Pathomechanisms of Anti–Cytosolic 5′-Nucleotidase 1A Autoantibodies in Sporadic Inclusion Body Myositis

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Objective: Sporadic inclusion body myositis (sIBM), an intractable progressive muscle disease, frequently occurs in older persons. sIBM pathogenesis may involve protein degradation dysfunction and immune abnormalities. Autoantibodies recognizing cytosolic 5′-nucleotidase 1A (cN1A) were found in plasma and serum from sIBM patients. However, whether anti-cN1A autoantibodies play a pathogenic role in sIBM is controversial. This study investigated the pathogenic properties of anti-cN1A autoantibodies in sIBM pathogenesis.

Methods: We developed a cell-based assay to detect anti-cN1A autoantibodies, which we found in serum from patients with neuromuscular diseases including sIBM. We also investigated the clinicopathological differences between sIBM patients with and without the autoantibodies. We used passive in vitro and in vivo immunization models to evaluate the pathogenic role of the autoantibodies.

Results: Of 67 patients with sIBM, 24 (35.8%) possessed anti-cN1A autoantibodies as determined via our cell-based assay. In the anti-cN1A–positive group, the percentage of patients with hepatitis C virus antibodies was significantly lower and the mean area of type 2 myofibers was significantly smaller compared with the autoantibody-negative group. In the in vitro passive immunization model, p62/SQSTM1 significantly increased in anti-cN1A–positive sIBM immunoglobulin G (IgG)-supplemented cells. In the in vivo passive immunization model, anti-cN1A–positive sIBM IgG-injected mice demonstrated p62/SQSTM1-positive sarcoplasmic aggregates in myofibers, associated with macrophage infiltration.

Interpretation: Our cell-based assay is useful for anti-cN1A autoantibodies detection. Patients with anti-cN1A autoantibodies demonstrated unique clinicopathological features. In vitro and in vivo passive immunization model results suggest that anti-cN1A autoantibodies may affect protein degradation in myofibers.

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Sporadic inclusion body myositis (sIBM) is the most common acquired inflammatory myopathy in patients older than 50 years, especially in Western countries. The number of sIBM patients has been increasing in Japan.[1] Long-term observational studies showed that most patients could not ambulate independently at 7 years after symptom onset, the result being that patients were wheelchair-bound after approximately 15 years.[2–4] To date, no known effective treatments are available for the disease. Although both protein degradation dysfunction and immune abnormalities have been implicated in the etiology of sIBM, the precise pathogenic mechanisms remain unclear.

Recently, 2 different groups identified autoantibodies against cytosolic 5′-nucleotidase 1A (cN1A) in plasma

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TABLE 1. Clinical Features of Patients with sIBM

Case	Sex	Age at Disease Onset, yr	CK, IU/l	Anti-cN1A Ab	Degree of Cell Infiltration	Myofibers with RV, %	Mean Area of Type 2 Fibers, μm^2	Diagnosis
1	F	40	341	−	Strong	10	1,981.3	PD
2	M	68	364	+	Strong	10	541.5	CD
3	M	74	510	−	Mild	1.5	2,204.8	CD
4	M	79	235	−	Strong	9.6	2,308.1	CD
5	M	77	563	+	Strong	4.2	680.3	CD
6	F	73	2,058	−	Mild	2.6	1,981.2	CD
7	F	49	911	−	Strong	0.4	1,835.2	CD
8	M	70	799	−	Strong	1.8	1,809.2	CD
9	F	73	340	+	Strong	3.9	1,278.2	CD

Degree of cell infiltration was classified as mild or strong according to the number of non-necrotic fibers that were invaded or surrounded by inflammatory cells per low-power field of view: $1 \leq$ mild < 3, strong ≥ 3.
Ab = antibody; CD = clinically defined; CK = creatine kinase; F = female; M = male; PD = pathologically defined; RV = rimmed vacuoles; sIBM = sporadic inclusion body myositis.

and serum samples from patients with sIBM.[5,6] Larman et al,[5] after utilizing a dot-blot assay, reported that moderate reactivity of anti-cN1A autoantibodies was 70% sensitive and 92% specific and that high reactivity was 34% sensitive and 98% specific for the diagnosis of sIBM. Pluk et al[6] used immunoprecipitation to demonstrate high concentrations of anti-cN1A autoantibodies in 33% of serum samples from sIBM patients. Measurement of anti-cN1A autoantibodies is thus a potentially valuable method for clinical diagnosis of sIBM, as well as a clue to clarifying the role of autoimmunity in sIBM pathogenesis.

In contrast, another study found anti-cN1A autoantibodies in a considerable proportion of sIBM patients but also in other patients with several different autoimmune diseases, including systemic lupus erythematosus and Sjögren syndrome, that were not associated with muscle diseases.[7,8] Thus, whether the autoantibodies play a pathogenic role in sIBM is still unclear.

The purposes of this study were therefore to confirm the usefulness of a new cell-based assay for detection of the anti-cN1A autoantibodies in the serum from patients with various neuromuscular diseases including sIBM; to determine whether clinicopathological differences exist between sIBM patients with and without the autoantibodies; and to investigate a possible pathogenic role of the autoantibodies by using passive immunization models.

Materials and Methods

Patients and Blood and Muscle Samples

All samples were collected after informed written consent was obtained from the patients and according to protocols approved by the Ethics Committee of Kumamoto University, Graduate School of Medical Sciences (Rinri No. 937 and No. 1124). For measurement of the anti-cN1A autoantibodies, serum samples were collected from 225 patients at Kumamoto University Hospital, Tohoku University Hospital, and Nara Medical University Hospital who had the following diseases: sIBM (n = 67), inflammatory myopathies other than sIBM (polymyositis [n = 36], dermatomyositis [n = 31], immune-mediated necrotizing myopathy associated with anti–signal recognition particle [SRP] autoantibody [n = 8]), noninflammatory muscle diseases (n = 41), fasciitis (n = 1), autoimmune diseases (n = 15), and neurogenic muscular atrophy (n = 16). Diagnoses of sIBM and other diseases were based on specific diagnostic criteria as described in previous reports.[4,9] Healthy control subjects (n = 10) were collected from members of the Department of Neurology, Kumamoto University.

Muscle samples from 9 patients with sIBM were evaluated via histology. All muscle samples were previously examined via routine histological techniques for diagnostic purposes. Fresh frozen samples were kept at −80°C until used. Table 1 summarizes clinical information available for the patients.

