Immune complexome analysis of a rich variety of serum immune complexes identifies disease-characteristic immune complex antigens in systemic sclerosis

Yuki Jimbayashi Kutsuna[a], Naoki Iwamoto[b*], Kunihiro Ichinose[b], Nozomi Aibara[c], Katsumi Nakashima[c], Hideki Nakamura[d], Yuta Koike[e], Hiroyuki Murota[e], Yukitaka Ueki[f], Hirotaka Miyamoto[g], Junya Hashizume[h], Yukinobu Kodama[h], Mikiro Nakashima[c], Atsushi Kawakami[b], and Kaname Ohyama[a,h**]

[a]Department of Molecular Pathochemistry, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

[b]Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

[c]Department of Pharmacy Practice, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

[d]Division of Hematology and Rheumatology, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan

[e]Department of Dermatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

[f]Sasebo Chuo Hospital, Sasebo, Japan

[g]Department of Pharmaceutics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

[h]Department of Hospital Pharmacy, Nagasaki University Hospital, Nagasaki, Japan

Corresponding authors: *N. Iwamoto, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto-machi, Nagasaki, 852-8501, Japan. E-mail: naoki-iwa@nagasaki-u.ac.jp; **K. Ohyama, Department of Hospital Pharmacy, Nagasaki University Hospital, 1-7-1 Sakamoto-machi, Nagasaki, 852-8501, Japan. E-mail: k-ohyama@nagasaki-u.ac.jp

Abbreviations: ANA: Anti-nuclear antibody; dcSSc: diffuse cutaneous SSc; ELISA: Enzyme-linked immuno sorbent assay; GWAS: genome-wide association studies; HDA: high disease activity; ICs: Immune complexes; lcSSc: limited cutaneous SSc; MED30: Mediator of RNA polymerase II transcription subunit 30; MS: mass spectrometry; RNAP: RNA polymerase; SSc: Systemic sclerosis; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; TPRX1: Tetra-peptide repeat homeobox protein 1

ABSTRACT

Systemic sclerosis (SSc) is an autoimmune disease characterized by vascular endothelial dysfunction and skin fibrosis. Recently, the presence and pathogenic role of immune complexes (ICs) of SSc patients were reported. However, the identities of antigens in these ICs are unknown. Therefore, we examined ICs in the serum of SSc patients to elucidate SSc pathogenesis. In this study, IC concentrations in serum samples from SSc and systemic lupus erythematosus (SLE) patients were measured by C1q enzyme-linked immunosorbent assays; immune complex analysis was used for comprehensive identification and comparison of antigens incorporated into ICs (IC-antigens). The expression patterns of SSc-specific IC-antigens in skin sections were investigated by immunohistochemistry. Compared with SLE patients who developed disease because of IC deposition, SSc patients had a greater number of IC-antigens and a smaller difference in IC concentrations, suggesting that SSc pathogenesis is affected by the proteins present in ICs. In contrast, the IC concentration and number of IC-antigens did not significantly differ according to the clinical phenotype of SSc. We identified 478 IC-antigens in SSc patients, including multiple RNAP II-associated proteins that were targeted by antibodies previously associated with SSc pathogenesis. The most frequently detected RNAP II-associated protein, RNA polymerase II transcription subunit 30 (MED30), was strongly expressed at lesion sites and reportedly regulates endothelial differentiation. Therefore, increased expression of MED30 in lesions may have an antigenic effect, and MED30 function may be impaired or inhibited by IC formation. RNAP II-associated proteins may SSc pathogenesis through mechanisms such as the MED30 pathway.

Keywords: immune complexes; immune complexome analysis; mediator of RNA polymerase II transcription subunit 30; vascular endothelial dysfunction; systemic sclerosis

1. Introduction

Systemic sclerosis (SSc) is a chronic systemic autoimmune disease characterized by vascular endothelial dysfunction, fibrotic lesions of the cutaneous and visceral organs, and immune activity [1]. There are two major clinical phenotypes of SSc according to the extent of skin involvement [2]: diffuse cutaneous SSc (dcSSc), in which skin lesions are observed on the trunk and extend to the elbows and/or knees, and limited cutaneous SSc (lcSSc), in which skin thickening is mainly restricted to the face, fingers, and forearms [3]. A key characteristic of SSc is the production of autoantibodies, and antinuclear antibodies (ANAs) (anti-RNA polymerase [RNAP], anti-DNA topoisomerase I, and anticentromere antibodies) have been detected in more than 95% of SSc patients [1,4].

In autoimmune diseases, antibodies generally recognize host proteins as antigens and form immune complexes (ICs). Excessive IC formation leads to inflammation, and IC deposition in tissues causes tissue damage. Because ICs induce humoral immune responses, it has been suggested that they are involved in the pathogenicity of autoimmune diseases. Especially, the formation and deposition of ICs on visceral organs has been implicated in the pathogenesis of systemic lupus erythematosus (SLE) [5]; ANAs (anti-double-stranded DNA and anti-Sm) have been detected in most SLE patients. However, the role of ICs in SSc, an autoimmune disease similar to SLE, is poorly understood.

Recent studies have demonstrated the pathogenic potential of ICs containing SSc-specific antibodies [1,4] and the presence of ICs in sera from SSc patients [1]. Furthermore, ICs from SSc patients induced inflammatory and profibrotic mediators by activating Toll-like receptors, suggesting the pathogenic potential of ICs from SSc patients [1]. The results of these studies also suggested that in addition to fibroblasts and endothelial cells, ICs might interact with several other cell types involved in SSc pathogenesis, including adipocytes, T cells, macrophages, and plasmacytoid dendritic

cells [4]. Furthermore, SSc-specific antibodies indirectly mediated fibrosis in SSc by forming ICs and interacting with soluble target antigens [3],[4]. Although a relationship between ICs and SSc pathogenesis has been suggested, the identification of constituent antigens within ICs has rarely been reported.

The aim of this study was to identify antigens in the serum of SSc patients and to explore SSc pathogenesis. We compared IC concentrations and the numbers of antigens incorporated into ICs (IC-antigens) between patients with SSc and patients with SLE, and between patients with dcSSc and patients with lcSSc, to investigate the associations between ICs and SSc pathogenesis. Next, we identified potentially pathogenic SSc-specific antigens by immune complexome analysis that enabled comprehensive identification of IC-antigens [6]. Finally, we investigated the expression patterns of specific IC-antigens (revealed by immune complexome analysis) in human skin sections by immunohistochemistry.

