newly established ELISA system was acceptable because the sensitivity and specificity of the system compared with RNA immunoprecipitation were 97.1% and 99.8%, respectively, even if anti-OJ-positive sera was not excluded. The prevalence of anti-ARS in our IIM cohort was comparable with previous reports [1,2]. It was noteworthy that 10.7% of IIP patients, and in particular, 12.1% of non-IPF patients were positive for anti-ARS antibodies and there were some differences between anti-ARS-positive and negative IIP patients in their clinical backgrounds and treatments. Anti-ARS-positive patients were treated significantly more frequently with GC or the combination of GC and immunosuppressants. However, we are not yet ready to recommend immunosuppressive therapy for anti-ARS-positive IIP patients because we have not yet collected enough data on their clinical response and prognosis. Although some of these anti-ARS-positive IIP patients might develop myopathy later, it suggests that the measurement of anti-ARS antibodies may be useful in stratifying patients into disease subsets, which may help in predicting their clinical course.

A line-blot assay for the detection of multiple MSAs/MAAs (EUROLINE Myositis Profile 3) has been used in which anti-Jo-1, PL-7, PL-12, EJ, and OJ are included. This system can detect and discriminate MSAs/MAAs without further anti-ARS tests, but it does not include anti-KS, which has a stronger association with IIP than myositis [30]. To address this point, our system can more efficiently detect anti-ARS and therefore, is the preferred assay to use for IIP patients than the line-blot assay, although our ELISA does not aim to discriminate specificity for each anti-ARS antibody.

In conclusion, our ELISA system using a mixture of five recombinant ARSs shows similar efficiency to RNA immunoprecipitation and makes it possible to more readily detect anti-ARS antibodies in patients with PM/DM and IIP, and can be widely applied in daily practice.

Supporting Information

Table S1 The list of approval by institutional review boards of all participating centers.
(XLSX)

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Author Contributions

Conceived and designed the experiments: TM RN. Performed the experiments: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Analyzed the data: RN YI. Contributed reagents/materials/analysis tools: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Wrote the paper: RN.

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The Journal of Immunology

GM-CSF but Not IL-17 Is Critical for the Development of Severe Interstitial Lung Disease in SKG Mice

Aoi Shiomi, Takashi Usui, Yuki Ishikawa, Masakazu Shimizu, Kosaku Murakami and Tsuneyo Mimori

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BRIEF REPORT

Autoantibodies to DNA Mismatch Repair Enzymes in Polymyositis/Dermatomyositis and Other Autoimmune Diseases: A Possible Marker of Favorable Prognosis

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Objective. **Myositis-specific autoantibodies (MSAs) are useful tools for identifying clinical subsets of patients with idiopathic inflammatory myopathies (IIMs). There have been few reports on antibodies to some DNA mismatch repair enzymes (MMREs) in patients with IIMs. This study was undertaken to determine the frequencies and clinical associations of antibodies to 7 types of MMREs (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, and PMS2) in patients with IIMs and other systemic autoimmune diseases.**

Methods. **Clinical data and serum samples were collected from 239 Japanese patients with IIMs (147 with adult dermatomyositis, 13 with juvenile dermatomyositis, 57 with polymyositis, and 22 with myositis overlap syndrome). One hundred patients with other diseases, including 40 with systemic lupus erythematosus (SLE), were assessed as disease controls. The presence of anti-MMRE antibodies in serum was examined by immunoprecipitation, enzyme-linked immunosorbent assay, and immunoprecipitation/Western blotting.**

Results. **Anti-MMRE antibodies were found in 15 patients with IIMs and 3 patients with SLE. They were restricted to MLH1, PMS1, MSH2, and PMS2, with simultaneous positivity for more than one of these antibodies occurring in some patients. Nine IIM patients with anti-MMREs also had other MSAs and their associated clinical features. All patients with anti-MMREs were still living at the time of the present analysis.**

Conclusion. **Anti-MMRE antibodies, which often coexist with other MSAs, may be serologic markers for good prognosis in IIMs.**

Idiopathic inflammatory myopathies (IIMs) are systemic autoimmune diseases that mainly affect muscle and/or skin. Various myositis-specific autoantibodies (MSAs) and myositis-associated autoantibodies (MAAs) have been described (1). MAAs have been reported in relation to myositis in overlap syndromes with other autoimmune diseases. In contrast, MSAs are exclusive to myositis, and ≥2 MSAs rarely coexist in a single patient.

DNA mismatch repair is one of several DNA repair pathways conserved from bacteria to humans. The primary function of mismatch repair is to eliminate the mismatch of base–base insertions and deletions that appear as a consequence of DNA polymerase errors during DNA synthesis. In humans, there are 2 sets of mismatch repair enzymes (MMREs), corresponding to homologs of the bacterial MutS and MutL systems (2). The human MutS homologs are MSH2, MSH3, and MSH6, and human MutL homologs include MLH1, MLH3, PMS1, and PMS2.

A 2001 report described the presence of autoantibodies to PMS1 in patients with IIM (3). Autoantibodies to PMS2 and MLH1 were also present in some patients. In 2005, anti-PMS1 and anti-MSH2 antibodies were found in Japanese patients with IIMs (4). In the present study, we evaluated the frequencies and clinical implications of autoantibodies to the 7 types of MMREs in patients with IIM and other autoimmune diseases.

