Screening and characterization of myositis-related autoantibodies in COVID-19 patients

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Abstract

An efficient host immune response against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, COVID-19) appears to be crucial for controlling and resolving this viral infection. However, many studies have reported autoimmune characteristics in severe COVID-19 patients. Moreover, clinical observations have revealed that COVID-19-associated acute distress respiratory syndrome shares many features in common with inflammatory myopathy including interstitial lung disease (ILD), most particularly rapidly progressive (RP)-ILD. This study explored this phenomenon by seeking to identify and characterize myositis-specific and related autoantibodies in 25 COVID-19 patients with mild or severe symptoms. Line blot analysis with the EUROLINE Myopathies Ag kit identified 9 (36%) patients with COVID-19 with one or more autoantibodies against several myositis-related antigens (Jo-1, Ku, Mi-2β, PL-7, PL-12, PM-Scl 75, PM-Scl 100, Ro-52, and SRP); no anti-MDA5 antibodies were detected. As the presence of antibodies identified by line blots was unrelated to disease severity, we further characterized the autoantibodies by radioimmunoassay, in which [^{35}S] methionine-labeled K562 cellular antigens were precipitated and visualized by gel electrophoresis. This result was confirmed by an immunoprecipitation assay and immunoblotting; 2 patients exhibited anti-Ku70 and anti-Ku80 antibodies.

Our data suggest that it is necessary to use more than one method to characterize and evaluate autoantibodies in people recovered from COVID-19, in order to avoid misinterpreting those autoantibodies as diagnostic markers for autoimmune diseases.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
SARS-COV-2 infection leads to excessive production of inflammatory cytokines, abnormal induction of autoantibodies, and acute distress respiratory syndrome in severe cases. These clinical features are also a clinical phenomenon of interstitial lung disease, which causes morbidity and mortality in patients with inflammatory myopathy.

WHAT QUESTION DID THIS STUDY ADDRESS?
We determined whether COVID-19 patients have myositis-specific and related autoantibodies and how levels of these autoantibodies may reflect the severity of SARS-CoV-2-induced lung injury.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
The combination of line blot assay, radioimmunoassay, and immunoprecipitation assay investigations provides accurate autoantibody data. As line blot identification of myositis-related autoantibodies was unrelated to disease severity, radioimmunoassay and immunoprecipitation assay data characterized anti-Ku70 and anti-Ku80 autoantibodies in COVID-19 patients.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
Our data suggest that it is necessary to carefully evaluate the presence of autoantibodies in people recovered after COVID-19 in order to avoid misinterpreting those autoantibodies as diagnostic markers for autoimmune diseases.

INTRODUCTION

Coronaviruses are a family of enveloped, positive-stranded RNA viruses that infect humans and animals and cause respiratory, gastrointestinal, or neurological diseases. The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, COVID-19) disease has been a pandemic since December 2019. At the time of writing this article, COVID-19-related deaths worldwide had reached 5.5 million.[1] An efficient host immune response involving innate and adaptive immunity against SARS-CoV-2 appears to be crucial for controlling and resolving this viral infection. Notably, the severity of COVID-19 may be associated with the excessive production of proinflammatory cytokines leading to a "cytokine storm" and subsequent acute lung injury/acute respiratory distress syndrome (ARDS).[2,3] A variety of autoimmune characteristics have been observed in cases of severe COVID-19, with the findings of lymphocytopenia (85.7%), elevated levels of C-reactive protein (94.7%), and interleukin-6 (89.5%), as well as anti-52 kDa SSA/Ro (Ro-52) antibodies (20%), anti-60 kDa SSA/Ro (Ro-60) antibodies (25%), and antinuclear antibodies (50%).[4]

Over several years of research, our clinical observations have revealed that COVID-19-associated ARDS shares many autoimmune features with interstitial pneumonia, particularly with a rapidly progressive (RP) interstitial lung disease (ILD), including melanoma differentiation-associated gene 5 (MDA5) antibody-associated RP-ILD.[5–7] These common features include ARDS, pathological lung images (as seen in high-resolution computed tomography [CT] images), and cytokine release syndrome characterized by participating immune cell populations and the release of cytokines.[3,8–10] ILD, especially RP-ILD, is a major cause of morbidity and mortality in patients with dermatomyositis (DM), because of ARDS.[11–13] Our research and other studies have shown that autoantibodies in DM are highly associated with ILD, particularly RP-ILD, and serve as prognosis markers for autoimmune-related ILD.[5,11,14–16] Growing evidence also indicates that antibody-mediated immunity has a fundamental role in the pathogenesis of RP-ILD, suggesting a pathological role for autoantibodies. MDA5 is a pattern recognition receptor responsible for the recognition of RNA viruses that consequently induces innate immune responses and the production of proinflammatory cytokines.[17] Although it is not clear as to the causal