2. Materials and methods

2.1. Patients

Serum samples were collected from 83 patients: SSc (n = 47; 11 dcSSc and 36 lcSSc; age, 43–84 years; 41 women; disease duration, 10.9 ± 9.4 years) and SLE (n = 36; 20 exhibited lupus nephritis). On the basis of their SLE disease activity index (*SLEDAI*) score, SLE patients were included in the high disease activity (HDA; SLEDAI > 10) group or not HDA (SLEDAI < 10) group [7]. There were 22 HDA SLE patients and 14 not HDA SLE patients. All patients fulfilled the 2013 ACR/EULAR criteria for diagnosis with SSc [8]. Sample collection and diagnostic assessment were performed at Nagasaki University Hospital and Sasebo Chuo Hospital. All experiments were performed in accordance with the Helsinki Declaration and with approval from the Institutional Review

Board of Nagasaki University Hospital (IRB approval no. 16042536 and 12012397). Each patient provided written informed consent for their participation in this study.

2.2. Immune complex concentration [C1q Enzyme-linked immunosorbent assay (ELISA)]

C1q ELISAs were performed as described by Stanilova et al. [9] with slight modification. In brief, flat bottomed polystyrene microtiter plates were incubated overnight at 4°C with 10 µg/ml C1q diluted in 100 mM sodium hydrogen carbonate buffer. After three washes, 100 µl of each serum sample, diluted 1:100 in phosphate-buffered saline (containing 2% BSA and 0.1% Tween 80) was incubated in the microtiter plates for 1 h at room temperature. After washing, 100 µl of goat anti-human IgG-Fc HRP (Bethyl Laboratories, Inc, Montgomery, TX, USA) diluted 1:160,000 in phosphate-buffered saline (containing 2% BSA and 0.1% Tween 80) was incubated in the microtiter plates for 1 h at room temperature. After 1 h of incubation with 100 µl of substrate solution (3,3',5,5'-tetramethylbenzidine), the developed color reaction was stopped with 2 M H_2SO_4, and the OD at 450 nm was measured on an ELISA plate reader (MULTISKAN JX, Thermo Fisher Scientific). All samples and standards were measured in duplicate, and the results were expressed as equivalents of heat-aggregated IgG.

2.3. Immune complexome analysis

ICs were purified using IC-capturing beads (protein A-coated magnetic beads [PureProteome®]). Under gentle mixing conditions, beads (40 µl) were incubated for 30 min at room temperature with 10 µl of patient serum diluted in 90 µl of phosphate-buffered saline; the liquid was then removed. In this analysis, we used papain, which digests antibodies at the hinge region, to selectively recover (elute) antigens and Fab from ICs collected on magnetic beads. This procedure was used to exclude non-specific

binding proteins from the liquid chromatography-tandem mass spectrometry (MS) analysis conducted for antigen identification. The detailed analytical procedures are described in our previous study [6] and Supplementary Data S1.

2.4. Immunohistochemistry

The skin expression patterns of mediator of RNAP II transcription subunit 30 (MED30), detected frequently and specifically in SSc patients by immune complexome analysis, were compared between patients with SSc and patients with SLE. Skin samples were collected from 11 patients: SSc (n = 7; three dcSSc and four lcSSc; age, 25–78 years; seven women; disease duration, 1.02 ± 1.0 years) and normal controls (n = 4; age, 31-80 years; two women). For immunohistochemistry, paraffin-embedded sections of human skin were used. Sections were deparaffinized and rehydrated by washing with xylene and a decreasing ethanol gradient. Then, sections were incubated for 10 min in 3% H_2O_2/H_2O to block endogenous peroxidase activity after epitope retrieval using a microwave. Thereafter, sections were stained using Simple Stain MAX-PO (R) (Nichirei Bioscience, Tokyo, Japan), in accordance with the manufacturer's instructions. Polyclonal rabbit anti-human MED30 (1:50) (Proteintech Group, Chicago, IL, USA) was used as the primary antibody. Negative controls were prepared using the same procedure, but the primary antibody was omitted. The color was developed by incubating the sections in 3,3'-diaminobenzidine and H_2O_2 for 10 min, followed by counterstaining in hematoxylin. Images were captured using a digital microscope (BZ-X700; Keyence, Osaka, Japan). For each section, the area of MED30-positive staining in a random area of deep dermis was quantified by a hybrid cell counting system mounted on the BZ-X700 microscope.

2.5. Statistical analysis

GraphPad Prism software (GraphPad, San Diego, CA, USA) was used for statistical analyses. ANOVA with Tukey's multiple comparison test was used to compare IC concentrations and numbers of IC-antigens between patients with SSc and patients with SLE, and between patients with dcSSc and patients with lcSSc. Student's unpaired *t*-test was used to evaluate the expression levels of MED30 in skin sections. All data were expressed as the mean ± standard deviation. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Comparison of IC concentrations and numbers of IC-antigens between patients with SSc and patients with SLE

We identified and compared 977 IC-antigens in serum samples from patients with SSc and patients with SLE. IC concentrations were compared between SSc patients and each of the two groups classified according to SLEDAI (Figure 1A). IC concentrations were significantly different between SSc patients and HDA SLE patients, but they were not significantly different between SSc patients and not HDA SLE patients (mean values: SSc, 10.6 ± 5.4 mg/ml; not HDA SLE, 12.3 ± 15.9 mg/ml; HDA SLE, 39.2 ± 40.2 mg/ml). The numbers of IC-antigens identified by immune complexome analysis were compared between SSc patients and each of the two groups classified according to SLEDAI (Figure 1B). The numbers of IC-antigens were not significantly different between SSc patients and HDA SLE patients, but they were significantly different between SSc patients and not HDA SLE patients (mean values: SSc, 59.1 ± 11.3; not HDA SLE, 48.9 ± 9.5; HDA SLE, 56.2 ± 15).

3.2. Comparison of IC concentrations and numbers of IC-antigens between patients with dcSSc and patients with lcSSc

We identified and compared 478 IC-antigens in serum samples from patients with dcSSc and patients with lcSSc. IC concentrations were compared between patients with dcSSc and patients with lcSSc (Figure 2A); IC concentrations were not significantly different between the two groups (mean value: dcSSc, 12.7 ± 6.7 mg/ml; lcSSc, 9.9 ± 4.8 mg/ml). The numbers of IC-antigens identified by immune complexome analysis were compared between patients with dcSSc and patients with lcSSc (Figure 2B); the numbers of IC-antigens were not significantly different between the two groups (mean value: dcSSc, 57.4 ± 10.4; lcSSc, 59.7 ± 11.5). However, a comparison of IC-antigen composition between patients with dcSSc and patients with lcSSc revealed that proteoglycan 4, which participates in boundary lubrication within articulating joints, was significantly more common among patients with lcSSc than among patients with dcSSc (lcSSc, 7/36; dcSSc, 0/11).