Cell-Based Assay for the Anti-cN1A Autoantibodies

Cells of the COS7 cell line were transduced with green fluorescent protein (GFP)-tagged cN1A expression vector (pCMV6-

Ac-GFP-human cN1A; Cat. No. RG224617; OriGene, Rockville, MD) by using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA). COS7-cN1A/GFP cells were incubated with human serum for 1 hour at room temperature after fixation with 4% paraformaldehyde and permeabilization with 5% donkey serum and 0.2% Triton-X in phosphate-buffered saline (PBS), washed, and then incubated with Alexa Fluor 594–conjugated donkey antihuman immunoglobulin G (IgG; H+L) antibody (Cat. No. 709-585-149; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. For the positive control, the cells were incubated with rabbit anti-cN1A (1:500 dilution; Cat. No. ab75101; Abcam, Cambridge, MA), washed, and then detected with Alexa Fluor 594–conjugated donkey antirabbit IgG antibody (1:200 dilution; Cat. No. A21207, Life Technologies). After the cells were washed, they were mounted with mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and analyzed qualitatively by means of a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). We defined the presence of the anti-cN1A autoantibodies on the basis of colocalization of GFP-labeled cN1A and Alexa Fluor 594–labeled human IgG under the following conditions: the negative control (omission of serum) showed no Alexa Fluor 594 signals, and the positive control (addition of rabbit anti-cN1A [1:500 dilution; Cat. No. ab75101, Abcam]) demonstrated a distinct signal of Alexa Flour 594 (Fig 1). To validate our cell-based assay method, we compared the results from the cell-based assay with those from a commercially available anti-cN1A enzyme-linked immunosorbent assay (ELISA) test (Cat. No. EA1675-4801G; Euroimmun, Lübeck, Germany) using our randomly selected 45 samples (20 with sIBM, 11 with polymyositis, 7 with dermatomyositis, 6 with other myopathy, and 1 from a healthy control).

Clinical Evaluation of sIBM Patients with and without the Anti-cN1A Autoantibodies

Patients with sIBM were investigated in terms of the following parameters: sex, age at disease onset, initial symptoms, presence or absence of dysphagia, distribution of muscle weakness (knee extension > hip flexion, and finger flexion > shoulder abduction), presence or absence of complications (autoimmune diseases, human T-cell lymphotropic virus-1 antibody, hepatitis C virus [HCV] antibody, and human immunodeficiency virus antibody), serum levels of creatine kinase, presence or absence of acute denervation findings of needle electromyography, respiratory function (percentage vital capacity and percentage forced vital capacity), inclusion body myositis functional rating scale (IBMFRS) scores,[10] and response to treatment (intravenous immunoglobulin and corticosteroids).

Histological Evaluation of Tissues from sIBM Patients with and without the Anti-cN1A Autoantibodies

Fresh frozen tissue sections were fixed with 4% paraformaldehyde and blocked with 5% normal donkey serum/0.1% Triton-X in PBS. For double labeling, combinations of mouse anti-p62/SQSTM1 (1:250 dilution; Cat. No. M162-3; Medical & Biological Laboratories, Nagoya, Japan) and rabbit anti-cN1A (1:400 dilution; Cat. No. ab75101, Abcam) were used. Immunolabeled proteins were visualized by using antimouse Ig antibody–conjugated Alexa Fluor 488 or antirabbit Ig antibody–conjugated Alexa Fluor 594 (1:200 dilution; Cat. Nos. A21202 and A21207, Life Technologies). The numbers of immunostained myofibers and fibers with rimmed vacuoles were expressed as a proportion of the total number of fibers after observation of 3 slices that contained > 300 muscle fibers in each staining assay. The mean area of type 2 myofibers was measured by using ImageJ software (NIH ImageJ analysis website: http://rsb.info.nih.gov/ij/) with serial adenosine triphosphatase staining.

Muscle tissues were stripped and lysed in radioimmunoprecipitation assay buffer. Each 10 μg sample was subjected to Western blotting by using rabbit anti–phosphorylated adenosine monophosphate–activated protein kinase (AMPK) α antibody (1:1,000 dilution; Cat. No. 2531; Cell Signaling Technology, Danvers, MA), anti-cN1A antibody (1:1,000 dilution; Cat. No. ab75101, Abcam), and glyceraldehyde 3-phosphate dehydrogenase (1:250 dilution; Cat. No. M171-3, Medical & Biological Laboratories). Band intensities were quantified by using NIH ImageJ software.

Investigation of the Pathogenic Effects of the Anti-cN1A Autoantibodies on Rhabdomyosarcoma Cells

RD cells (human rhabdomyosarcoma cells) were supplied by the Japanese Collection of Research Bioresources Cell Bank (National Institutes of Biomedical Innovation, Health, and Nutrition, Osaka, Japan). IgG was extracted from serum samples obtained from sIBM patients with (n = 3) and without (n = 3) the anti-cN1A autoantibodies and from healthy subjects without the autoantibodies (n = 3) by using HiTrap Protein G HP columns (GE Healthcare UK, Buckinghamshire, UK). Each IgG sample was added, to a final concentration of 7mg/ml, to the culture medium of RD cells in triplicate. After incubation of samples for 48 hours, cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) calorimetric assay (Promega, Madison, WI). Immunofluorescence analyses were performed with 4% paraformaldehyde-fixed cells with the primary antibody rabbit anti-p62/SQSTM1 (1:250 dilution; Cat. No. PM045, Medical & Biological Laboratories) and the secondary antibody antirabbit Ig antibody–conjugated Alexa Fluor 488 (1:200 dilution; Cat. No. A21206, Life Technologies). The immunolabeled cells were examined with a fluorescence microscope (BZ-9000; Keyence). For Western blotting, the primary antibodies used here were as follows: mouse anti–β-actin antibody (1:10,000; Cat. No. A5441; Sigma, St Louis, MO), rabbit anti-p62/SQSTM1 (1:1,000 dilution; Cat. No. PM045, Medical & Biological Laboratories), mouse anti-LC3 (1:1,000 dilution; Cat. No. M186-3, Medical & Biological Laboratories), and rabbit anti-cN1A (1:500 dilution; Cat. No. ab75101, Abcam). Band intensities were quantified by using NIH ImageJ software.