relationship between anti-MDA5 antibodies and DM-ILD, anti-MDA5 antibodies are strongly associated with RP-ILD.[11] COVID-19-associated mortality differs amongst different age groups,[18] which suggests the involvement of an age-related immune regulatory factor. Since autoantibody production increases with aging[19] and MDA5 is an innate immune sensor for coronaviruses,[20] it is possible that pre-existing or viral-induced anti-MDA5 antibodies or other myositis-related autoantibodies may play a role in the pathogenesis of SARS-CoV-2-induced lung injury.

Although various anti-immune or anti-inflammatory inhibitors, interleukin-6 (IL-6) inhibitors, IL-1 inhibitors, tumor necrosis factor alpha inhibitors, corticosteroids, and intravenous immunoglobulin have all been suggested as therapeutic options for COVID-19 patients,[2,21] immunosuppression for hyperinflammation in COVID-19 could be a double-edged sword due to the lack of a prognostic marker for disease severity. Thus, factors are urgently needed that can predict progression towards severe acute lung injury. In this study, the combination of line blot assay, radioimmunoassay, and immunoprecipitation assay investigations provided accurate autoantibody data. Line blot identification of myositis-related autoantibodies was unrelated to disease severity. Radioimmunoassay and immunoprecipitation assay data characterized anti-Ku70 and anti-Ku80 autoantibodies in COVID-19 patients.

METHODS

Cell culture

K562 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco) medium containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco) and maintained at 37°C in a humified 5% CO_2 atmosphere. Cells were subcultured every 2 to 3 days. *Mycoplasma* contamination testing was performed using a MycoAlert kit (Lonza).

Patients and methods

Twenty-five COVID-19 Hispanic or Caucasian sera samples were purchased from Boca Biolistics in the USA. We included COVID-19 patients without pre-existing conditions, as described in Table 1. All patients were documented as having SARS-CoV-2 infection by either the Cepheid GeneXpert or Roche Cobas system. SARS-CoV-2-associated immunoglobulin G (IgG) was measured by the SARS-CoV-2 IgG assay (Architect). Nine sera samples from myositis-specific antibody-related ILD patients and 2 healthy controls were collected by Dr. Joung-Liang Lan.

The ILD reference samples were collected from patients diagnosed with ILD in China Medical Hospital, Taiwan. Each ILD reference sample positively reacts with a corresponding indicated antigen, as confirmed by both the EUROLINE Autoimmune Inflammatory Myopathies 16 Ag (IgG) (DL 1530–1601-4 G) kit and radioimmunoprecipitation assays conducted in our laboratory (used for research or diagnostic purposes). The EUROLINE Autoimmune Inflammatory Myopathies 16 Ag (IgG)(DL 1530–1601-4 G) kit screened for related autoantibodies in the 25 COVID-19 patient sera samples, following the manufacturer's instructions: EJ, glycyl-tRNA synthetase; Jo-1, histidyl-tRNA synthetase; Ku, DNA binding protein; MDA5, melanoma differentiation-associated protein 5; Mi-2α, chromodomain-helicase-DNA-binding protein (CHD) 3; Mi-2β, chromodomain-helicase-DNA-binding protein (CHD) 4; NPX2, nuclear matrix protein 2; OJ, isoleucyl-tRNA synthetase multienzyme complex; PM-Scl 75, polymyositis-scleroderma 1 complex protein (75 kD); PM-Scl 100, polymyositis-scleroderma 1 complex protein (100 kD); PL-7, threonyl-tRNA synthetase; PL-12, alanyl-tRNA synthetase; Ro-52, tripartite motif-containing protein 21 (TRIM21); SAE1, small ubiquitin-like modifier 1 activating enzyme; SRP, signal recognition particle; TIF1γ, transcriptional intermediary factor 1 gamma. The EUROLineScan program was used to evaluate the signal intensity from each line blot. Based on the signal intensity, we could classify the results into four groups: 0–5 (0, negative); 6–10 ([+], borderline); 11–25 or 26–50 (+ or ++, positive); and >50 (+++, strongly positive). The borderline (+) results should be evaluated as an increase, but negative.