PATIENTS AND METHODS

Patients. Serum samples from 239 Japanese patients (56 male, 183 female) with IIM (147 with adult dermatomyo-

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sitis [DM], 13 with juvenile DM, 57 with polymyositis [PM], and 22 with myositis overlap syndrome) were analyzed for this study. The sera were from 144 patients seen at Nagoya University Hospital between 1994 and 2013 and 95 patients seen at Kyoto University Hospital between 2004 and 2013. They were obtained at the time of diagnosis (except for 2 samples obtained at a later time point from patients with juvenile DM) and were from consecutive patients in most cases (samples from some consecutively diagnosed patients were not available for study). One hundred patients with other autoimmune diseases (40 with systemic lupus erythematosus [SLE], 20 with systemic sclerosis [SSc], 20 with rheumatoid arthritis [RA], and 20 with Sjögren's syndrome [SS]) were assessed as disease controls. All 57 patients with PM and 95 of the patients with adult DM fulfilled the Bohan and Peter criteria (5); 52 patients fulfilled the Sontheimer criteria for clinically amyopathic DM (CADM) (6). The patients with SLE, RA, and SSc met the respective American College of Rheumatology classification criteria for these diseases (7–9). SSc was classified as diffuse cutaneous or limited cutaneous according to the criteria of LeRoy et al (10). SS was diagnosed based on Japanese diagnostic criteria (11). Clinical information was collected retrospectively by medical chart review. The study was conducted with the approval of the ethics committees of the Nagoya University Graduate School of Medical Science and the Kyoto University Graduate School of Medical Science.

Laboratory tests and serologic assays. Serum samples were screened for antibodies against SSA, SSB, U1 RNP, Sm, CENP-B, and double-stranded DNA (dsDNA) using commercial enzyme-linked immunosorbent assay (ELISA) kits (MBL). In addition, anti–Mi-2, anti–transcription intermediary factor 1γ (anti–TIF-1γ), anti–melanoma differentiation–associated protein 5 (anti–MDA-5), anti–nuclear matrix protein 2 (anti–NXP-2), and anti–aminoacyl–transfer RNA synthetase (anti-aaRS) antibodies were investigated by protein and RNA immunoprecipitation (12) and/or immunoprecipitation with recombinant protein (13).

Immunoprecipitation and ELISA using recombinant protein. The full-length complementary DNA (cDNA) clones of 7 human MMREs (Flexi ORF Clone) were purchased from Promega. Biotinylated recombinant protein was produced from the cDNA, using a T7 Quick Coupled Transcription/Translation System (Promega) according to our previously described protocol (13). Briefly, 800 μl TnT Quick Master Mix, 20 μl 1 m*M* methionine, 30 μl biotin-lysyl–transfer RNA, 120 μl water, and 30 μl DNA (1 μg/μl) were mixed and then incubated for 60 minutes at 30°C. Immunoprecipitation was performed using in vitro translation and transcription (TnT) products as previously described (13).

Anti-MMRE antibodies were also tested by antigen-capture ELISA according to our previously described methods (14). Briefly, a 96-well Immobilizer Streptavidin Plate (Thermo Scientific Nunc) was incubated with 1 μl/well of TnT reaction mixture including biotinylated recombinant protein. Wells were then incubated with sera (1:1,000 dilution) and probed with horseradish peroxidase–conjugated anti-human IgG antibody (1:30,000 dilution; Dako). After incubation with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce), relative luminescence units (RLU) were determined using a GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU minus background was used for data analysis. The RLU of the samples was converted into units using a standard curve created with a prototype positive serum.

Immunoprecipitation/Western blotting. Immunoprecipitation assays were performed using extracts of the leukemia cell line K562 as previously described (12), with minor modifications. Patient serum (10 μl) was bound to 10 μl of protein G–Sepharose Fast Flow (GE Healthcare Japan) in 500 μl of immunoprecipitation buffer (10 m*M* Tris HCl [pH 8.0], 500 m*M* NaCl, 0.1% Nonidet P40) and incubated for 2 hours at 4°C, followed by washing 5 times with immunoprecipitation buffer. Antibody-coated Sepharose beads were mixed with 100 μl K562 cell extracts derived from 10^6 cells and rotated at 4°C for 2 hours. After 5 washes, the beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer, and samples were fractionated by SDS–polyacrylamide gel electrophoresis followed by Western blotting. Polyclonal antibodies to MLH1, MSH2, PMS1, and PMS2 were purchased from Santa Cruz Biotechnology.

Statistical analysis. Statistical significance was assessed by Fisher's exact test, Mann-Whitney U test, or log rank test, as appropriate. Data were evaluated using SPSS Statistics (IBM). *P* values less than 0.05 were considered significant.

RESULTS

Detection of anti-MMRE antibodies in patients with IIM and other autoimmune diseases. Serum samples from 239 patients with IIM were screened for anti-MMREs using immunoprecipitation with recombinant proteins. PMS1, MLH1, MSH2, and PMS2 recombinants were immunoprecipitated by 10, 9, 3, and 2 sera, respectively, as determined by TnT immunoprecipitation (Figure 1). They were confirmed to react with the corresponding proteins, since the precipitates were recognized by polyclonal antibodies to these proteins in Western blotting (Figure 1). MLH3, MSH3, and MSH6 were not reactive with any sera from the IIM patients, although their recombinants were produced (Figure 1).

In all of the IIM sera, the presence of each of the 7 anti-MMRE antibodies was also examined by ELISA. With positivity defined as an RLU value more than 5 SD above the mean in 20 healthy controls, these analyses demonstrated that all of the sera that were positive for antibodies to PMS1, MLH1, MSH2, and PMS2 by immunoprecipitation were positive by ELISA as well (data available from the corresponding author upon request). As in the immunoprecipitation analyses, no sera were found by ELISA to be positive for anti-MLH3, anti-MSH3, or anti-MSH6 antibodies.