3.3. Identification of IC-antigens specific for SSc patients by immune complexome analysis

Of the 977 IC-antigens, 478 IC-antigens were only present in SSc patients. Of these 478 IC-antigens, MED30, tetra-peptide repeat homeobox protein 1 (TPRX1), ATP-dependent RNA helicase DHX29, fibrinogen beta chain, and dermcidin were independently identified in samples from five or more patients; they were also found exclusively in SSc patients (Table 1). Of these five antigens, MED30 and TPRX1 were detected with the highest frequency (30%). The frequencies of MED30 and TPRX1 were not significantly different between patients with dcSSc and patients with lcSSc. Additionally, we identified RNAP II subunits and RNAP II-associated proteins in serum ICs from patients with SSc and patients with SLE (Table 2). In total, 10 RNAP II subunits and RNAP II-associated proteins were identified in patients with SSc and patients with SLE. Six of the 10 proteins were detected specifically in SSc patients; MED30 was

detected in 14 SSc patients, and each of the other proteins was only detected in one SSc patient. MED30 and TPRX1 MS data, as well as MS/MS spectra assigned to the unique peptides of these proteins, are shown in Supplementary Figure S1.

3.4. In situ expression levels of MED30 in skin sections from patients with SSc

We investigated the *in situ* expression levels of MED30 in skin sections by immunohistochemistry. MED30 expression was observed in epidermal lesions in skin sections from SSc patients and normal controls; however, the expression levels in fibroblasts and vascular structures in the deep dermis were higher in SSc patients than in normal controls (Figure 3A, B, and C). Quantification analysis of the deep dermis using a hybrid cell count system confirmed the qualitative staining results (Figure 3D).

4. Discussion

Normally, ICs are efficiently removed by mononuclear phagocytes and therefore do not accumulate in the body. However, when the increase in ICs is faster than the rate of IC clearance, ICs are deposited on tissues and initiate an immune cascade involving complement activation and macrophage induction, which leads to tissue injury and clinical symptoms [10]. Additionally, when antigens form ICs with antibodies, IC-associated antigens may lose their original molecular functions [11]. Therefore, the identification of IC-antigens in the serum of affected patients may help to elucidate the corresponding disease pathogenesis.

After measuring IC concentrations in patients with SSc and patients with SLE, we measured the numbers of IC-antigens in serum (Figure 1). The order of numbers of IC-antigens and the order of IC concentrations between patients with SSc and patients with SLE were not the same. Our results suggest that many types of antigens form small numbers of ICs in SSc patients, whereas certain IC-antigens were present in excessive

numbers in SLE patients. Because ICs are the main pathogenic factor in SLE and an increased number of ICs is characteristic of SLE [5], an unexpectedly rich variety of ICs may contribute to SSc pathogenesis through IC-induced inflammatory reactions. Therefore, the comprehensive identification of SSc-specific IC-antigens is important to clarify SSc pathogenesis.

Vascular endothelial dysfunction is presumed to cause most symptoms of SSc because microvascular damage and dysfunction are the earliest morphological and functional markers of SSc [12]. In terms of SSc subtypes, dcSSc is a greater concern because of its rapid progression and early internal-organ involvement, which are life-threatening and associated with high mortality. In patients with dcSSc, serum levels of angiogenesis enhancers and inhibitors significantly change during periods of disease activity [13]. Additional hallmarks of SSc pathogenesis during disease progression include proliferation of skin fibroblasts, leading to myofibroblast transition, and proliferation of vascular smooth muscle cells, which causes intimal hyperplasia in small arteries [14]. Although early dcSSc (within the first 3–5 years after symptom onset) is a particularly important topic [15], our study only included two patients with early dcSSc; therefore, we could not explore the IC-antigen profile in patients with early dcSSc. In terms of SSc clinical phenotype, our study showed that the proportion of patients who exhibited more than one of the five SSc-specific IC-antigens (shown in Table 1) was higher among patients with dcSSc (82%) than among patients with lcSSc (61%). Moreover, IC concentrations tended to be higher in patients with dcSSc than in patients with lcSSc (Figure 2A). These results suggested that the identified IC-antigens and IC concentrations may have influenced the observed differences in pathogenesis, but these differences were not statistically significant. Furthermore, although multiple studies have shown that proteoglycans have an effect on skin fibrosis [16],[17], we could not determine

whether proteoglycan 4 is involved in the pathogenesis of lcSSc.

In this study, we presumed that frequently detected IC-antigens were related to the pathogenesis of SSc. Five IC-antigens were frequently detected as IC-antigens specific to SSc patients (Table 1). Here, we discuss the potential association of the most frequently identified IC-antigens with the pathogenesis of SSc. TPRX1 was specifically and frequently detected in patients with SSc. However, information about this protein and its relationship to SSc is not clear. In contrast, MED30 regulates endothelial differentiation by an alternative splicing mechanism [18]. We focused on MED30 because abnormal endothelial differentiation may be involved in SSc pathogenesis. MED consists of more than 30 protein complexes that interact with RNAP II to promote the assembly, activation, and regeneration of transcription complexes on core promoters during the initiation and re-initiation phases of transcription [18]. MED30 is localized in the head submodule of MED [19], which is partially conserved and exhibits the most extensive contact with RNAP II [18]. In addition to MED30, we identified RNAP II subunits and RNAP II-associated proteins that form ICs in SSc patients (Table 2). Previous studies have indicated that ANAs have pathogenic roles in SSc; these roles vary according to antibody type, autoantigen specificity, and clinical phase of the disease [20]. Although anti-RNAP III antibodies are used in assays for the detection of ANAs [21], anti-RNAP antibodies in the serum of patients with SSc might recognize multiple subunits of RNAP I, II, and III [22], [23]. The findings in previous studies and our current results suggest that anti-RNAP antibodies preferentially form ICs against these RNAP II subunits and RNAP II-associated proteins in SSc patients. In terms of MED30 pathogenicity, a missense mutation in MED30 reportedly causes progressive cardiomyopathy in homozygous mice [24]; the expression of MED30 promoted the proliferation and invasion of gastric cancer cells *in vitro* [25]. The protein antigenicity of MED30 has been associated with gene

mutations, structural misfolding, degradation, proteolytical cleavage, or overexpression. In this study, we explored MED30 expression at lesion sites in SSc patients (Figure 3). We found that MED30 was more strongly expressed in fibroblasts and vascular structures in skin sections from SSc patients, compared with skin sections from normal controls. These findings suggest that the increased expression of MED30 has an antigenic effect at the lesion site; MED30 function may be impaired or inhibited by IC formation. Considering that SSc-specific antibodies have been reported to indirectly mediate fibrosis [4], and that anti-RNAP antibodies in the serum of patients with SSc may recognize RNAP I, II, and III [22], [23], we suspect that RNAP II-associated proteins (Table 2) affect SSc pathogenesis through mechanisms such as the MED30 pathway.