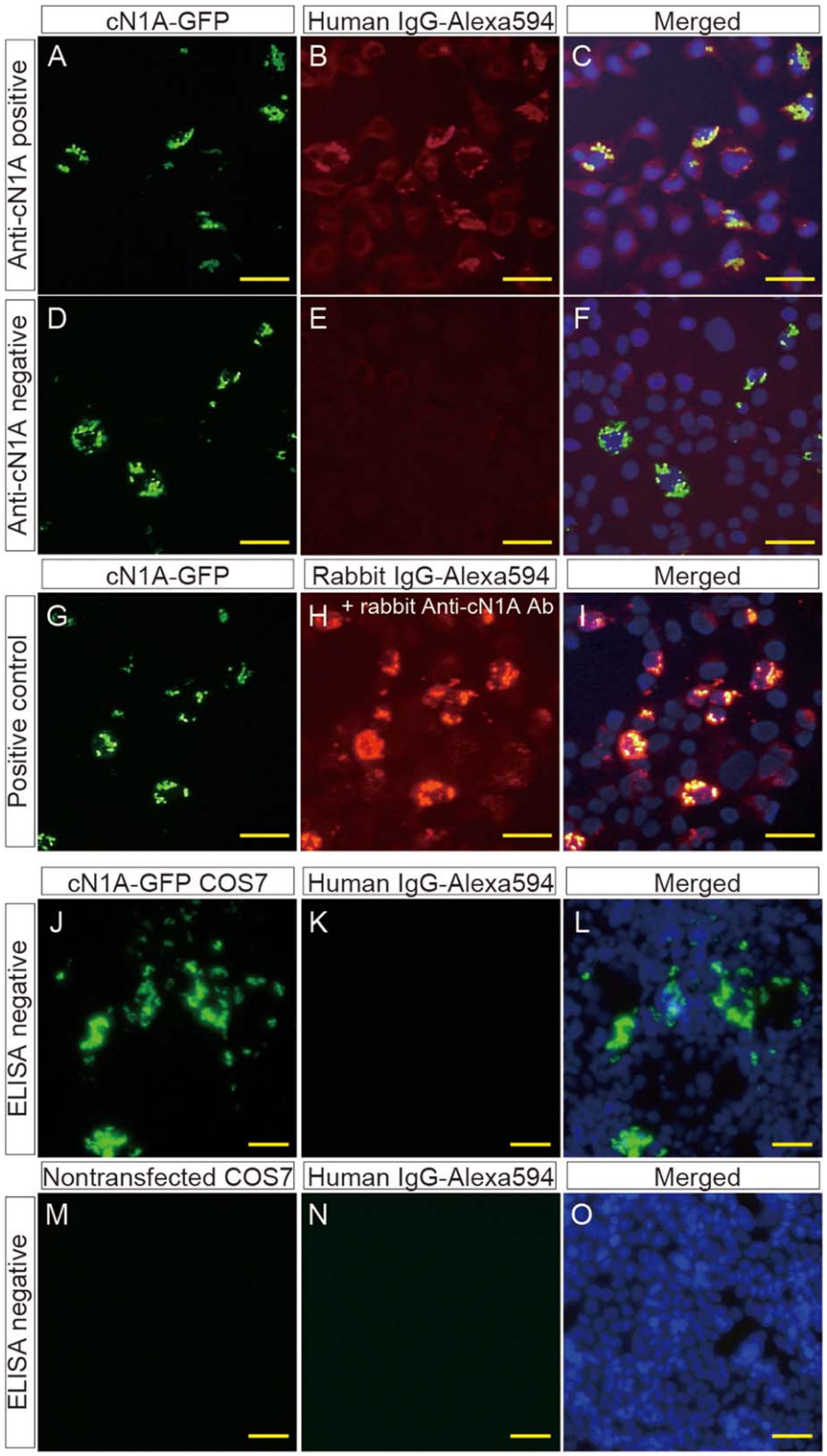

FIGURE 1: (A–F) Representative immunofluorescence results for cN1A–green fluorescent protein (GFP; A, D) and human IgG–Alexa Fluor 594 (B, E) in anti-cN1A–positive (A–C) and anti-cN1A–negative (D–F) samples. (G–I) The positive control images for cN1A-GFP (G) and rabbit IgG–Alexa Fluor 594 (H) in COS7-cN1A/GFP cells incubated with rabbit anti-cN1A antibody. (J–O) Immunofluorescence results for cN1A-GFP (J, M) and human IgG–Alexa Fluor 594 (K, N) in COS7-cN1A/GFP cells (J–L) and non-transfected COS7 cells (M–O) incubated with samples that tested negative for anti-cN1A autoantibodies using enzyme-linked immunosorbent assay (ELISA) assay. No signals for Alexa Fluor 594 were detected, suggesting that nonspecific binding of serum IgG to the COS7-cN1A/GFP cells was negligible. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Merged images (C, F, I, L, O) are composed of cN1A-GFP, Alexa Fluor 594, and DAPI images. Scale bars = 50 μm.

TABLE 2. Percentage of Patients with Various Diseases Whose Serum Samples Were Positive for the Anti-cN1A Autoantibodies

Patients with Disease or Subjects	Mean Age ± SD	Patients Examined, No.	Positive Patients, No.	Positive, %
sIBM	67.5 ± 9.9	67	24	35.8
Polymyositis	58.6 ± 16.3	36	5	13.9
Immune-mediated necrotizing myopathy	55.3 ± 9.8	8	2	25
Dermatomyositis	52.8 ± 15.8	31	4	12.9
Other myopathies	49.9 ± 17.9	41	2	4.9
Fasciitis	90	1	0	0
Autoimmune diseases (SLE, Sjögren syndrome)	56.9 ± 18.8	15	0	0
Neurogenic muscular atrophy	67.9 ± 11.2	16	0	0
Healthy control subjects	28.2 ± 7.2	10	0	0
Total		225	37	

SD = standard deviation; sIBM = sporadic inclusion body myositis; SLE = systemic lupus erythematosus.

Investigation of the Pathogenic Properties of the Anti-cN1A Autoantibodies by Using Passive Immunization of Mice

IgG samples (10mg each) from sIBM patients with (n = 3) and without (n = 3) the anti-cN1A autoantibodies and from healthy subjects without the autoantibodies (n = 3) as mentioned above were injected intraperitoneally into 3 C57BL/6J mice each after administration of 300 μg/g body weight cyclophosphamide. As a negative control, PBS was injected into 3 mice. After the injections, the body weight of each group was measured every other day, and motor activity was analyzed by means of the rotarod test and the mesh hanging test, according to the literature.[11,12] Four weeks after the injection, mice were killed and analyzed by means of routine histological techniques and immunohistochemistry with rabbit anti-p62/SQSTM1 (1:500 dilution; Cat. No. PM045, Medical & Biological Laboratories), rabbit antihuman Ig (1:500 dilution; Cat. No. ab109489, Abcam), and rabbit anti-CD68 (H-255; 1:100 dilution; Cat. No. sc-9139; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies were as follows: donkey antirabbit Ig antibody–conjugated Alexa Fluor 488 (1:200 dilution; Cat. No. A21206, Life Technologies) for p62, or goat antirabbit Ig antibody–conjugated horseradish peroxidase (1:200 dilution; Cat. No. P0448; Dako, Santa Clara, CA) for human Ig and CD68.

Statistical Analysis

All values are expressed as mean ± standard deviation unless stated otherwise. Differences among means were analyzed with the Mann–Whitney test, chi-square test, or 1-way analysis of variance by using Prism 6 software (GraphPad Software, La Jolla, CA).

Results

Anti-cN1A Autoantibodies in Various Diseases

To confirm the usefulness of our newly developed cell-based assay for the detection of the anti-cN1A autoantibodies, we first analyzed serum obtained from patients with various neuromuscular diseases including sIBM. Of 67 sIBM patients who met the ENMC 2010 diagnostic criteria,[4] the anti-cN1A autoantibodies were detected in 24 (35.8%) patients with sIBM (Table 2). In patients with other inflammatory muscle diseases, the anti-cN1A autoantibodies were present in 5 of 36 (13.9%) patients with polymyositis, in 4 of 31 (12.9%) patients with dermatomyositis, and in 2 of 8 (25.0%) patients with necrotizing myopathy associated with anti-SRP autoantibodies (see Table 2). Among sIBM patients with mild cell infiltration, no patients had the autoantibodies (see Table 1). In contrast, the antibody was not detected in patients with fasciitis, autoimmune disease (systemic lupus erythematosus and Sjögren syndrome), or neurogenic muscular atrophy (such as motor neuron diseases), or in healthy controls. Thus, the reactivity of anti-cN1A autoantibodies was 35.8% sensitive and 91.7% specific for the diagnosis of sIBM in our cell-based assay. With regard to differentiation of sIBM from other inflammatory muscle diseases and neurogenic atrophy, the specificity of the anti-cN1A autoantibodies was 85.3% and 100%, respectively.