Autoantibody purification

All sera in this study were used to purify the autoantibodies for immunoprecipitation. Sera were centrifuged at 17,970***g*** and 4°C for 5 min to remove impurities. The sera were then incubated with protein G beads (GeneTex) and protein A beads (GE Healthcare) at a ratio of 1:1 in NP-40 buffer (for cytosol antigens: 150 mM NaCl [Sigma], 20 mM, pH 8.0 Tris–HCl [Sigma], 5 mM EDTA [Sigma] and 0.1% NP-40 [Sigma]; for nucleus antigens: 300 mM NaCl, 20 mM, pH 8.0 Tris–HCl, 5 mM EDTA and 0.3% NP-40) at 4°C for 1 h. The beads were conjugated with autoantibodies and washed once with the same buffer and were then ready to use in immunoprecipitation investigations.

Radioimmunoassay

Cells were seeded into a 10 cm dish at a concentration of 6×10^5 cells/ml and cultured overnight with RPMI medium

TABLE 1 Clinical characteristics of 25 COVID-19 patients

Characteristic		All cases (N = 25)	Disease severity of COVID-19	
			Mild cases (n = 12)	Severe cases (n = 13)
Demographics	Average age, years (mean ± SD)	53.08 ± 12.58	51 ± 13.33	55 ± 12.06
	Gender, M/F	16/9	7/5	9/4
	Duration of hospitalization (n)	None (2), <15 days (9), >15 days (14)	None (2), <15 days (9), >15 days (1)	>15 days (13)
	Duration of mechanical ventilation (n)	None (12), <5 days (6), >5 days (7)	None (12)	<5 days (6), >5 days (7)
	SARS-CoV-2 IgG-positive S/C (mean ± SD)	7.35 ± 1.44	7.84 ± 1.47	6.82 ± 1.26
	Smoker, n (%)	8 (32)	3 (25)	5 (38.46)
	Other chronic diseases, n (%)	7 (28)	0 (0)	7 (53.85)
Symptoms, n (%)	Nausea or vomiting	10 (40)	2 (16.67)	8 (61.54)
	Difficulty breathing	14 (56)	4 (33.33)	10 (76.92)
	Cough	13 (52)	10 (83.33)	3 (23.08)
	General malaise	11 (44)	4 (33.33)	7 (53.85)
	Sore throat	6 (24)	3 (25)	3 (23.08)
	Headache	11 (44)	5 (41.67)	6 (46.15)
	Diarrhea	6 (24)	4 (33.33)	2 (15.38)
	Fever	23 (92)	10 (83.33)	13 (100)
	Loss of smell and taste	3 (12)	2 (16.67)	1 (7.69)
	Weight loss	1 (4)	0 (0)	1 (7.69)
	Chills	3 (12)	3 (25)	0 (0)
	Muscle pain	2 (8)	2 (16.67)	0 (0)
	Abdominal pain	1 (4)	1 (8.33)	0 (0)
	Shortness of breath	2 (8)	2 (16.67)	0 (0)
	Runny nose	1 (4)	1 (8.33)	0 (0)
	Fatigue	1 (4)	1 (8.33)	0 (0)

Abbreviations: F, female; IgG, immunoglobulin G; M, male; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation.

containing [^{35}S]methionine (EasyTag™ Methionine, L-[^{35}S]-, PerkinElmer). On the second day, cells were lysed with NP-40 buffer (for cytosol antigens: 150 mM NaCl, 20 mM, pH 8.0 Tris–HCl, 5 mM EDTA and 1% NP-40; for nucleus antigens: 300 mM NaCl, 20 mM, pH 8.0 Tris–HCl, 5 mM EDTA and 1% NP-40) and cleared by centrifugation at 17,970 g and 4°C for 15 min. The cell lysates were cleaned with protein G beads for 30 min then centrifuged at 5870g and 4°C for 5 min to remove the beads. Cell lysates were incubated with beads conjugated with the autoantibodies purified from patient sera in NP-40 buffer, identical to the buffer used in the purification process, at 4°C for 4 h. Beads were washed four times with the same NP-40 buffer. Proteins were eluted into dye containing 2-mercaptoethanol (2ME, Sigma) at 100°C for 10 min and resolved with gel electrophoresis using NuPAGE 4%–12%

Bis-Tris Gel (Invitrogen™NOVEX™). Gels were dried on a heater at 60°C for 6 h. High-performance chemiluminescence film (GE Healthcare) visualized signals from [^{35}S]methionine of the protein. Films were exposed to gels in a cassette at −80°C for 7 or 14 days.

Immunoprecipitation and immunoblotting

K562 cells were lysed with NP-40 buffer (150 mM NaCl, 20 mM, pH 8.0 Tris–HCl, 5 mM EDTA, and 1% NP-40) and cleared by centrifugation at 17,970 g and 4°C for 15 min. Cell lysates (50 μl) were prepared as the control for immunoprecipitation. The remaining cell lysates were cleaned with protein G beads for 20 min then centrifuged at 5870 g and 4°C for 1 min to remove the beads.