Additionally, we compared SSc-characteristic IC-antigens with SSc susceptibility genes, which were identified using a candidate gene approach or in genome-wide association studies; we found only one instance of overlap between antigens and genes (nitric oxide synthase and *NOS*). A nitric oxide synthase-incorporated IC was detected in only one SSc patient. This finding is consistent with the lower heritability of SSc compared with other autoimmune diseases (e.g., rheumatoid arthritis [26]); it is also consistent with the pathological contributions of environmental factors, such as exposure to chemical compounds [27]. Such exposure can trigger chemical modification of various proteins, leading to protein abnormality and antigenicity.

This study had the following limitations. Antigens specifically detected in a disease group were considered promising candidate pathogenic molecules because our method screens for disease-associated IC-antigens; however, the clinical benefits of these specific antigens should be validated by further analysis using a large number of patients. The sensitivity of individual disease-specific IC-antigens may be improved by developing ELISAs that specifically detect individual ICs with high specificity and sensitivity [28]. Moreover, the presence of autoantibodies that bind to SSc-specific antigens should be

confirmed. Nevertheless, the accurate detection of such autoantibodies requires information about epitope sequences in the antigen structure. Although our method detects and identifies antigens that form ICs *in vivo*, it does not provide epitope sequence information.

Acknowledgments

We thank J. Ludovic Croxford, PhD, and Ryan Chastain-Gross, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Funding statement

This work was supported by JSPS KAKENHI [Grant number 21K08439] and THE KATO MEMORIAL TRUST FOR NAMBYO RESEARCH.

Authors' contributions statement

Yuki Jimbayashi Kutsuna: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft. Naoki Iwamoto: Conceptualization, Methodology, Investigation, Resources, Data Curation, Writing - Original Draft, Funding acquisition. Kunihiro Ichinose: Resources, Data Curation. Nozomi Aibara: Formal analysis, Investigation, Writing - Original Draft. Katsumi Nakashima: Formal analysis, Investigation. Hideki Nakamura: Data Curation. Yuta Koike: Resources. Hiroyuki Murota: Resources, Data Curation. Yukitaka Ueki: Resources. Hirotaka Miyamoto: Data Curation. Junya Hashizume: Formal analysis, Data Curation. Yukinobu Kodama: Data Curation. Mikiro Nakashima: Methodology, Investigation. Atsushi Kawakami: Methodology, Data Curation. Kaname Ohyama: Conceptualization, Methodology, Investigation, Data Curation, Writing - Original Draft, Supervision, Project administration, Funding acquisition.

Declarations of competing interest

The authors have declared no conflicts of interests

Data availability statement

All study data are included in the article. Some study data are available upon reasonable request to the corresponding author.

References

[1] E. Raschi, C.B. Chighizola, L. Cesana, D. Privitera, F. Ingegnoli, C. Mastaglio, P.L. Meroni, M.O. Borghi, Immune complexes containing scleroderma-specific autoantibodies induce a profibrotic and proinflammatory phenotype in skin fibroblasts, Arthritis Research and Therapy. 20 (2018) 1–18. https://doi.org/10.1186/s13075-018-1689-6.

[2] E.C. LeRoy, J. Medsger T.A., Criteria for the classification of early systemic sclerosis, Journal of Rheumatology. 28 (2001) 1573–1576.

[3] C. Corallo, S. Cheleschi, M. Cutolo, S. Soldano, A. Fioravanti, N. Volpi, D. Franci, R. Nuti, N. Giordano, Antibodies against specific extractable nuclear antigens (ENAs) as diagnostic and prognostic tools and inducers of a profibrotic phenotype in cultured human skin fibroblasts: Are they functional?, Arthritis Research and Therapy. 21 (2019) 1–11. https://doi.org/10.1186/s13075-019-1931-x.

[4] E. Raschi, D. Privitera, C. Bodio, P.A. Lonati, M.O. Borghi, F. Ingegnoli, P.L. Meroni, C.B. Chighizola, Scleroderma-specific autoantibodies embedded in immune complexes mediate endothelial damage: an early event in the pathogenesis of systemic sclerosis, Arthritis Research and Therapy. 22 (2020). https://doi.org/10.1186/s13075-020-02360-3.

[5] K.A. Davies, A.M. Peters, H.L.C. Beynon, M.J. Walport, Immune complex processing in patients with systemic lupus erythematosus. In vivo imaging and clearance studies, Journal of Clinical Investigation. 90 (1992) 2075–2083. https://doi.org/10.1172/JCI116090.

[6] N. Aibara, C. Kamohara, A.K. Chauhan, N. Kishikawa, Y. Miyata, M. Nakashima, N. Kuroda, K. Ohyama, Selective, sensitive and comprehensive detection of immune complex antigens by immune complexome analysis with papain-digestion and elution, Journal of Immunological Methods. 461 (2018) 85–90. https://doi.org/10.1016/j.jim.2018.06.021.

[7] M. Petri, M. Genovese, E. Engle, M. Hochberg, Definition, incidence, and clinical description of flare in systemic lupus erythematosus. A prospective

cohort study, Arthritis & Rheumatism. 34 (1991) 937–944. https://doi.org/10.1002/art.1780340802.

[8] F. Van Den Hoogen, D. Khanna, J. Fransen, S.R. Johnson, M. Baron, A. Tyndall, M. Matucci-Cerinic, R.P. Naden, T.A. Medsger, P.E. Carreira, G. Riemekasten, P.J. Clements, C.P. Denton, O. Distler, Y. Allanore, D.E. Furst, A. Gabrielli, M.D. Mayes, J.M. Van Laar, J.R. Seibold, L. Czirjak, V.D. Steen, M. Inanc, O. Kowal-Bielecka, U. Müller-Ladner, G. Valentini, D.J. Veale, M.C. Vonk, U.A. Walker, L. Chung, D.H. Collier, M.E. Csuka, B.J. Fessler, S. Guiducci, A. Herrick, V.M. Hsu, S. Jimenez, B. Kahaleh, P.A. Merkel, S. Sierakowski, R.M. Silver, R.W. Simms, J. Varga, J.E. Pope, 2013 classification criteria for systemic sclerosis: An american college of rheumatology/European league against rheumatism collaborative initiative, Arthritis and Rheumatism. 65 (2013) 2737–2747. https://doi.org/10.1002/art.38098.

[9] S.A. Stanilova, E.S. Slavov, Comparative study of circulating immune complexes quantity detection by three assays - CIF-ELISA, C1q-ELISA and anti-C3 ELISA, Journal of Immunological Methods. 253 (2001) 13–21. https://doi.org/10.1016/S0022-1759(01)00370-2.