To validate our cell-based assay method, we compared the results from the cell-based assay with those from anti-cN1A ELISA testing using our randomly selected 45 samples. The reactivity of anti-cN1A

TABLE 3. Clinical Comparison between Anti-cN1A Ab-Positive and Ab-Negative Cases

Characteristics	Ab-Positive Cases, n = 24	Ab-Negative Cases, n = 43	*p*
M:F	16:8	24:19	0.385
Age at onset, yr	61.6 ± 9.0	62.9 ± 12.2	0.389
Initial symptoms, %			
Lower limb	78.3	88.1	0.444
Upper limb	17.4	7.1	
Others	4.3	4.8	
Dysphagia, %	38.1	32.5	0.662
Distribution of muscle weakness, %			
Knee extension > hip flexion	84.6	60.0	0.133
Finger flexion > shoulder abduction	75.0	69.0	0.669
Complications, %			
Autoimmune diseases	14.3	19.0	0.714
HTLV-1 Ab	4.8	9.7	0.514
HCV Ab	4.5	26.5	0.036
HIV Ab	0	0	
Serum levels of creatine kinase, IU/l	412.3 ± 259.9	641.6 ± 819.5	0.284
Acute denervation on EMG, %	78.6	80.0	0.916
Respiratory function, %			
VC	93.8 ± 18.7	90.8 ± 15.4	0.659
FVC	93.3 ± 18.6	84.8 ± 15.8	0.447
IBMFRS scores	26.6 ± 9.3	29.9 ± 4.9	0.382
Positive response to treatment, %			
IVIg	50.0	40.0	0.714
Corticosteroid	71.4	50	0.377

Ab = antibody; EMG = electromyography; F = female; FVC = forced vital capacity; HCV = hepatitis C virus; HIV = human immunodeficiency virus; HTLV-1 = human T-cell lymphotropic virus-1; IBMFRS = inclusion body myositis functional rating scale; IVIg = intravenous immunoglobulin; M = male; VC = vital capacity.

autoantibodies was 45% sensitive and 88% specific for the diagnosis of sIBM in the ELISA assay (Supplementary Table 1). In contrast in our cell-based assay, the sensitivity and specificity of the anti-cN1A autoantibodies was 45% and 96%, respectively (see Supplementary Table 1). As to concordance between ELISA and cell-based assay, 10 patients tested positive for both ELISA and cell-based assay, whereas 33 patients tested negative for the assays (Supplementary Table 2). Only 2 patients were positive for ELISA although cell-based assay showed negative. No patient tested positive for cell-based assay but negative for ELISA.

These observations may suggest that sensitivities are equivalent between both assays, but specificity is higher in cell-based assay than in the ELISA assay.

Clinical Features of sIBM Patients with and without the Anti-cN1A Autoantibodies

We next evaluated the clinical differences of sIBM patients with and without the autoantibodies. We found no significant difference in the male-to-female ratio, age at onset of disease, initial symptoms, presence of dysphagia, distribution of muscle weakness, serum level of creatine kinase, presence of acute denervation as determined

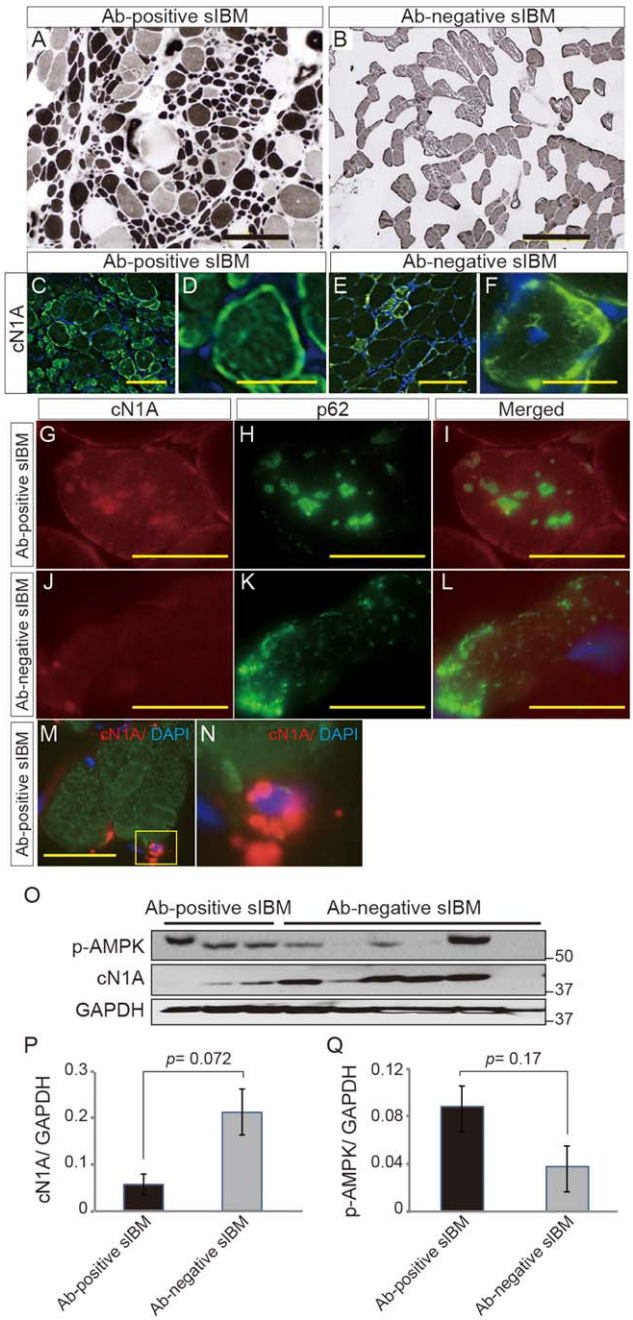

FIGURE 2: (A, B) Adenosine triphosphatase pH 10.6 stain on muscle specimens from anti-cN1A–positive (A) and anti-cN1A–negative (B) sporadic inclusion body myositis (sIBM) patients. Ab = antibody. (C–F) Immunofluorescence for cN1A on muscle specimens from anti-cN1A–positive (C, D) and anti-cN1A–negative (E, F) sIBM patients. (G–N) Representative immunofluorescence results for cN1A (red) and p62/SQSTM1 (p62, green) in biopsied muscle specimens obtained from sIBM patients with (G–I, M, N) and without (J–L) the anti-cN1A Ab. (M, N) Myonuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). N is a higher-magnification image of the boxed area in M. Scale bars = 200 μm (A, B), 100 μm (C, E), 25 μm (D, F), 50 μm (G–M). (O) Immunoblots of phosphorylated adenosine monophosphate–activated protein kinase (p-AMPK), cN1A, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) obtained by using biopsied muscle specimens from anti-cN1A–positive and anti-cN1A–negative sIBM patients. (P) Quantification of cN1A band intensity normalized to GAPDH. (Q) Quantification of p-AMPK band intensity normalized to GAPDH.