Positivity for antibodies to PMS1, MLH1, MSH2, and PMS2 was also assessed by ELISA in the sera of disease controls and healthy controls (data available from the corresponding author upon request). The only

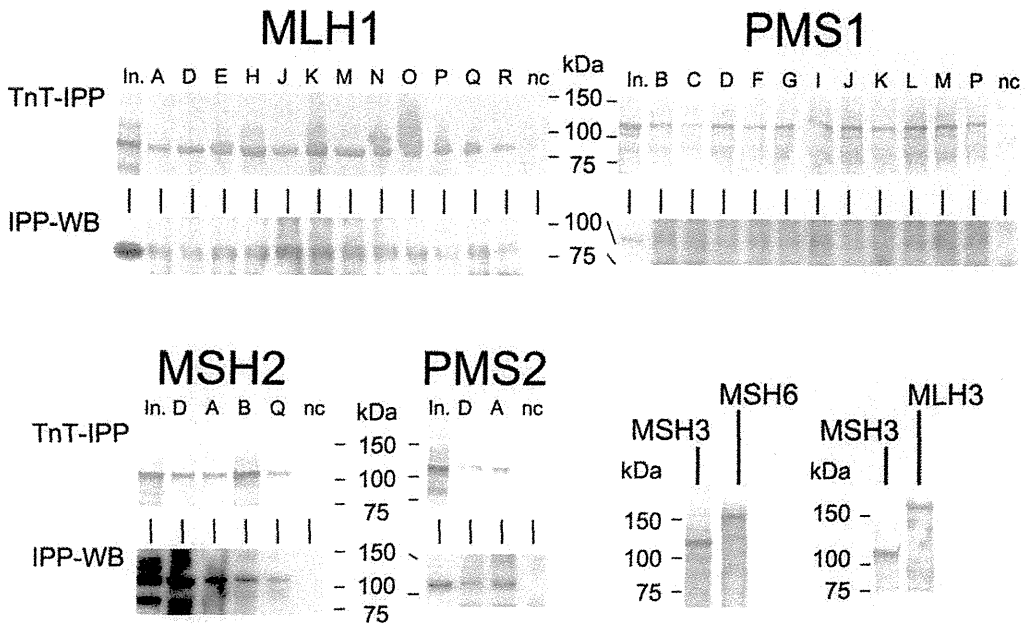

Figure 1. Detection of antibodies to DNA mismatch repair enzyme (anti-MMRE). In translation and transcription immunoprecipitation (TnT-IPP) experiments, biotinylated recombinant MLH1, PMS1, MSH2, and PMS2 were assessed by immunoprecipitation. Recombinant proteins were subjected to 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with streptavidin–alkaline phosphatase and substrate. In immunoprecipitation/Western blotting (WB) experiments, immunoprecipitates from K562 cell extracts with human sera were probed with polyclonal antibodies to MLH1, PMS1, MSH2, and PMS2. The input (In.) was half the dose (or a full dose for immunoprecipitation/Western blotting of MLH1), of the biotinylated proteins or cell extract used for the immunoprecipitation assay. Biotinylated recombinant MSH3, MSH6, and MLH3 were also subjected to 4–20% SDS-PAGE and analyzed by immunoblotting with streptavidin–alkaline phosphatase and substrate; no serum samples immunoprecipitated these recombinants. Lanes A–R correspond to anti-MMRE–positive patients shown in Table 1. nc = normal control serum.

positive results in these assays were found in 3 sera from patients with SLE. All 3 SLE sera were reactive with MLH1; 1 was additionally reactive with PMS1, and another was additionally reactive with MSH2. The presence of antibodies to MLH1, PMS1, and MSH2 in these 3 patients with SLE was confirmed by immunoprecipitation/Western blotting (Figure 1).

Longitudinal study of anti-MMRE antibodies coexisting in individual patients. Among a total of 18 IIM or SLE patients with anti-MMRE, 8 were positive for at least 2 types of anti-MMRE antibodies (Table 1). Patterns of reactivity with 4 MMREs (MLH1, PMS1, MSH2, and PMS2) and their combinations were heterogeneous among patients and were not associated with the specific disease or disease subset (data available from the corresponding author upon request). The coexistence of anti-MLH1 and anti-PMS1 antibodies was found most frequently (5 patients). All patients who were positive for anti-MSH2 and/or anti-PMS2 were also positive for anti-MLH1 and/or anti-PMS1.

To further investigate the associations of antibodies to different MMREs, we obtained longitudinal serum samples from 7 patients who were positive for >1 type of anti-MMRE and examined antibody titers by ELISA (data available from the corresponding author upon request). In patient D, who was positive for 4 different anti-MMREs, titers of all 4 decreased similarly over time. Titers of anti-MLH1 changed in parallel to those of anti-PMS1 in patients J, K, M, and P and in parallel to those of anti-MSH2 in patient Q. In patient B, titers of anti-PMS1 changed similarly to titers of anti-MSH2.

Clinical and laboratory profiles of patients with anti-MMRE antibodies. Of the 239 patients with IIM, 15 were positive for at least 1 anti-MMRE antibody: 5 (5.3%) of 95 adults with DM, 3 (5.3%) of 57 adults with PM, 2 (3.8%) of 52 adults with CADM, 2 (15.4%) of 13 juvenile patients with DM, and 3 (13.6%) of 22 adults with myositis overlap (Table 1). The antibody frequency was higher among female IIM patients (15 of 183) than among male patients (0 of 56) ($P < 0.026$). Muscle symptoms and arthralgia were seen in 12 and 10 patients, respectively, while internal malignancy was not found. Among adult patients with DM including CADM, anti-

Table 1. Clinical and laboratory features of the IIM patients and disease control patients who were found to be positive for antibodies to MMRE*

	Patient																	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Age, years	48	56	70	22	19	28	40	30	47	25	15	4	40	44	63	38	15	28
Sex	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Diagnosis	PM	PM	PM	DM	DM	DM	DM	DM	CADM	CADM	JDM	JDM	PM–SSc	PM–SSc	PM–SLE	SLE	SLE	SLE
Anti-MLH1	+	–	–	+	+	–	–	+	–	+	+	–	+	+	+	+	+	+
Anti-PMS1	–	+	+	+	–	+	+	–	+	+	+	+	+	–	–	+	–	–
Anti-PMS2	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Anti-MSH2	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Other autoantibodies	–	Jo-1, SSA	–	–	–	TIF-1γ	MDA-5	MDA-5	PL-7	MDA-5	TIF-1γ	TIF-1γ	–	CENP-B	PL-12, SSA, dsDNA	U1 RNP, SSA, dsDNA	U1 RNP, Sm, SSA, dsDNA	U1 RNP, SSA
Muscle symptoms†	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	+	–	–
Highest CK level, IU/liter	339	393	3,405	1,263	150	6,554	209	220	202	101	130	65	1,016	195	437	252	87	95
Gottron's sign	–	–	–	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–
Heliotrope rash	–	–	–	–	+	+	–	+	–	+	+	+	–	–	–	–	–	–
Mechanic's hands	–	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	–	–
Arthralgia	–	+	+	+	+	–	+	+	–	+	–	–	+	+	+	+	+	+
ILD	–	+	+	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
Malignancy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