[10] S. Jancar, M.S. Crespo, Immune complex-mediated tissue injury: A multistep paradigm, Trends in Immunology. 26 (2005) 48–55. https://doi.org/10.1016/j.it.2004.11.007.

[11] T. Mutoh, T. Shirai, T. Ishii, Y. Shirota, F. Fujishima, F. Takahashi, Y. Kakuta, Y. Kanazawa, A. Masamune, Y. Saiki, H. Harigae, H. Fujii, Identification of two major autoantigens negatively regulating endothelial activation in Takayasu arteritis, Nature Communications. 11 (2020) 1–13. https://doi.org/10.1038/s41467-020-15088-0.

[12] M. Cutolo, A. Sulli, V. Smith, Assessing microvascular changes in systemic sclerosis diagnosis and management, Nature Reviews Rheumatology. 6 (2010) 578–587. https://doi.org/10.1038/nrrheum.2010.104.

[13] N. Del Papa, F. Pignataro, The Role of endothelial progenitors in the repair of vascular damage in systemic sclerosis, Frontiers in Immunology. 9 (2018) 1–10. https://doi.org/10.3389/fimmu.2018.01383.

[14] E. Kissin, J.H. Korn, Apoptosis and myofibroblasts in the pathogenesis of systemic sclerosis., Current Rheumatology Reports. 4 (2002) 129–135. https://doi.org/10.1007/s11926-002-0008-y.

[15] A.L. Herrick, S. Assassi, C.P. Denton, Skin involvement in early diffuse cutaneous systemic sclerosis: an unmet clinical need, Nature Reviews Rheumatology. 18 (2022) 276–285. https://doi.org/10.1038/s41584-022-00765-9.

[16] G. Westergren-Thorsson, L. Cöster, A. Åkesson, F.A. Wollheim, Altered dermatan sulfate proteoglycan synthesis in fibroblast cultures established from skin of patients with systemic sclerosis, Journal of Rheumatology. 23 (1996).

[17] M. KITABATAKE, H. ISHIKAWA, H. MAEDA, Immunohistochemical demonstration of proteoglycans in the skin of patients with systemic sclerosis, British Journal of Dermatology. 108 (1983). https://doi.org/10.1111/j.1365-2133.1983.tb03962.x.

[18] M. Rienzo, J. Nagel, A. Casamassimi, A. Giovane, S. Dietzel, C. Napoli, Mediator subunits: Gene expression pattern, a novel transcript identification and nuclear localization in human endothelial progenitor cells, Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 1799 (2010) 487–495. https://doi.org/10.1016/j.bbagrm.2010.05.001.

[19] A. Casamassimi, C. Napoli, Mediator complexes and eukaryotic transcription regulation: An overview, Biochimie. 89 (2007) 1439–1446. https://doi.org/10.1016/j.biochi.2007.08.002.

[20] C. Kayser, M.J. Fritzler, Autoantibodies in systemic sclerosis: Unanswered questions, Frontiers in Immunology. 6 (2015) 2–7. https://doi.org/10.3389/fimmu.2015.00167.

[21] Y. Okano, V.D. Steen, T.A. Medsger, Autoantibody reactive with RNA polymerase III in systemic sclerosis, Annals of Internal Medicine. 119 (1993). https://doi.org/10.7326/0003-4819-119-10-199311150-00007.

[22] M. Kuwana, Y. Okano, J. Kaburaki, T.A. Medsger, T.M. Wright, Autoantibodies to RNA polymerases recognize multiple subunits and demonstrate cross-reactivity with rna polymerase complexes, Arthritis and Rheumatism. 42 (1999) 275–284. https://doi.org/10.1002/1529-0131(199902)42:2<275::AID-ANR9>3.0.CO;2-P.

[23] G.R. Harvey, A.L. Rands, N.J. Mchugh, Anti-RNA polymerase antibodies in systemic sclerosis (SSc): Association with anti-topoisomerase I antibodies and identification of autoreactive subunits of RNA polymerase II, Clinical and Experimental Immunology. 105 (1996) 468–474. https://doi.org/10.1046/j.1365-2249.1996.d01-798.x.

[24] P. Krebs, W. Fan, Y.-H. Chen, K. Tobita, M.R. Downes, M.R. Wood, L. Sun, X. Li, Y. Xia, N. Ding, R.M. Evans, B. Beutler, Lethal mitochondrial cardiomyopathy in a hypomorphic Med30 mouse mutant is ameliorated by ketogenic diet, Proceedings of the National Academy of Sciences of the United States of America. 108 (2011) 19678–19682. https://doi.org/10.1073/pnas.1117835108.

[25] Y.J. Lee, M.E. Han, S.J. Baek, S.Y. Kim, S.O. Oh, MED30 regulates the proliferation and motility of gastric cancer cells, PLoS ONE. 10 (2015) 1–15. https://doi.org/10.1371/journal.pone.0130826.

[26] P.S. Tsou, A.H. Sawalha, Unfolding the pathogenesis of scleroderma through genomics and epigenomics, Journal of Autoimmunity. 83 (2017) 73–94. https://doi.org/10.1016/j.jaut.2017.05.004.

[27] A. Makol, M.J. Reilly, K.D. Rosenman, Prevalence of connective tissue disease in silicosis (1985-2006)-a report from the state of michigan surveillance system for silicosis, American Journal of Industrial Medicine. 54 (2011) 255–262. https://doi.org/10.1002/ajim.20917.

[28] J.H. Rho, P.D. Lampe, High-throughput screening for native autoantigen-autoantibody complexes using antibody microarrays, Journal of Proteome Research. 12 (2013) 2311–2320. https://doi.org/10.1021/pr4001674.

Figure legends

Figure 1. Comparison of IC concentration and numbers of IC-antigens between the patients with SSc and SLE.

Classification of SLE patients by SLE disease activity index (*SLEDAI*). Using the SLEDAI score system, SLE patients were classified with high disease activity (HDA; SLEDAI > 10) or not HDA (SLEDAI < 10). Comparison of **A**: IC concentrations and **B**: number of IC-antigens between the SSc, not HDA SLE, and HDA SLE groups. *p<0.005 between patient groups by ANOVA with Tukey's multiple comparison test.

Figure 2. Comparison of IC concentration and numbers of IC-antigens between the patients with dcSSc and lcSSc.

Comparison of **A**: IC concentrations and **B**: number of IC-antigens between dcSSc and lcSSc. *p<0.005 between patient groups by ANOVA with Tukey's multiple comparison test.

Figure 3. MED30 expression in the skin.