with electromyography, respiratory function, IBMFRS score, and response to treatment (Table 3). However, the percentage of patients with HCV antibodies was significantly higher in the anti-cN1A–negative group compared with the autoantibody-positive group ($p = 0.036$; see Table 3). For patients with other complications, such

TABLE 4. Pathological Comparison between Anti-cN1A Ab-Positive and Ab-Negative Cases

Variable	Ab-Positive Cases, n = 3	Ab-Negative Cases, n = 6	*p*
Myofibers with RV, %	6.0 ± 2.8	4.3 ± 3.9	0.57
Cases with severe cell infiltration, %	100	66.6	0.32
Cases with congophilic materials, %	0	16.7	0.52
Cases with colocalization of cN1A and p62, %	100	50.0	0.17
Cases with perinuclear localization of cN1A, %	66.7	16.7	0.17
Mean area of type 2 myofibers, μm^2	833.3 ± 319.6	2,020.0 ± 182.0	0.00043

Ab = antibody; RV = rimmed vacuoles.

as autoimmune diseases, and for human T-cell lymphotropic virus-1 and human immunodeficiency virus carriers, no significant difference was observed for both groups.

Histological Features of sIBM Patients with and without the Anti-cN1A Autoantibodies

To assess the pathological differences between sIBM patients with and without the autoantibodies, 9 (3 with the autoantibodies and 6 without the autoantibodies) of 67 patients with sIBM were analyzed regarding histological features. We compared the groups with regard to the percentage of myofibers with rimmed vacuoles, degree of inflammatory cell infiltration, presence of congophilic materials, localization of cN1A protein, and mean area of type 2 myofibers. We observed no significant differences in the percentage of myofibers with rimmed vacuoles, degree of cell infiltration, and presence of congophilic materials between the groups. One important finding was the significantly smaller mean area of type 2 myofibers in the anti-cN1A–positive group than in the autoantibody-negative group ($p = 0.00043$; Fig 2, Table 4). Regardless of the presence or absence of the autoantibodies, all sIBM cases showed a certain level of cN1A-positive signals in the sarcoplasm and sarcoplasmic membranes. In the autoantibody-positive group, cN1A-positive signals were often observed in the perinuclear regions of myofibers. Also, colocalization of cN1A with p62/SQSTM1, a ubiquitin-binding adaptor protein, was observed more frequently in the perinuclear regions of myofibers from seropositive sIBM patients.

We then investigated and compared the expression levels of cN1A and phosphorylated AMPK in biopsied muscle specimens in seropositive and seronegative sIBM patients. The cN1A expression level tended to decrease and the level of phosphorylated AMPK tended to increase in muscles of anti-cN1A–positive sIBM patients compared with anti-cN1A–negative sIBM patients, although a statistically significant difference was not observed ($p = 0.072$ and 0.17, respectively; see Fig 2O–Q).

Pathogenic Properties of IgG Isolated from sIBM Patients with anti-cN1A autoantibodies

To explore a possible pathogenic role of the anti-cN1A autoantibodies, we utilized in vitro and in vivo passive immunization models. We first analyzed the cytotoxic effect of IgG with anti-cN1A autoantibodies on the cell viability of RD cells by using the MTS calorimetric assay. However, we found no significant difference in cell viability among autoantibody-positive sIBM IgG and autoantibody-negative sIBM IgG-supplemented cultures, and the control IgG-supplemented culture in our experimental conditions (data not shown). We next investigated the expression levels of the autophagy-related proteins p62/SQSTM1 and LC3, because sarcoplasmic aggregation of both proteins has been widely known as a pathological hallmark of sIBM.[13,14] The level of p62/SQSTM1 expression was significantly higher in anti-cN1A–positive sIBM IgG-supplemented cells compared with the autoantibody-negative sIBM IgG-supplemented, control IgG-supplemented, or PBS-supplemented cells or naive cells (Fig 3). The ratio LC3-I/LC3-II was not different among the experimental groups (data not shown). In contrast, the level of cN1A expression significantly decreased in anti-cN1A–positive sIBM IgG-supplemented cells compared with the autoantibody-negative sIBM IgG-supplemented, control IgG-supplemented, or PBS-supplemented cells or naive cells. Immunofluorescence analysis of p62/SQSTM1 revealed abundant dotlike aggregations in the cytoplasm of anti-cN1A–positive sIBM IgG-supplemented cells, whereas the autoantibody-negative sIBM IgG-supplemented,