The supernatant was split equally between each group. Patient sera samples were added and the mixtures were incubated by rotation at 4°C for 2.5 h (150 mM NaCl, 20 mM, pH 8.0 Tris–HCl, 5 mM EDTA, and 0.1% NP-40). The mixtures were incubated with beads at 4°C for another 30 min. Supernatant was removed after centrifugation. Beads were washed three times with the same NP-40 buffer. Proteins were eluted into dye at 60°C for 10 min. After centrifugation, dyes containing proteins were moved without the beads to new Eppendorf tubes. 2-ME was added into protein samples and the mixtures were incubated at 95°C for 10 min to denature the proteins. Protein samples were resolved on Bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Immobilon Western Chemiluminescent HRP (Millipore) visualized proteins on the ChemiDoc™ MP imaging system (BIO-RAD). Ku-70 polyclonal antibody (10723-1-AP), Ku-80 polyclonal antibody (16389-1-AP), and TARS polyclonal antibody (14773-1-AP) were purchased from the Proteintech Group, Inc.

Statistical analysis

Patients were classified into two groups based on time required for mechanical ventilation: mild cases and severe cases. The χ^2 test was used to compare categorical variables. A two-tailed $p < 0.05$ was considered statistically significant.

Ethics committee approval

Twenty-five Hispanic or Caucasian COVID-19 sera samples were purchased from Boca Biolistics in the USA, under approval from an Independent Investigational Review Board, Inc., for Boca Biolistics in the USA (SOP 10–00414 Rev E). Nine sera samples from myositis-specific antibody-related ILD patients and two healthy controls were collected by Dr. Joung-Liang Lan, under approval from the Ethics Committee of China Medical University Hospital in Taiwan (CMUH104-REC3-093).

RESULTS

Patient characteristics

Clinical parameters of the 25 patients with COVID-19 are shown in Table 1. All patients had a positive molecular diagnosis of SARS-CoV-2 and were separated into two groups based on mechanical ventilation requirements (mild, $n = 12$; severe, $n = 13$). Sixteen patients were men and 9 were women, with an average age of 53 years. In the mild group, 2 patients were not hospitalized and only 1 patient was hospitalized for more than 15 days. In the severe group, all 13 patients were hospitalized for more than 15 days. Eight of the 25 patients were smokers; 3 were in the mild group and 5 in the severe group. Fever was the most common symptom (92% of all patients had an average body temperature of 38.8°C); only 2 patients in the mild group did not have a fever. Breathing difficulties afflicted 33% of patients in the mild group and 77% of the severe group. All COVID-19 patients were positive for IgG antibody against SARS-CoV-2.

Positivity of myositis-related autoantibodies identified by line blotting

To determine whether autoantibodies against myositis-specific or related antigens are present in patients with COVID-19, an immunoblot analysis of sera from all patients was performed using the commercial EUROLINE kit, which contains myositis-specific or related antigens, including MDA5. The EUROLINE kit is a clinical diagnosis kit that is routinely used to detect autoantibodies in autoimmune patients; healthy individuals have a relatively very low incidence of positive results. Corresponding specificities with and without anti-SSA/Ro52 were 62.34% and 96.10%, respectively.[22,23] Table 2 demonstrates that the entire study cohort displayed autoantibodies against myositis-specific and related antigens, including Jo-1, Ku, Mi-2β, PL-7, PL-12, PM-Scl 75, PM-Scl 100, Ro-52, and SRP. Some 28% had one autoantibody, 4% had two autoantibodies, and 4% had three or more autoantibodies. In total, 36% of patients had one or more myositis-related autoantibodies. Autoantibodies in individual patients are shown in Table 3. Despite the high prevalence of myositis-related autoantibodies, these did not relate to disease severity (Table 2). These data suggest that while patients with COVID-19 may be highly positive for myositis-related autoantibodies, these are not associated with disease severity.