Sections of SSc skin (A) and normal skin (B) were stained with MED30. Positive staining of MED30 appears as a brown color in epidermal lesions (A, B upper) and deep dermis (A, B lower). C, Negative control staining of SSc skin. Sections were counterstained with eosin (A-C). Quantitative analysis of MED30 staining in the deep dermis from SSc (n=7) and normal controls (n=4) was performed by a hybrid cell counting system (D). MED30 expression was determined relative to normal skin sections defined as 1. Values are given as the means ± standard deviation. *p<0.05 versus normal control.

Supporting Information

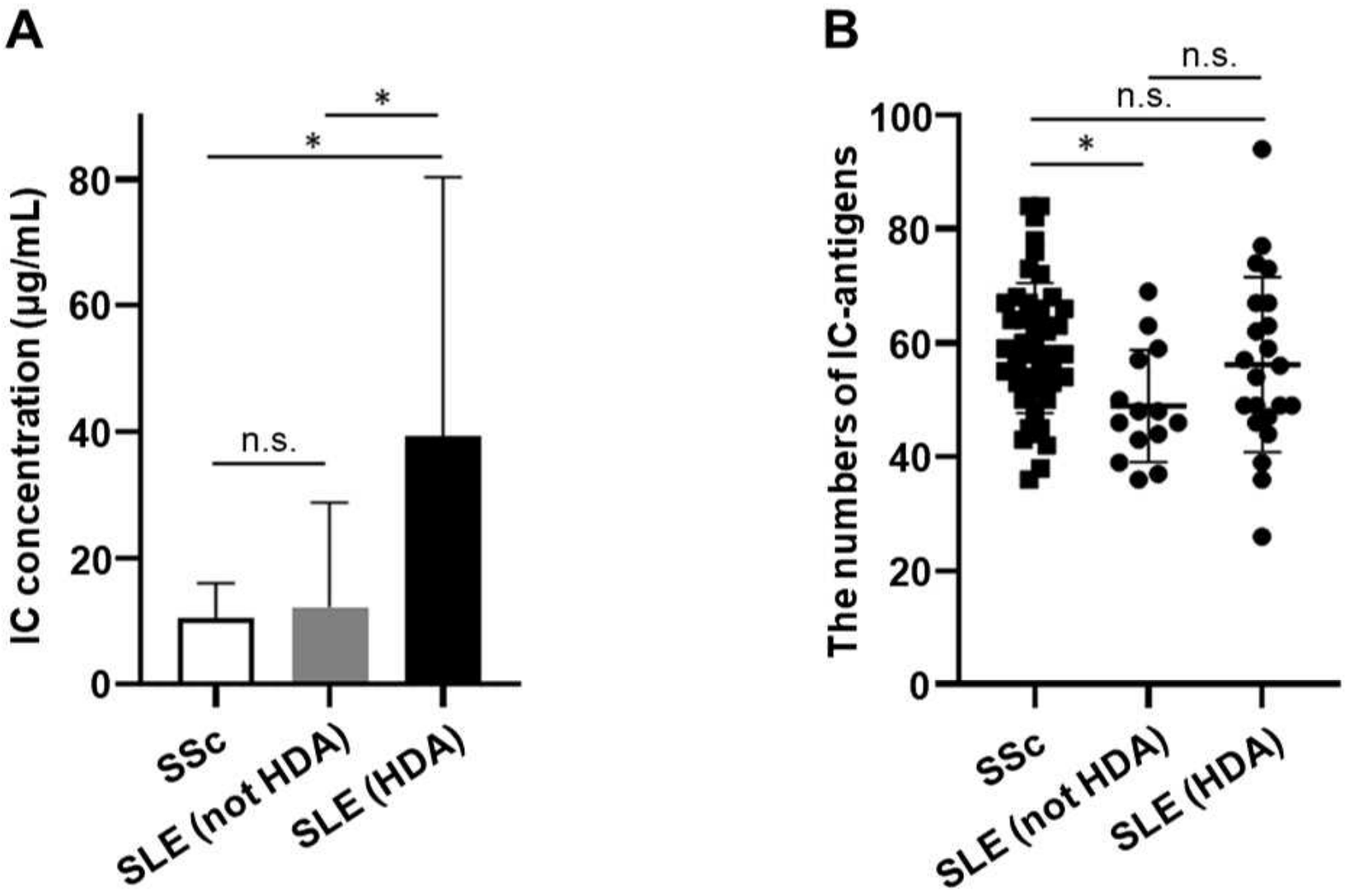
Additional Supporting Information may be found in the online version of this article at the publisher's website.

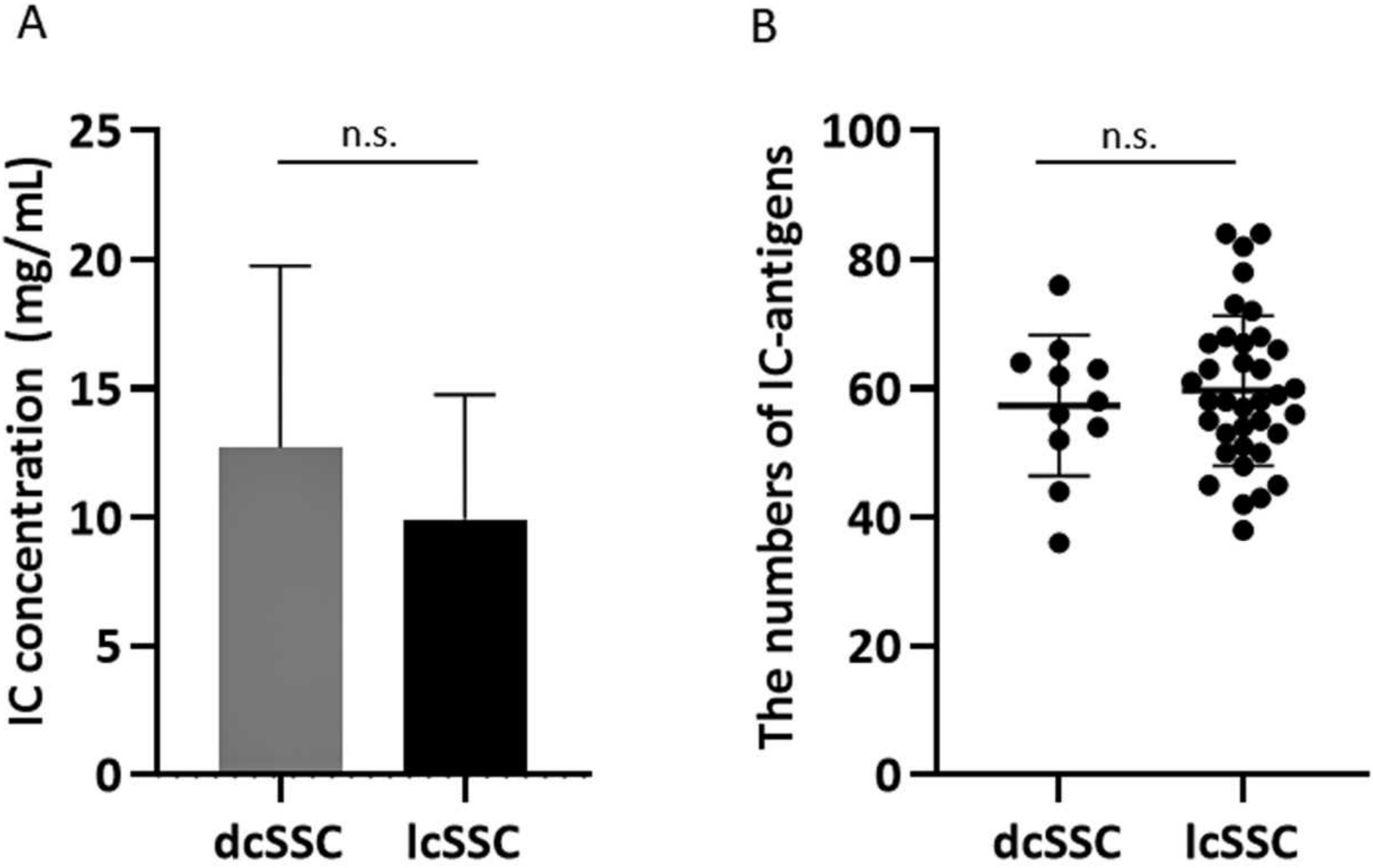
Table 1. SSc-specific IC-antigens that were detected in more than five SSc patients.