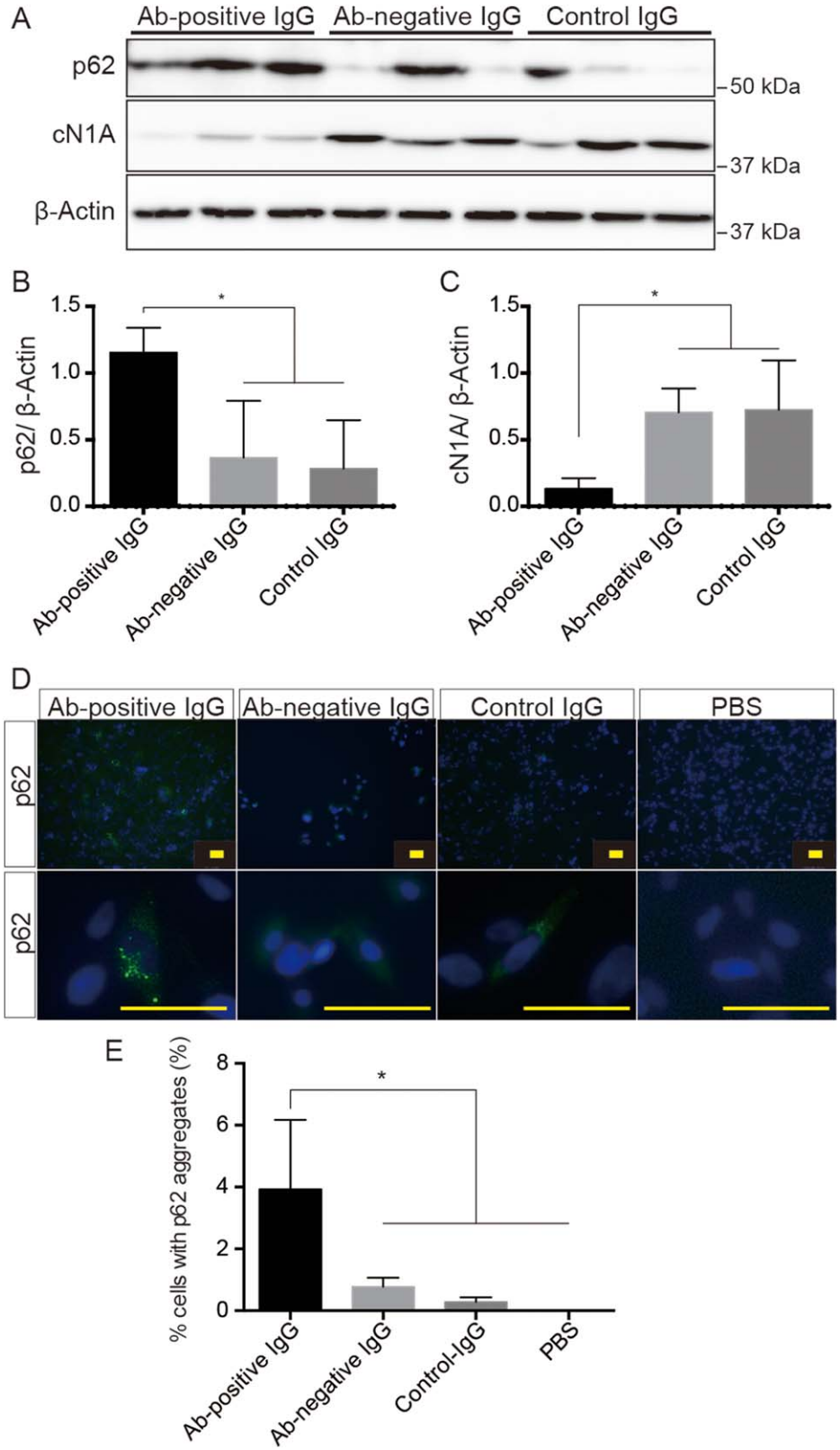

FIGURE 3: (A) Immunoblots of p62/SQSTM1 (p62), cN1A, and β-actin obtained by using samples from anti-cN1A autoantibodies (Ab)-positive sporadic inclusion body myositis (sIBM) IgG-supplemented cells, Ab-negative sIBM IgG-supplemented cells, or control IgG-supplemented cells; n = 9 per group. (B) Quantification of p62 band intensity normalized to β-actin; n = 9 per group, *$p < 0.05$. (C) Quantification of cN1A band intensity normalized to β-actin; n = 9 per group, *$p < 0.05$. (D) Immunofluorescence analysis of p62 via RD cells supplemented with anti-cN1A–positive sIBM IgG, anti-cN1A–negative sIBM IgG, control IgG, or phosphate-buffered saline (PBS). Bottom panel shows enlargements of the images in the top panel. The cytoplasm of anti-cN1A–positive sIBM IgG-supplemented cells had abundant dotlike aggregations. Scale bars = 50 μm. (E) Percentage of cells with p62-positive aggregates in RD cells supplemented with anti-cN1A–positive sIBM IgG, Ab-negative sIBM IgG, control IgG, or PBS; *$p < 0.05$.

control IgG-supplemented, or PBS-supplemented cells showed merely faint p62/SQSTM1 signals. The percentage of myofibers with dotlike p62/SQSTM1 patterns was significantly higher in the autoantibody-positive sIBM IgG-supplemented cells.

We then used an in vivo passive immunization model to confirm the pathogenic properties of the anti-cN1A autoantibodies. Histological analysis demonstrated a fiber size variation with small angulated fibers in mice injected with anti-cN1A–positive sIBM IgG, whereas mice receiving the autoantibody-negative sIBM IgG, control IgG, or PBS showed normal muscle histology (Fig 4). Modified Gomori trichrome staining revealed no specific abnormalities, such as rimmed vacuoles, in all groups. Of particular note is that the anti-cN1A–positive sIBM IgG-injected mice exclusively demonstrated p62/SQSTM1-positive sarcoplasmic aggregates in myofibers. We were unable to obtain histological evidence that injected IgG crossed into the muscle tissues of the autoantibody-positive or autoantibody-negative sIBM IgG-injected and control IgG-injected mice. However, CD68-positive macrophages frequently infiltrated the endomysium of the autoantibody-positive sIBM IgG-injected mice.

In addition, we studied the effect of anti-cN1A–positive sIBM IgG on motor activities as measured by body weight change, the rotarod test, and the mesh hanging test. We found no significant differences in body weight change and the rotarod and mesh hanging tests in the antibody-positive sIBM IgG-injected, autoantibody-negative sIBM IgG-injected, control IgG-injected, and PBS-injected groups under our experimental conditions (Fig 5).

Discussion

Anti-cN1A Autoantibodies in Various Diseases

We herein report our development of a new cell-based assay method for detection of the anti-cN1A autoantibodies. Since Salajegheh et al[15] first reported detection of the autoantibodies in plasma and serum samples from patients with sIBM, and then Larman et al[5] and Pluk et al[6] identified the target as cN1A, several studies using different techniques have suggested the involvement of the anti-cN1A autoantibodies in the pathogenesis of sIBM. The techniques used by these studies included immunoprecipitation,[5,6,15] immunoblotting with human skeletal muscle extracts[5,6,15] or lysates of cN1A-expressing human embryonic kidney 293 cells,[7,16] and ELISAs of a recombinant full-length cN1A protein[16,17] or synthetic peptides derived from cN1A.[8] Also, Greenberg[17] examined not only IgG but also IgM and IgA anti-cN1A autoantibodies isotypes by using immunoblots and ELISAs. They found that combination assays for all autoantibody isotypes improved the diagnostic sensitivity up to 76%. Although these studies showed that the anti-cN1A autoantibody had diagnostic utility for differentiating sIBM from other types of myositis and other neuromuscular diseases,[18] the sensitivities and specificities for the diagnosis of sIBM varied as a result of the different methods of assay. In measuring anti–aquaporin-4 antibody, cell-based assays have been more sensitive than have ELISAs.[19] Our new cell-based assay seemed equivalent to previously proven methods in terms of sensitivity for the diagnosis of sIBM. To firmly establish the usefulness of anti-cN1A autoantibodies measurement for a more accurate diagnosis of sIBM, additional research is required to standardize antibody detection methods while considering the epitopes recognized by the antibody and Ig isotypes.

Clinicopathological Features of sIBM Patients with and without the Anti-cN1A Autoantibodies

Until now, whether sIBM patients with the anti-cN1A autoantibodies manifest unique clinicopathological features had not been fully investigated. Recently, results from a small sample size study suggested that seropositivity for the anti-cN1A autoantibodies was associated with greater motor and functional disability in sIBM; also, patients with the autoantibodies showed more prominent bulbar, facial, and respiratory symptoms.[15] However, these findings are inconsistent with our observations, which may be attributed to the different assay methods used, sample sizes, or patients' backgrounds.