Radioimmunoassay confirmation of myositis-related autoantibodies

To further verify the presence of autoantibodies in the sera of patients with COVID-19, we performed the radioimmunoassay using immunoprecipitation of [35S]methionine-labeled K562 cellular antigens to confirm the molecular pattern of each antigen. In this experiment, we used sera from patients with RP-ILD as the positive control of each

TABLE 2 Statistical analysis of autoantibodies present in sera from COVID-19 patients

		All cases (n = 25)	Disease severity of COVID-19		χ^2 (with Yates' Correction)
			Mild cases (n = 12)	Severe cases (n = 13)	p value
AutoAbs, n (%)	EJ	0 (0)	0 (0)	0 (0)	N/A
	Jo-1	1 (4)	0 (0)	1 (7.69)	0.9674
	Ku	2 (8)	1 (8.33)	1 (7.69)	0.4973
	MDA5	0 (0)	0 (0)	0 (0)	N/A
	Mi-2α	0 (0)	0 (0)	0 (0)	N/A
	Mi-2β	1 (4)	1 (8.33)	0 (0)	0.9674
	NPX2	0 (0)	0 (0)	0 (0)	N/A
	OJ	0 (0)	0 (0)	0 (0)	N/A
	PM-Scl 75	2 (8)	2 (16.67)	0 (0)	0.4256
	PM-Scl 100	1 (4)	0 (0)	1 (7.69)	0.9674
	PL-7	3 (12)	2 (16.67)	1 (7.69)	0.9411
	PL-12	1 (4)	0 (0)	1 (7.69)	N/A
	Ro-52	1 (4)	0 (0)	1 (7.69)	0.9674
	SAE1	0 (0)	0 (0)	0 (0)	N/A
	SRP	1 (4)	1 (8.33)	0 (0)	0.9674
	TIF1γ	0 (0)	0 (0)	0 (0)	N/A
Presence of 1 autoAb, n (%)		7 (28)	3 (25)	4 (30.77)	0.7510
Presence of 2 autoAbs, n (%)		1 (4)	0 (0)	1 (7.69)	0.4973
Presence of 3 or more autoAbs, n (%)		1 (4)	1 (8.33)	0 (0)	0.9674

Note: The EUROLINE Autoimmune Inflammatory Myopathies Ag (IgG) kit screened for related autoantibodies (autoAbs) in the 25 COVID-19 patient sera samples, following the manufacturer's instructions: EJ, glycyl-tRNA synthetase; Jo-1, histidyl-tRNA synthetase; Ku, DNA binding protein; MDA5, melanoma differentiation-associated protein 5; Mi-2α, chromodomain-helicase-DNA-binding protein (CHD) 3; Mi-2β, chromodomain-helicase-DNA-binding protein (CHD) 4; NPX2, nuclear matrix protein 2; OJ, isoleucyl-tRNA synthetase multienzyme complex; PM-Scl 75, polymyositis-scleroderma 1 complex protein (75 kD); PM-Scl 100, polymyositis-scleroderma 1 complex protein (100 kD); PL-7, threonyl-tRNA synthetase; PL-12, alanyl-tRNA synthetase; Ro-52, tripartite motif-containing protein 21 (TRIM21); SAE1, small ubiquitin-like modifier activating enzyme 1; SRP, signal recognition particle; TIF1γ, transcriptional intermediary factor 1 gamma.

antigen that was recognized by autoantibodies. The signal of each antigen was identified with the correct molecular weight using sera from patients with RP-ILD (Figure 1), which is consistent with previously published data. One or more specific protein bands in each lane of ILD patients served as positive controls to demonstrate the presence of the specific autoantibody against each Ag (Figure 1; the solid arrowheads indicate specific antigens). We subsequently tested all positive samples identified by the EUROIMMUN kit, as shown in Figure 1. In contrast to the data from the previous screening, we could not detect any obviously positive signals in sera from patients with COVID-19 (Figure 1). Only two samples, COVID-19-6 and COVID-19-20 (Figure 1a; lanes 3 and 5 and Figure 1b; lanes 1 and 3), with the empty arrowhead in Figure 1, displayed a background similar to that of RP-ILD patients (ILD-PL-7 and ILD-Ku) with both anti-PL-7 (Figure 1a; lane 6 and Figure 1b; lane 4) and anti-Ku (Figure 1a; lane 7 and Figure 1b; lane 5) autoantibodies. Notably, immunoprecipitation detected many unidentified

protein bands in patients with COVID-19 that did not correspond with the myositis-related autoantibodies we screened, which implies that there could be other unknown autoantibodies present in those patients. Using immunoprecipitation followed by immunoblotting revealed two patients (#6 and #20) with anti-Ku or anti-PL-7 antibodies, who had previously displayed the same antibodies. Immunoblots (Figure 2a,b) identified anti-Ku70 and anti-Ku80 antibodies in both patients. In contrast, we did not observe anti-PL-7 antibodies (Figure 2c). These data suggest considerable inconsistency between the three diagnostic methods, with the radioimmunoassay showing the greatest accuracy in identifying autoantibodies in patients with COVID-19.