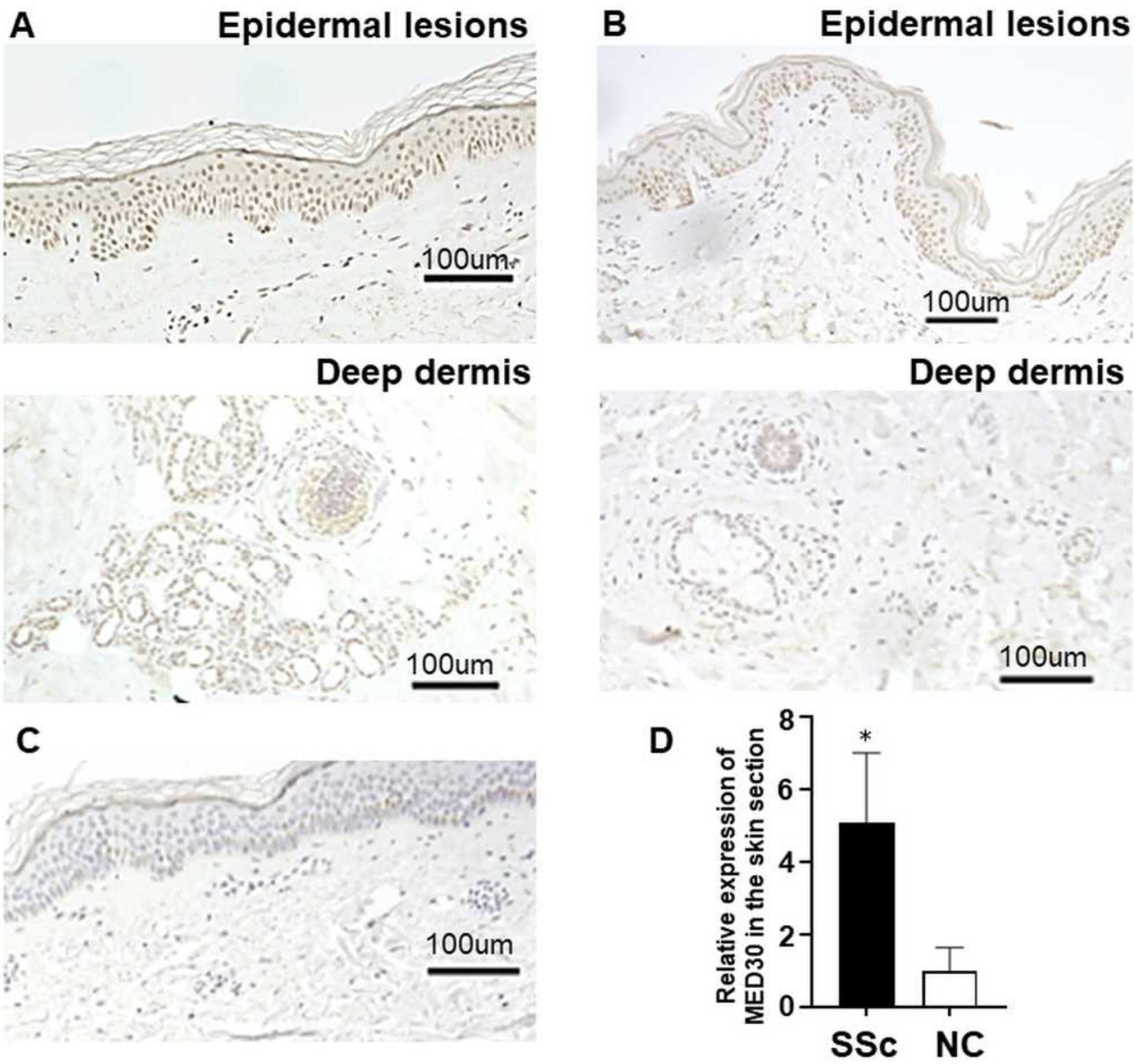
Accession	Description	Frequency (n=47)
Q96HR3	Mediator of RNA polymerase II transcription subunit 30	14
Q8N7U7	Tetra-peptide repeat homeobox protein 1	14
Q7Z478	ATP-dependent RNA helicase DHX29	8
P02675	Fibrinogen beta chain	5
P81605	Dermcidin	5

Table 2. List of identified RNAP II subunits and RNAP II-associated proteins in serum immune complexes from systemic sclerosis and systemic lupus erythematosus patients

Accession	Description	SSc (n=47)	SLE (n=36)
Q96HR3	Mediator of RNA polymerase II transcription subunit 30	14	
A6NLF2	RNA polymerase II transcription factor SIII subunit A3-like-2	1	
O75448	Mediator of RNA polymerase II transcription subunit 24	1	
P24928	DNA-directed RNA polymerase II subunit RPB1	1	
Q71F56	Mediator of RNA polymerase II transcription subunit 13-like	1	
Q8N7H5	RNA polymerase II-associated factor 1 homolog	1	1
Q9UHV7	Mediator of RNA polymerase II transcription subunit 13		1
O60244	Mediator of RNA polymerase II transcription subunit 14		1
Q6EEV4	DNA-directed RNA polymerase II subunit GRINL1A, isoforms 4/5		1
Q86YW9	Mediator of RNA polymerase II transcription subunit 12-like protein		1