Uruha et al[20] recently reported that a significantly higher number of patients with sIBM (28%) had the anti-HCV antibody compared with patients with polymyositis and the general aged Japanese population, which suggests a possible pathomechanistic link between sIBM and HCV infection. In our study, 26.5% of sIBM patients without the anti-cN1A autoantibodies had anti-HCV antibodies, a finding that resembles that in the Uruha et al[20] report. In contrast, our result that the percentage of cases with HCV antibodies was significantly lower in the anti-cN1A–positive group compared with the autoantibody-negative group suggests a distinct pathogenic mechanism for sIBM with and without the anti-cN1A autoantibodies.

Pathogenic Properties of IgG Isolated from sIBM Patients with anti-cN1A autoantibodies

In our in vitro and in vivo passive immunization models, anti-cN1A–positive sIBM IgG resulted in intracytoplasmic aggregation of p62/SQSTM1. Sarcoplasmic aggregation of autophagy-related proteins such as p62/SQSTM1

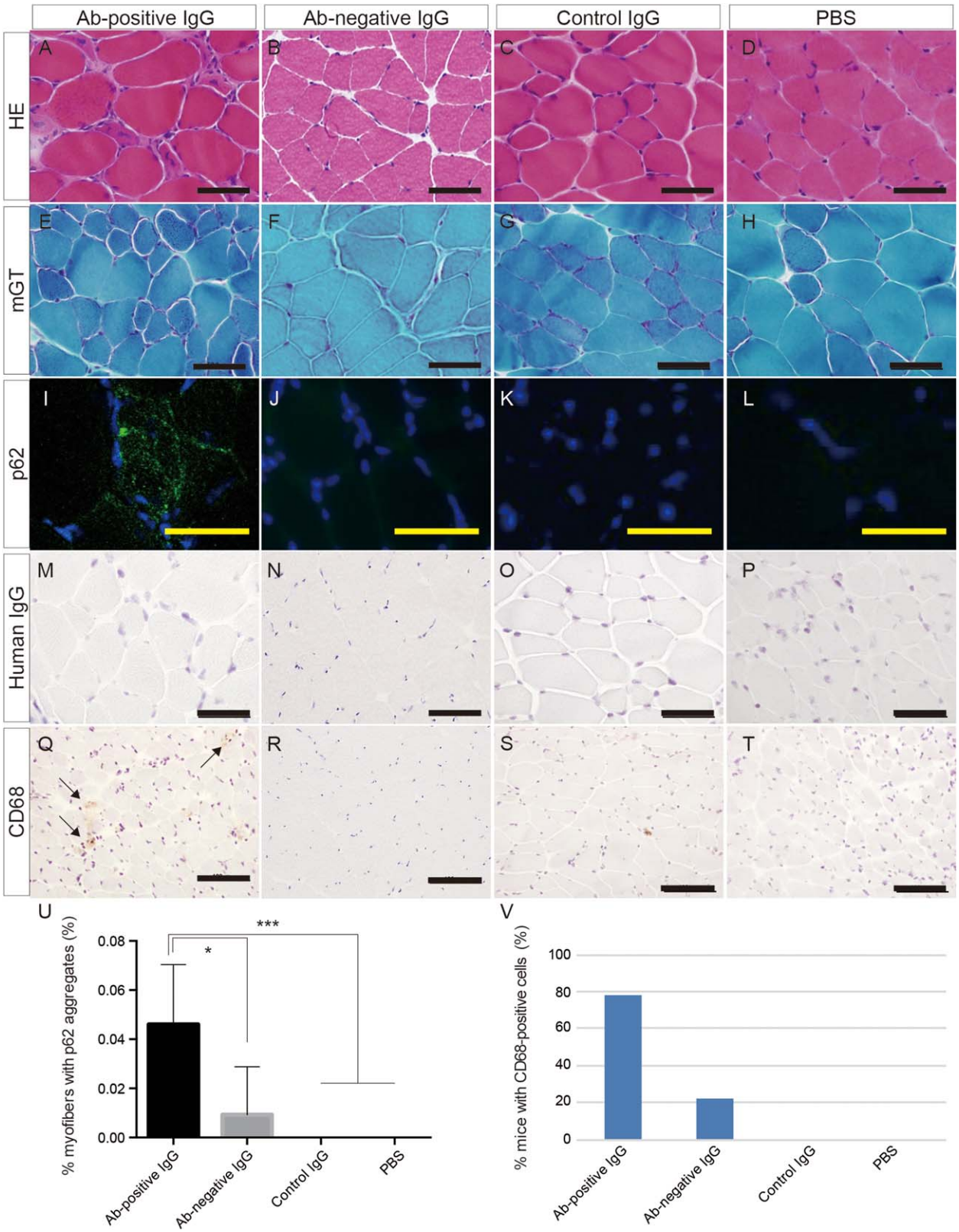

FIGURE 4: (A–T) Representative histological staining with hematoxylin-eosin (HE; A–D), modified Gomori trichrome (mGT; E–H), p62/SQSTM1 (p62; I–L), human IgG (M–P), and CD68 (Q–T) in upper proximal muscles (biceps brachii) from mice injected with anti-cN1A–positive sporadic inclusion body myositis (sIBM) IgG (A, E, I, M, Q), anti-cN1A–negative sIBM IgG (B, F, J, N, R), control IgG (C, G, K, O, S), or phosphate-buffered saline (PBS; D, H, L, P, T). Ab = autoantibody. Scale bars = 50 μm. Arrows indicate CD68-positive macrophages. (U) Percentage of myofibers with p62-positive aggregates in mice injected with anti-cN1A–positive sIBM IgG, Ab-negative sIBM IgG, control IgG, or PBS; *$p < 0.05$, ***$P < 0.001$. (V) Percentage of mice infiltrated with CD68-positive cells in mice injected with anti-cN1A–positive sIBM IgG, Ab-negative sIBM IgG, control IgG, or PBS.