DISCUSSION

Recent studies have suggested that SARS-CoV-2 infection stimulates the production of many autoantibodies,

TABLE 3 Results of antibody recognition using the EUROLINE Autoimmune Inflammatory Myopathies Ag (IgG) kit and sera from 25 COVID-19 patients

		EJ	Jo-1	Ku	MDA5	Mi-2α	Mi-2β	NPX2	OJ	PM-Scl 75	PM-Scl 100	PL-7	PL-12	Ro-52	SAE1	SRP	TIF1γ
Severe cases	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	5	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	6	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–
	7	–	–	–	–	–	–	–	–	–	–	–	–	+++	–	–	–
	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	9	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
	10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	13	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Mild cases	14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	17	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	20	–	–	+	–	–	+	–	–	+	–	+	–	–	–	–	–
	21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	23	–	–	–	–	–	–	–	–	++	–	–	–	–	–	+	–
	24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

–, negative; + or ++, positive; +++, strongly positive.
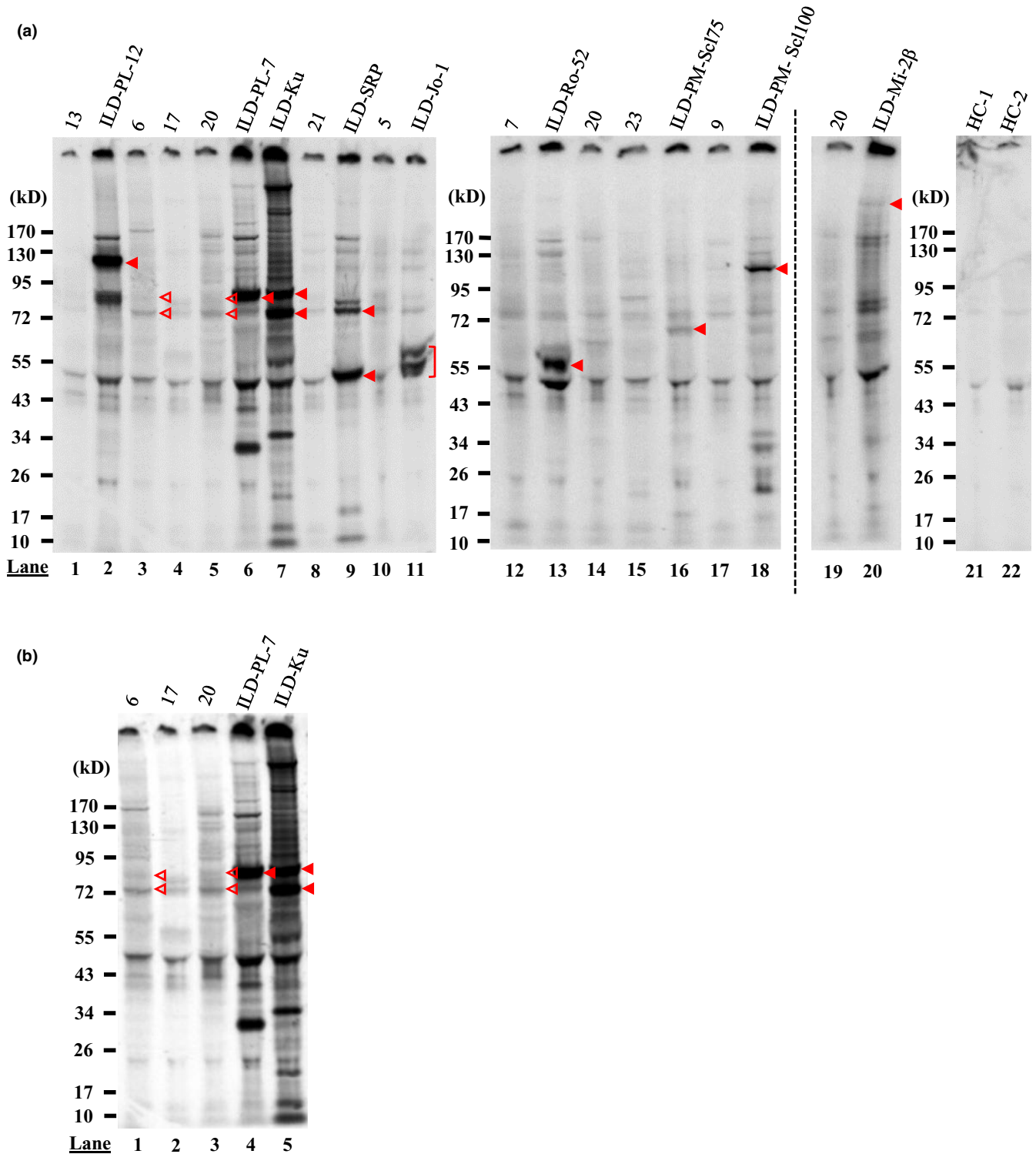
FIGURE 1 Immunoprecipitation analysis of antigens recognized by autoantibodies purified from sera of either interstitial lung disease patients (ILD) or COVID-19 patients (COVID). [35S]Methionine-labeled antigens were extracted from K562 cells after overnight incubation with [35S]methionine-containing medium. Arrowheads and parentheses indicate positions of autoantibody-recognized antigens (red solid arrowheads and red parentheses: recognized by autoantibodies of sera from ILD patients; red empty arrowheads: recognized by autoantibodies of sera from COVID-19 patients). Molecular weight is indicated on the left-hand side of each figure. HC, healthy control. Left (a), exposed for 7 days. Right (a), exposed for 14 days. (b) exposed for 7 days. Dashed line: removal of the lane due to repeated data of lane 3 (patient 6).
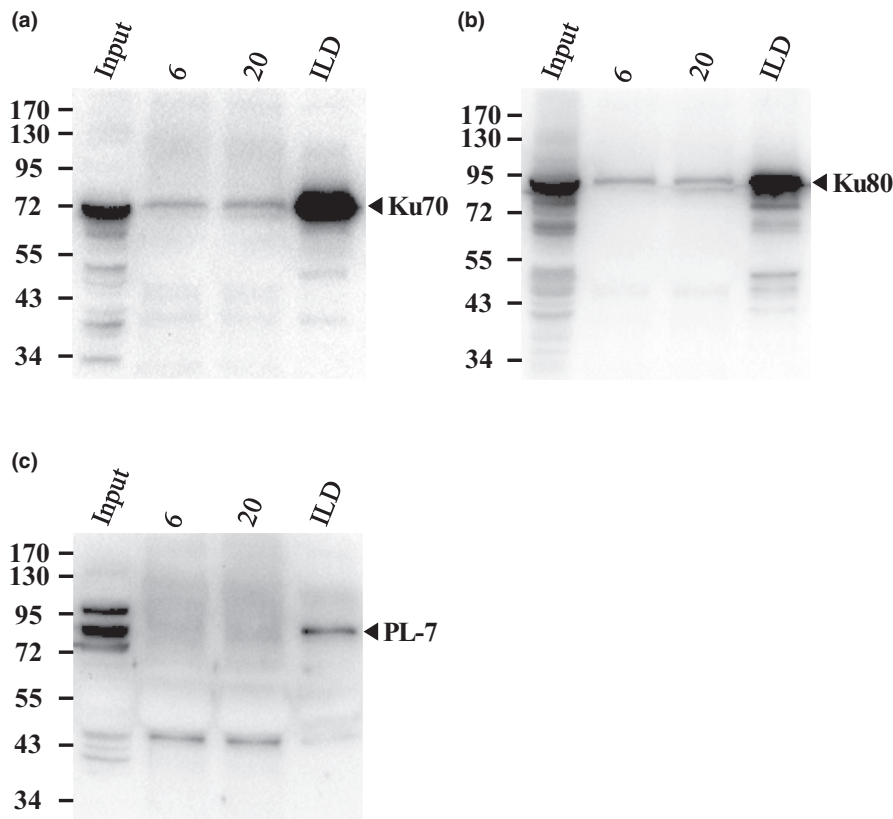
FIGURE 2 Immunoprecipitation followed by Western blot was performed to visualize specific proteins recognized by autoantibodies from patient sera. (a) Immunoprecipitation of Ku70 protein. (b) Immunoprecipitation of Ku80 protein. (c) Immunoprecipitation of PL-7 protein. Molecular weight is indicated on the left-hand side of each figure.