* IIM = idiopathic inflammatory myopathy; MMRE = DNA mismatch repair enzyme; PM = polymyositis; DM = dermatomyositis; CADM = clinically amyopathic DM; JDM = juvenile DM; SSc = systemic sclerosis; SLE = systemic lupus erythematosus; TIF-1γ = transcription intermediary factor 1γ; MDA-5 = melanoma differentiation–associated protein 5; dsDNA = double-stranded DNA; CK = creatine kinase; ILD = interstitial lung disease.
† Muscle weakness and/or myalgia.

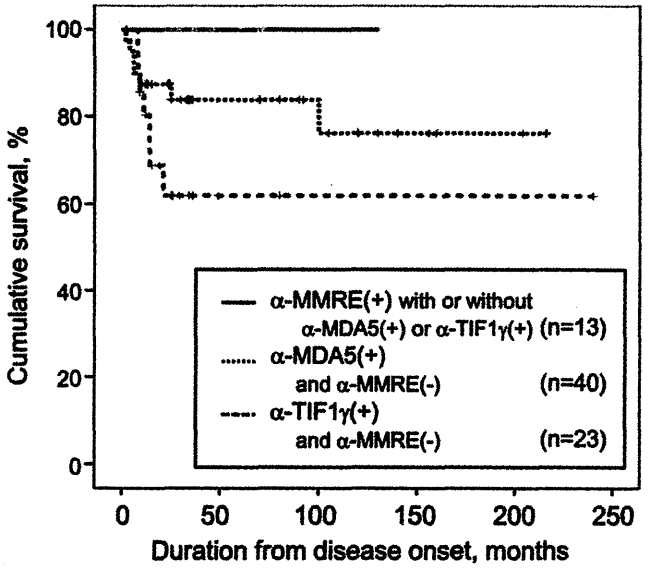

Figure 2. Cumulative survival rates from the time of disease onset in 76 Japanese patients with polymyositis/dermatomyositis (including clinically amyopathic dermatomyositis and myositis overlap syndrome) who were positive for serum anti–mismatch repair enzyme (anti-MMRE), anti–melanoma differentiation–associated protein 5 (anti-MDA-5), or anti–transcription intermediary factor 1γ (anti–TIF-1γ). The analysis did not include anti-MMRE–positive patients with juvenile dermatomyositis or systemic lupus erythematosus, although these patients were also still alive at the time of the analysis. The anti-MMRE–positive group included some patients who were also positive for anti–MDA-5 (n = 3) or anti–TIF-1γ (n = 1); no patients in the other 2 groups were positive for anti-MMRE. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38866/abstract.

MMRE–positive patients were younger than antibody-negative patients ($P < 0.001$).

MSAs or systemic autoimmune disease–associated autoantibodies were found in 13 of the 18 IIM or SLE patients who were anti-MMRE positive. The prevalences of anti-MMREs in anti–MDA-5–positive patients (3 of 43 [7%]) and in anti–TIF-1γ–positive patients (3 of 30 [10%]) were not significantly higher than the prevalence of anti-MMREs in anti–Jo-1–positive patients (1 of 22 [4.5%]). Anti-aaRS and anti–MDA-5 antibodies were found in 3 patients and 2 patients, respectively, all of whom had interstitial lung disease. Three patients with anti–TIF1-γ were either juvenile or young adult patients with DM. Patients who were positive for anti–U1 RNP and/or anti-dsDNA were all diagnosed as having SLE. Of the 5 patients who were positive for at least 1 anti-MMRE antibody but for no other autoantibodies, all had IIMs.

Since all of the patients with anti-MMREs were still living at the time of the present analysis, we compared the survival rates among 3 groups: 1) patients who were positive for anti-MMRE with or without anti–MDA-5 or anti–TIF-1γ (excluding those with SLE or juvenile DM), 2) patients who were positive for anti–MDA-5 and negative for anti-MMRE, and 3) patients who were positive for anti–TIF-1γ and negative for anti-MMRE (Figure 2). Cumulative survival rates were lower among patients with anti–MDA-5 and in patients with anti–TIF-1γ compared to patients with anti-MMRE ($P = 0.136$ [not significant] and $P = 0.016$, respectively).

DISCUSSION

The present study is the first in which autoantibodies to 7 different types of MMREs were investigated. There are 2 previous reports describing the detection of anti-MMRE antibodies in patients with IIMs (3,4). Casciola-Rosen reported 6 patients with IIM and anti-MMRE positivity (3); anti-PMS1, anti-MLH1, and anti-PMS2 antibodies were found in 4, 3, and 2 patients, respectively. Japanese investigators initially detected anti-PMS1 and anti-MSH2 antibodies in a patient with pancreatic cancer (4). Subsequently, they detected anti-PMS1 in 13.5% of pancreatic cancer patients and in 6.7% of PM/DM patients by immunoprecipitation with TnT protein, and anti-MSH2 in 8.1% of pancreatic cancer patients and in 4.9% of PM/DM patients by immunoblotting with bacterial recombinant protein. Although detailed clinical information on the PM/DM patients was not reported, it was noted that 1 of the anti-PMS1–positive PM/DM patients had breast cancer.