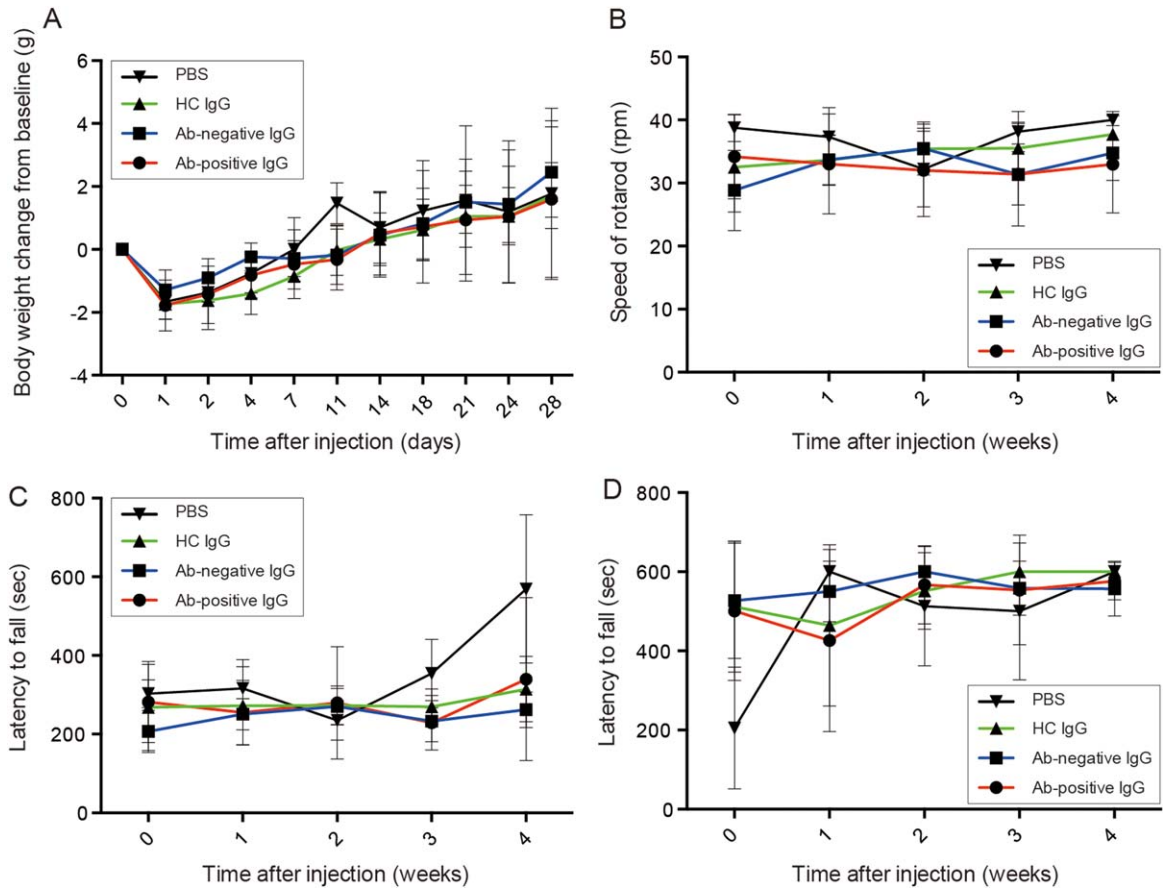

FIGURE 5: (A) Body weight change, (B) speed at fall on rotarod test, (C) latency to fall on rotarod test, and (D) latency to fall on mesh hanging test results for mice injected with anti-cN1A–positive sporadic inclusion body myositis (sIBM) IgG, anti-cN1A–negative sIBM IgG, healthy control (HC) IgG, or phosphate-buffered saline (PBS); n = 9 per group. Ab = antibody.

and LC3 has been known to be a pathological hallmark of sIBM.[13,14] Of note, our preliminary data showed that abundant accumulation of p62/SQSTM1 was observed in the myofibers from patients with immune-mediated necrotizing myopathy with both anti-cN1A and anti-SRP autoantibodies (Fig 6). Thus, the potential relationship between intracytoplasmic accumulation of p62/SQSTM1 and cN1A autoantibodies can be raised.

The finding that cN1A expression levels were reduced in the muscle tissues from sIBM associated with the cN1A autoantibodies as well as lysates from the autoantibodies-positive IgG-supplemented cells may provide a clue to elucidate the possible pathogenic role of the autoantibodies on muscle degeneration. cN1A, which is highly expressed in skeletal muscles, has pleiotropic roles in several cellular processes including physiological control of energy balance, metabolic regulation, and cell replication.[21] Of particular significance is the dephosphorylation of noncyclic nucleoside monophosphates to produce nucleosides and inorganic phosphates. A gene-silencing study revealed that cN1A knockdown increased phosphorylation of the metabolic stress-sensing protein kinase AMPK.[22] Activated AMPK turns on catabolic pathways that produce adenosine triphosphate (ATP) as it simultaneously turns off anabolic pathways that consume energy (ATP).[23] Activation of AMPK resulted in upregulation of muscle-specific ubiquitin ligases muscle atrophy F-box and muscle RING finger 1, which are typical markers in catabolic conditions of skeletal muscles.[24] At this point, we have not confirmed an increase in phosphorylated AMPK in the passive immunization models that we used here. However, reduced cN1A expression via supplementation with anti-cN1A–positive IgG may lead to stimulation of catabolic pathways followed by AMPK activation.

Limitations

In this article, we utilized a new cell-based assay to detect the anti-cN1A autoantibodies. To validate our cell-based assay method, we compared the results from the cell-based assay with those from anti-cN1A ELISA test using a limited number of samples. To establish our assay as a tool for a more accurate diagnosis and a helpful treatment index of sIBM, quantitative evaluation—measuring more samples

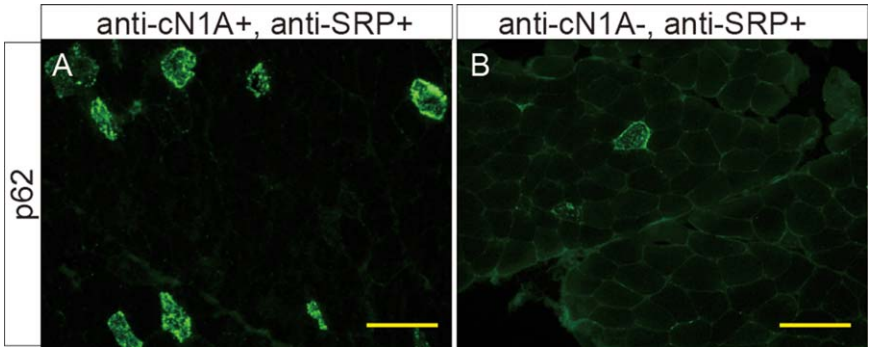

FIGURE 6: Representative immunofluorescence results for p62 in (A) both anti-cN1A and anti–signal recognition particle (SRP)–positive, and (B) anti-cN1A–negative but anti-SRP–positive samples. Scale bars = 50 μm.

at various disease stages—is also required for autoantibody detection, compared to previous formats, such as an ELISA or dot-blot assay. Moreover, a greater number of samples should be used to analyze pathogenic properties of IgG isolated from sIBM patients with anti-cN1A autoantibodies. The pathophysiology by which the anti-cN1A autoantibodies cause dysfunction of the protein degradation system should be investigated more carefully, by using not only passive models but also active immunization models. Furthermore, the mechanisms by which the anti-cN1A autoantibodies access myofibers should be determined.

Conclusions

Our cell-based assay is valuable for detection of the anti-cN1A autoantibodies. Patients with the anti-cN1A autoantibodies manifested unique clinicopathological features. Data from in vitro and in vivo passive immunization models suggested that the anti-cN1A autoantibodies may affect the protein degradation system in myofibers.

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Author Contributions

Conception and design of the study: N.T., S.Y., Y.A. Acquisition and analysis of data: N.T., S.Y., X.Z., M.K., Z.Z., T.D., Y.Mat., S.N., Y.Mae., K.S., N.S., M.A. Drafting the text or preparing the figures: N.T., S.Y.

Potential Conflicts of Interest

Nothing to report.

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