including anti-Ro52 antibody (20%), anti-Ro60 antibody (25%), antinuclear antibody (50%),[4,24,25] and anti-type I interferon (IFN) antibody.[26] Autoantibodies against type I IFNs are highly related to life-threatening SARS-CoV-2 infection,[26] suggesting the importance of autoantibodies in immune dysregulation. We therefore screened for the presence of these myositis-related autoantibodies in order to understand whether or not they are involved in the pathogenesis of COVID-19 or serve as prognostic markers for these patients. Consistent with a previous publication that used dot blot analysis,[4] we identified myositis-related autoantibodies anti-Ro 52 and anti-Jo-1. We also found other autoantibodies, including anti-Ku, anti-Mi-2β, anti-PL-7, anti-PL-12, anti-PM-Scl 75, anti-PM-Scl 100, and anti-SRP antibodies and 36% of the patients in our cohort had one or more myositis-related autoantibodies identified by line blots with the EUROLINE Myopathies Ag kit. However, none of these autoantibodies was related to disease severity. We therefore decided to further characterize the pattern of autoantigens. After analyzing these antibodies by radio-immunoassay and immunoprecipitation assay, we discovered that the results were inconsistent between the line blot analysis and radioimmunoassay findings. The molecular patterns suggested that some autoantibodies did not reflect the correct molecular weight, which implies that these antibodies may not target myositis-related antigens. Previous studies have revealed the