In our study, anti-MLH1, anti-PMS1, anti-MSH2, and anti-PMS2 antibodies were detected, whereas no subject was positive for anti-MSH2, anti-HSH6, or anti-MLH3. Interestingly, nearly half of the antibody-positive patients (8 of 18) had multiple anti-MMRE antibodies, with the titers of the different antibodies changing in parallel within individual patients. We performed homology searches, using Pearson's online *lalign* program (http://www.ch.embnet.org/software/LALIGN_form.html), among amino acid structures of MSH1, PMS1, PMS2, and MSH2. These revealed no significantly homologous regions in long stretches among the 4 proteins. However, we found a highly homologous sequence with a 12–amino acid stretch ($T^{Y}/_{F}GFRGEAL^{A}/G/_{S}S^{I}/_{L}$) at the N-terminus of MLH1, PMS1, and PMS2, which MSH2 does not have. Thus, we cannot completely exclude the possibility of cross-reactivities among MLH1, PMS1, and/or PMS2.

Since only patients with IIM (n = 5) were positive for anti-MMRE but not for other autoantibodies, anti-MMREs can be considered as MAAs. The clinical features of the other 13 patients were closely associated with coexistent autoantibodies. In a previous study, 1 DM patient also had anti–Mi-2 and 1 PM patient had serologic evidence of SLE (autoantibodies to poly[ADP-ribose] polymerase and to catalytic subunit of DNA-dependent protein kinase) (3). Several MSAs have been discovered in recent years, and some of these autoantibodies may have been concomitantly present, but not tested for, in IIM patients who were found to be positive for anti-MMRE before the other MSAs were identified.

All of the IIM patients in whom anti-MMREs had been identified were still living at the time of the present analysis. The cumulative survival rate among these patients was significantly better than among patients with anti–TIF-1γ, which is a serologic marker for cancer-associated DM (1,13); the increased survival among patients with anti-MMREs compared to those with anti–MDA-5, which was originally defined as a serologic marker for CADM complicated by rapidly progressive interstitial lung disease (1,12,13), was not significant. There are conflicting reports regarding outcomes among anti–MDA-5 antibody–positive patients with IIM (15), but recent therapeutic advances are resulting in improved survival for this group (Muro Y, et al: unpublished observations).

In summary, anti-MMREs are considered to be myositis-associated antibodies, but clinical subsets are strongly influenced by coexistent autoantibodies. Inclusion of a larger number of disease control patients in the present study would likely have improved our ability to assess this in greater detail. Further study is needed to investigate whether the antibodies described herein might have prognostic, in addition to diagnostic, value.

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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. Muro 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. Muro, Nakashima, Mimori, Akiyama.
Acquisition of data. Muro, Nakashima, Hosono, Sugiura.
Analysis and interpretation of data. Muro, Nakashima, Hosono, Sugiura, Mimori, Akiyama.

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GM-CSF but Not IL-17 Is Critical for the Development of Severe Interstitial Lung Disease in SKG Mice

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Interstitial lung disease (ILD) is a common complication and sometimes a prognostic factor of connective tissue diseases (CTDs) in humans. However, suitable animal model of severe CTD-associated ILD (CTD-ILD) has been limited. In this study, we showed that zymosan-treated SKG mice developed not only arthritis but also chronic–progressive ILD with high mortality over several months. The pathological and clinical features of ILD in zymosan-treated SKG mice were similar to that of human severe CTD-ILD. ILD in this mouse was characterized by massive infiltration of Th17 cells, GM-CSF–producing $CD4^+$ T cells, and $CD11b^+$ $Gr1^+$ neutrophils with fibrosis. Naive SKG T cells were skewed to differentiate into GM-CSF–producing cells, and GM-CSF secreted by T cells enhanced IL-6 and IL-1β production by macrophages, which in turn enhanced differentiation of IL-17A– and/or GM-CSF–producing T cells and infiltration of neutrophils into lung. Neutralization of GM-CSF completely blocked the development of this ILD, and the blocking of IL-6 signaling resulted in partial prevention of it, whereas neutralization of IL-17A did not. In contrast, the progression of arthritis was inhibited by the neutralization of GM-CSF and slightly by the neutralization of IL-17A, but not by the blocking of IL-6 signaling. These data suggested zymosan-treated SKG mice could be a useful mouse model of severe CTD-ILD, and GM-CSF, rather than IL-17A or IL-6, contributed to the development of ILD in zymosan-treated SKG mice, indicating that neutralization of GM-CSF would be a useful therapeutic strategy for severe CTD-ILD. ***The Journal of Immunology*****, 2014, 193: 849–859.**

Connective tissue diseases (CTDs) are systemic disorders that share certain clinical characteristics, including inflammation of the joints, serosal membranes, connective tissues, and blood vessels in various organs. The lung is a particularly vulnerable target organ of the CTDs, and 20–60% of CTDs complicates ILD (1). The CTD-associated interstitial lung disease (CTD-ILD) sometimes exhibits a severe and progressive ILD with high morbidity and mortality. Many ILDs are characterized by the accumulation of inflammatory cells within the lung, followed by the progressive deposition of extracellular matrix and the subsequent destruction of lung airspaces (2, 3). Analyses of inflammatory cells using bronchoalveolar lavage (BAL) of CTD-ILD patients showed an accumulation of neutrophils with or without increased percentages of lymphocytes (4, 5), and neutrophils were reported as important effector cells and associated with poor outcome of CTD-ILD (5–8). Immunohistochemical analysis of open lung biopsy specimens obtained from ILD patients also revealed that the density of plasma cells, neutrophils, macrophages, $CD3^+$, $CD4^+$, and $CD8^+$ T cells is significantly increased in the interstitium of ILD (3), and a previous report showed that the increased number of $CD4^+$ T cells in lung tissue was the characteristic of CTD-ILD (9). These reports suggested that not only lymphoid cells but also myeloid cells contribute to the pathogenesis of CTD-ILD. However, BAL and lung biopsy in patients with severe ILD are invasive and risky examinations; we still do not have enough information about severe ILD, and there are still many unknown mechanisms underlying the development of severe CTD-ILD. As a result of these difficulties, there have been few advancements in therapy for severe ILD for more than half a century. The major cause of these stagnations is the lack of suitable animal models for severe CTD-ILD. Although the pathogenesis of ILD has been investigated using animal models such as the murine bleomycin-induced pulmonary fibrosis model, most of these ILD models exhibit not progressive, but acute and transient disease (10). Moreover, most of the animal models are not related to autoimmunity, are fibrosis dominant, and do not fully recapitulate the histologic pattern of CTD-ILD (10). Therefore, it has been difficult to examine the entire pathogenesis of severe CTD-ILD and the effects of therapeutic interventions.

SKG mice, a mutant of the gene encoding ZAP70, spontaneously develop $CD4^+$ T cell–mediated autoimmune arthritis and also develop extra-articular manifestations, including ILD (11, 12). For this point, ILD in SKG mice could be a good candidate of a murine CTD-ILD model. However, the histological analyses in previous reports were not detailed, and the chronicity, prognosis, or etiology of ILD in SKG mice has yet to be clarified. The mutation of ZAP70 in SKG mice alters the sensitivity of T cells to positive and negative selection in the thymus, leading to production of potentially autoimmune T cells (11, 13). Injection of zymosan, a crude extract of β-glucans or purified β-glucans, activates innate immunity via TLR and Dectin-1 and drives differentiation and expansion of Th17 cells (14). In addition, complement activation

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; CTD, connective tissue disease; ILD, interstitial lung disease; NSIP, nonspecific interstitial pneumonia; PAP, pulmonary alveolar proteinosis.

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by β-glucans also contributes to promote Th17 differentiation in synergy with GM-CSF (15). These activated Th17 cells are reported to be responsible for the development of SKG arthritis, as adoptively transferred IL-17A$^{+/+}$ CD4$^+$ T cells did and IL-17A$^{-/-}$ CD4$^+$ T cells did not induce arthritis (13). In contrast, neutralizing IL-17 itself has proven to be a rather unsatisfactory method for blocking Th17 cell–mediated disease, giving only partial efficacy, which suggests that additional factors may be more important in Th17-mediated disease (16–18). Recent reports demonstrated that not IL-17A, but GM-CSF was critical for the pathogenesis of Th cells in experimental autoimmune encephalomyelitis, which had been known as a Th17-mediated disease (19, 20). Similarly, the other recent report showed CD4$^+$ T cell–derived GM-CSF contributed to inflammatory aortic aneurysms in a genetic model of Smad3 deficiency (21). Therefore, GM-CSF may also be a major contributor to triggering other autoimmune diseases previously known as Th17-mediated disease, such as SKG arthritis and/or its extra-articular manifestations.

In this study, we described that zymosan-treated SKG mice developed chronic–progressive and fatal ILD over several months. Because this ILD shared many histological characteristics with CTD-ILD and showed remarkable chronicity and severity, it is a useful model to understand severe CTD-ILD. Furthermore, we found that the blocking of IL-17A, GM-CSF, or IL-6 signals differently modified ILD and arthritis in this mouse. Among them, the neutralization of GM-CSF completely blocked the development of this ILD. These results suggested that the neutralization of GM-CSF would be a potential strategy for treatment of severe CTD-ILD.

Materials and Methods

Mice

SKG (CLEA Japan) and BALB/c mice (Japan SLC) at 7–12 wk were used. All mice were kept in specific pathogen-free conditions within the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All mice were treated in accordance with the guidelines for animal care approved by the Animal Experimentation Committee of Kyoto University (MedKyo 11052).

Induction of arthritis and ILD in SKG mice

Seven- to eight-week-old female mice were given a single i.p. injection of 7.5 mg/20 g body weight of zymosan (Sigma-Aldrich) suspended in 0.5 ml PBS or 0.5 ml PBS as a control, as previously described (14).

Clinical assessment of arthritis

Joint swelling was monitored by inspection and scored as follows: 0, normal; 1, mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits; 2, moderate redness and swelling of ankle or wrist; 3, severe redness and swelling of the entire paw, including digits; and 4, maximally inflamed limb with involvement of multiple joints. Clinical scores were represented as the total score of four paws.

Histology and histological score of the lung

The left lung and the other organs were inflated with 4% paraformaldehyde and paraffin embedded. Cryostat sections were stained with H&E and also subjected to Masson's trichrome staining. All images were captured using a Keyence BZ-9000. The severity of ILD was evaluated by measuring diffusely affected area, as previously reported (22). To score the mean of affected area with cellular infiltration precisely and exclude subjective assessment, three lung images per one mouse were randomly sampled, and all image processing was conducted by blinded method. The obtained lung images were converted to 8-bit grayscale, and the minimum and maximum points of the grayscale of each image were resettled 0 and 255 points, respectively, according to their histograms. The mean densities of the whole lung areas were then calculated by Image J software (Wayne Rasband). Each histological score of lung was described as the mean score of three images.

ELISA

Cytokines were measured by ELISA kits (eBioscience), according to the manufacturer's instructions. We confirmed that the existence of neutralizing/blocking Abs did not interfere with ELISA. To measure mouse anti-rat IgG Abs (Abs against anti-mouse GM-CSF Ab), the Coster 9018 ELISA plates were coated with 0.5 μg/ml rat anti-mouse GM-CSF Ab (eBioscience) in carbonate buffer and incubated overnight at 4°C. Subsequently, the plate was washed with 0.05% Tween 20 containing PBS and blocked with assay diluent (eBioscience) for 1 h. Samples and mouse anti-rat IgG (H+L; Jackson ImmunoResearch Laboratories) as standards were diluted with assay diluent and added to the wells after washing. After 1-h incubation at room temperature, the plate was washed and 0.01 μg/ml peroxidase-conjugated F(ab′)$_2$ fragment of rabbit anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories) was added as detection Ab and then incubated at room temperature for 1 h. After extensive washing, tetramethylbenzidine solution (eBioscience) was added, and, subsequently, 1 M H_3PO_4 was added to stop the reaction. The OD values at 550 nm were subtracted from those at 450 nm, and the values of samples were determined.