inconsistency between the results of the line blot and immunoprecipitation.[27–29] Moreover, many previously reported that autoantibodies, such as anti-Ro-52 antibody and anti-Jo-1 antibody,[4] may share the same features and thus require further characterization with, for instance, a radioimmunoassay, which differs from the line blots, to confirm specific antigens.

There is growing evidence that antibody-mediated immunity has a fundamental role in the pathogenesis of autoimmune-related ILD.[5,14,15] Although the causal relationship between autoantibodies and ILD remains to be clarified, our research group and others have shown that myositis-related autoantibodies are highly associated with ILD, particularly RP-ILD.[5,11,14–16] Other studies have also shown that SARS-CoV-2 infection can trigger autoimmune responses,[4,25] which may not only relate to COVID-19-associated mortality but may also shape immune regulation to an autoimmune disease-prone status. As depicted in Figure 1, the normal controls have a clear background without autoantibodies, whereas COVID-19 patients developed many autoantibodies against different cellular antigens that are very similar to those in autoimmune RP-ILD patients, who have a strong background of high-level autoantibodies. Although a specific autoantibody for COVID-19 remains to be identified, our findings support the hypothesis that SARS-CoV-2 infection can trigger immune dysregulation and this dysregulation may persist in patients even after they recover from the

virus infection. More studies are required to understand whether this may become an important risk factor for autoimmune diseases, especially for RP-ILD.

The assessment of both anti-aminoacyl-tRNA synthetase and anti-MDA5 autoantibodies is useful for predicting the clinical course and prognosis of DM/DM-ILD patients.[30] Anti-aminoacyl-tRNA synthetase antibodies, including anti-Jo-1, anti-PL-7, anti-PL-12, anti-EJ, and anti-OJ antibodies, are associated with a wide spectrum of autoimmune diseases, including myositis and ILD.[30] Moreover, anti-aminoacyl-tRNA synthetase antibodies are associated with a better prognosis in DM/DM-ILD patients,[31,32] whereas anti-MDA5 antibodies are associated with a poorer prognosis in these patients.[33] Despite some evidence demonstrating the presence of anti-MDA5 antibodies in patients infected with SARS-CoV-2, very few reports have suggested a possible pathological role of anti-MDA5 antibodies during this infection.[34] However, as we show in Table 1, no positive patients in our cohort were found to have anti-MDA5 antibodies. In contrast, some patients presented with anti-Ku70 and anti-Ku80 autoantibodies. The genetic background of our Hispanic and Caucasian cohort may mean that these patients rarely have the human leukocyte antigens (HLA) DRB1*0101/*0405 and DRB1*0401/*12:02, which are found in the Han Chinese and Japanese and are highly associated with the presence of anti-MDA5 antibodies.[35,36] This genetic difference may explain the absence of anti-MDA5 antibodies in our report, although Wang et al.[34] have reported finding that 48.2% (132/274) of COVID-19 patients in China presented with anti-MDA5 autoantibodies, identified by immunoprecipitation and enzyme-linked immunosorbent assay (ELISA). Isotope-labeled protein immunoprecipitation with serum or plasma is the gold standard, showing high sensitivity for determining the existence of an autoantibody. Differences in genetic backgrounds and methodologies might explain this controversial finding. In this study, we have evaluated the presence of myositis-related antibodies, anti-Ku70 and anti-Ku80, using multiple methods to confirm whether these antibodies contribute to the prognosis of COVID-19 patients. Several reports have suggested the presence of autoantibodies in COVID-19 patients,[25,26,37,38] but have lacked further confirmation by either radioimmunoassay or immunoprecipitation assay. On the strength of our study results, we recommend that investigations into these autoantibodies are revised, using the gold standard method of radioimmunoassay.

AUTHOR CONTRIBUTIONS

J.-L.H. and J.-L.L. wrote the manuscript. J.-L.H. designed the research. K.-F.T. performed the research. J.-T.H., Y.-H.L., and C.-K.C. analyzed the data. J.-L.L., D.-Y.C., and P.-R.H. contributed new reagents.

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