Isolation of lung-infiltrating cells

Mice were euthanized by exsanguination under chloroform-anesthesia, and then the lungs were perfused with PBS via hearts to remove blood cells. The right upper lobe was minced and digested in collagenase D (Roche Applied Science) for 30 min at 37°C. Following digestion, 100 μl 100 mM EDTA was added and incubated for 5 min at 37°C. Subsequently, digested lungs were filtered through a 100-μm nylon filter and the cells were collected.

Intracellular cytokine staining

Cell stimulation with PMA and ionomycin and intracellular cytokine staining were performed, as previously described (23). Pacific Blue–conjugated anti-CD3 (17A2), allophycocyanin-Cy7–conjugated anti-CD4 (RM4-5), PE-conjugated anti-F4/80 (CI:A3-1), allophycocyanin-conjugated anti–IL-17A (TC11-18H10.1), PE-Cy7–conjugated anti–IFN-γ (XMG1.2), PE-conjugated anti–IL-6 (MP5-20F3), and PE-conjugated anti–IL-4 (11B11) were purchased from BioLegend; PerCP-Cy5.5–conjugated anti-CD8 (53-6.7), PerCP-Cy5.5–conjugated anti-CD11b (M1/70), PE-Cy7–conjugated anti-CD45R (B220) (RA3-6B2), FITC-conjugated anti-Gr1 (RB6-8C5), FITC-conjugated anti–GM-CSF (MP1-22E9), and FITC-conjugated anti–TNF-α (MP6-XT22) were purchased from eBioscience. Data of flow cytometry were acquired on LSR Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Cell culture

We prepared single-cell suspensions of fresh splenocytes, and naive CD4$^+$ T cells were sorted by MACS CD4$^+$ T cell–negative selection (Miltenyi Biotec), followed by BD FACS Aria II (BD Biosciences) for CD4$^+$CD8$^-$ CD62L$^+$ cells. A total of 1.25×10^5 CD4$^+$CD62L$^+$ T cells was stimulated with 4 μg/ml anti-CD3 (2C11; eBioscience) and 5 μg/ml anti-CD28 (BioLegend) and cultured with 5 U/ml human rIL-2 (PeproTech) in 1:1 mixed medium of RPMI 1640 medium and DMEM supplemented with 10% FCS and 50 μM 2-ME, penicillin, and streptomycin for neutral condition (Th0), or with the following cytokines and neutralizing Abs for the desired polarization: IL-12 (1 ng/ml; PeproTech) and anti–IL-4 (20 μg /ml; eBioscience) for Th1 condition; IL-4 (50 ng/ml; Peprotech) and anti–IFN-γ (10 μg/ml; BioLegend) for Th2 condition; and IL-6 (20 ng/ml; BioLegend), TGF-β (10 ng/ml; WAKO), and anti–IFN-γ (10 μg/ml) for Th17 condition.

A total of 4×10^4 resident peritoneal macrophages was sorted by BD FACSAria II for forward light scatter^{high}, side light scatter^{high}, CD11b^{high} cells from lavage of the peritoneal cavity with 5 ml PBS with 2 mM EDTA and cultured with 100 ng/ml LPS (Sigma-Aldrich), 1 μg/ml zymosan (Sigma-Aldrich), and with or without GM-CSF (WAKO). In ex vivo culture, 1×10^6 fresh splenocytes were cultured with 5 μg/ml Con A, 5 μg/ml LPS, 10 μg/ml zymosan, or 100 μg/ml curdlan (Sigma-Aldrich).

Hydroxyproline measurement

The right middle lobe was homogenized in 1 ml PBS and centrifuged at 2000 rpm for 10 min. The supernatants were incubated with equal volume of 12 N HCl at 110°C for 8 h. Then lung collagen levels were evaluated by quantifying hydroxyproline, as previously described (24).

Treatment study

Mouse anti-mouse IL-17A Ab, rat anti-mouse GM-CSF Ab, and their isotype Abs, mouse IgG1κ and rat IgG2aκ, respectively, were purchased

from eBioscience. SKG mice received 12 weekly i.p. injections of 100 μg these Abs or PBS from the day of zymosan injection. MR16-1 (rat anti-mouse IL-6R Ab) was provided by Chugai Pharmaceutical (Kanagawa, Japan). The isotype Abs of MR16-1, rat IgG1κ, were purchased from eBioscience. To prevent the production of anti–MR16-1 Ab, mice were i.p. injected with 2 mg MR16-1 on the day of zymosan injection, and then followed by 11 weekly i.p. injections of 0.5 mg MR16-1, as previously reported (25). After 12 wk, mice were euthanized and analyzed.

Statistical analysis

All analyses were performed using GraphPad Prism 5 software (GraphPad Software). The Mann–Whitney *U* test was performed for two-group comparisons. Kruskal Wallis test was performed for multiple group comparisons. Log-rank test was performed for analysis of survival curves. The p values <0.05 were considered statistically significant. Error bars in all figures indicate SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

Zymosan-treated SKG mice develop chronic–progressive and fatal ILD over several months

We found that zymosan-treated SKG mice gradually lost their weight and showed progressive debilitation to death, and this was not observed in zymosan-treated BALB/c mice or PBS-treated SKG mice (Fig. 1A, 1B). Histological analysis revealed that zymosan-treated SKG mice at the terminal phase had severe ILD characterized by massive infiltration of inflammatory cells with massive fibrosis without honeycombing, which was not observed in zymosan-treated BALB/c mice (Fig. 1C, 1D). Although SKG mice were also reported to develop dermatitis and ileitis, the infiltration of inflammatory cells in these tissues and the other organs was mild (Fig. 1F, Supplemental Fig. 1). Time series analyses revealed the ILD in zymosan-treated SKG mice showed temporally uniform and chronic interstitial cellular infiltration with fibrosis, which started in peribronchovascular lesion and accompanied follicular bronchiolitis, which was commonly seen in CTD-ILD (26), in the early phase (Fig. 1E, 4 and 6 wk). These histological characteristics were in accordance with the nonspecific interstitial pneumonia (NSIP) in CTDs. In the later phase, the infiltration of inflammatory cells was gradually increased, and massive infiltration was extended to both peribronchiolar and alveolar spaces (Fig. 1E, 8 and 12 wk). The overlap of the histological pattern and the progressive exacerbation of ILD in this mouse also showed a marked similarity to CTD-ILD (27). The histological score of zymosan-treated SKG mice started to increase from the sixth week after zymosan injection and reached a peak at the twelfth week, but these changes were not observed in zymosan-treated BALB/c mice (Fig. 1G). Furthermore, increase of histological scores well correlated with the body weight losses and their elevated mortality (Fig. 1A, 1B, 1G). These findings indicated that the possible leading cause of death was respiratory failure by progression of ILD. Collectively, these results indicated that zymosan triggered chronic inflammatory cell infiltration into the lung and consequently developed chronic–progressive and fatal ILD, which had similar characteristics with severe CTD-ILD.

ILD in zymosan-treated SKG mice is characterized by the infiltration of IL-17A– or GM-CSF–producing T cells as well as CD11b$^+$Gr1$^+$ neutrophils

Next, we further investigated the characteristics of ILD in zymosan-treated SKG mice. After 18 wk from zymosan or PBS treatment, the histological scores of the lungs revealed marked increases in zymosan-treated SKG mice (Fig. 2A). The development of fibrosis in zymosan-treated SKG mice was confirmed by hydroxyproline estimation of lungs (Fig. 2B). The histological scores of zymosan-treated BALB/c mice also slightly increased compared with those of PBS-treated BALB/c mice; however, zymosan-treated SKG mice had statistically higher histological score compared with zymosan-treated BALB/c mice, and the pulmonary hydroxyproline level of zymosan-treated BALB/c mice was statistically not different from that of PBS-treated BALB/c mice (Fig. 2A, 2B). Infiltrated cell counts in lungs were measured by flow cytometry and revealed that zymosan-treated SKG mice had increased number of total cells, IL-17A–producing CD4$^+$ T cells, GM-CSF–producing CD4$^+$ T cells, and CD11b$^+$ Gr1$^+$ neutrophils compared with PBS-treated SKG mice. Notably, the majority of GM-CSF–producing CD4$^+$ T cells in zymosan-treated SKG mice are IL-17A–nonproducing (GM-CSF$^+$ IL-17A$^-$) cells, although a small number of GM-CSF/IL-17A–double-producing (GM-CSF$^+$ IL-17A$^+$) CD4$^+$ T cells is also increased (Fig. 2C, 2D). The number of CD4$^+$ T cells, CD11b$^+$Gr1$^-$ macrophages/monocytes, and IL-6–producing macrophages/monocytes (CD11b$^+$ Gr1$^-$ IL-6$^+$) of zymosan-treated SKG mice also tended to be higher than that of PBS-treated SKG mice, but they were not statistically significant. Some fractionations of lung-infiltrating cells in zymosan-treated BALB/c mice also seemed to be higher than those in PBS-treated BALB/c mice; however, it was not statistically significant except for the number of CD8$^+$ T cells. Serum IFN-γ and IL-6 were increased in zymosan-treated SKG mice compared with PBS-treated SKG mice. Serum IL-17A was also higher in zymosan-treated SKG but not statistically significant. Both serum GM-CSF and IL-4 were not detected in all groups (Fig. 2E). These results implied the possibility that IL-17A–producing CD4$^+$ T cells as well as GM-CSF–producing CD4$^+$ T cells, which may be distinct Th cells from Th17 cells, and IL-6–producing macrophages/monocytes contributed to the development of ILD in zymosan-treated SKG mice, and these upregulated cytokines contributed to neutrophil infiltration into the lung.

IL-6 and IL-1β production from macrophages of SKG and BALB/c mice is functionally equivalent

Zymosan is widely known as a strong activator of innate immunity and induces secretion of proinflammatory cytokines such as IL-6 or IL-1β by macrophages (28). Intraperitoneally injected zymosan is basically thought to activate residential peritoneal macrophages and then initiates systemic inflammatory process. The differentiation of Th17 cells is known to be driven by macrophage-derived IL-6 in SKG mice (15), and GM-CSF-production by CD4$^+$ T cells is reported to be regulated by inflammasome-derived IL-1β (29). However, because SKG mice are mutants of gene encoding ZAP70 in T cells, the ability of SKG macrophages to drive IL-17A– or GM-CSF–producing T cell differentiation itself is considered equivalent to that of BALB/c macrophages. Therefore, SKG T cells themselves must have a high potential to differentiate into IL-17A– or GM-CSF–producing cells. To assess this hypothesis, we analyzed the ability of macrophages from SKG and BALB/c mice to induce the differentiation of IL-17A– or GM-CSF–producing T cells. The FACS-sorted peritoneal macrophages of SKG or BALB/c mice were cultured with zymosan or LPS for 72 h, and the concentrations of IL-6 and IL-1β in the culture supernatants revealed there was no difference between SKG and BALB/c (Fig. 3A). We also analyzed the effect of GM-CSF at the various concentrations to enhance these cytokine productions from macrophages in the presence or absence of LPS or zymosan. Again, IL-6 and IL-1β productions of SKG and BALB/c macrophages were equally and significantly enhanced by GM-CSF (Fig. 3). Taken together, these results indicated that the differences of the phenotype of zymosan-treated SKG or BALB/c mice do not depend on the qualitative differences of their macrophages.