A
IC concentration (μg/mL)
0
20
40
60
80
*
*
n.s.
SSc
SLE (not HDA)
SLE (HDA)
B
The numbers of IC-antigens
0
20
40
60
80
100
*
n.s.
n.s.
SSc
SLE (not HDA)
SLE (HDA)


A
IC concentration (mg/mL)
25
20
15
10
5
0
n.s.
dcSSC
lcSSC
B
The numbers of IC-antigens
100
80
60
40
20
0
n.s.
dcSSC
lcSSC


A
Epidermal lesions
100um
B
Epidermal lesions
100um
Deep dermis
100um
Deep dermis
100um
C
100um
D
Relative expression of MED30 in the skin section
8
6
4
2
0
*
SSc
NC

Supporting Information

Supplementary Data S1

Immune complexome analysis

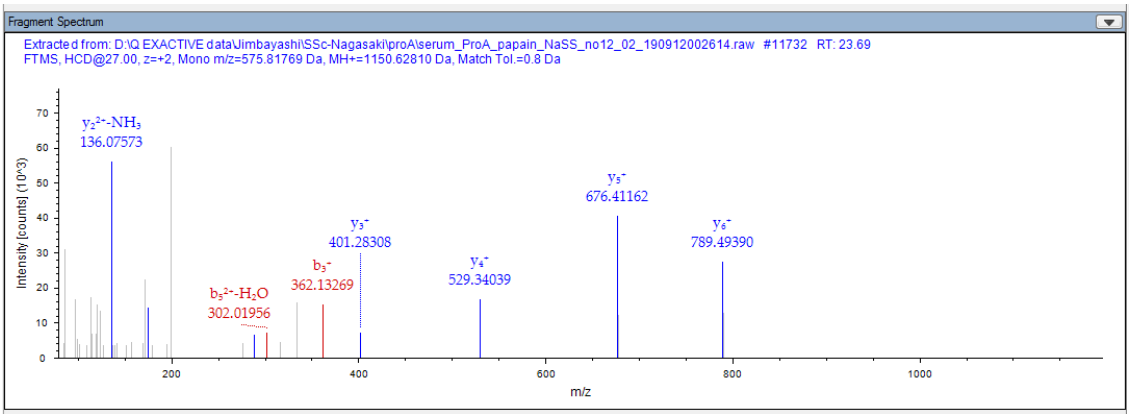
The beads were washed three times with 500 µL PBS, re-suspended in 50 µL of 0.01 mg/mL papain solution (0.04 M EDTA, 0.04 M L-cysteine) and incubated at 37°C for 30 min. The supernatant was transferred to another tube, 50 µL of 0.06 M iodoacetamide dissolved in PBS was added to stop papain digestion, then 100 µL of 10 mM dithiothreitol was added and the sample was further incubated at 56°C for 45 min. Iodoacetamide (100 µL, 55 mM) was added to the mixture and incubated at room temperature in the dark for 30 min. Ammonium hydrogen carbonate (100 µL, 50 mM) and 100 µL of ultrapure water were added, followed by trypsin in 0.05% acetic acid to achieve a final concentration of 4 mg of trypsin/L, then the mixture was incubated overnight at 37°C. We then added 12 µL of 10% TFA and mixed the beads for 2 min to stop digestion. This aliquot (about 500 µL) was vacuum-reduced to approximately 80 µL and stored at 4°C for subsequent analysis by nano-LC-MS/MS.

The peptide mixture (5 µL) was injected into the injection loop of a nano-precolumn (Acclaim PepMap^{TM} 100, 75 µm x 2 cm, nano Viper, C18, 3 µm, 100 Å, Thermo Fisher Scientific, Waltham, MA, USA) and washed with 0.1% trifluoroacetic acid in 2% acetonitrile. An MS/MS instrument (Q-Exactive series, Thermo Fisher Scientific) equipped with a nano-LC system (EASY-nLC^{TM}, Thermo Fisher Scientific) was used for analysis. Peptides were separated on a nano-LC column (C18, 75 µm i.d. × 125 mm, 3 µm particle, 100 Å pore size, Nikkyo Technos, Tokyo, Japan) and ion-sprayed into the MS/MS instrument using a spray voltage of 2.0 kV. Separation was performed using mobile phase A (0.1% formic acid in 1% acetonitrile) and mobile phase B (0.1% formic acid in 90% acetonitrile) with gradient elution at a flow rate of 300 nL/min from 5% to 35% mobile phase B in 20 min, and 100% mobile phase B held for 10 min. All spectra were measured with an overall mass/charge ratio range of 400–1500. Full MS scans were acquired with a mass resolution of 70,000. The AGC target value was 1.00E+06. Tandem mass spectra were acquired with a mass resolution of 17,500 and the AGC target value was 5.00E+04. The collision energy was normalized to 27%. The ion selection threshold was 2.00E+04 counts, and the maximum allowed ion accumulation times were 100 ms for full MS scans and 50 ms for tandem mass spectra. The dynamic exclusion time was set to 15 s. The transfer capillary temperature was set at 250°C. Spectra were searched against sub-databases from the public nonredundant protein database UniProt Knowledgebase (human, 2015.01.29 download) with the following search parameters: mass type, monoisotopic precursor and fragments; enzyme, trypsin (KR); enzyme limits, full enzymatic cleavage allowing up to two trypsin missed cleavages sites; peptide tolerance, 10 ppm; fragment ion tolerance, 0.8 Da; ion and ion series calculated, B and Y ions; static modification, C (carbamidomethylation, +57.021 Da); and differential modifications, M (oxidation, +15.995 Da), N, and Q (deamidation, +0.984 Da). MS/MS data were extracted using Proteome Discoverer ver. 1.4.0.288 (Thermo Fisher Scientific). The filter criteria to identify peptides assigned to a certain protein (single, double, and triple charged peptides with correlation factor [XCorr] and protein probability [P]) were flexibly adjusted using the Percolator system (1% false discovery rate). Each sample was subjected to a single pretreatment process and three

replicate nano-LC–MS/MS analyses. In this study, proteins other than antibodies were treated as IC-antigens and were identified by more than one unique peptide. Also, we defined the proteins that were detected in one or more than one of three replicates. Data processing was performed in Excel to identify specific IC-antigens. Performance of the nano-LC–MS/MS system was checked daily prior to measurements by confirming the peak shapes of fully digested peptides derived from bovine serum albumin (40 fmol) and the number (>1000) of proteins from a HeLa protein digest standard (100 ng, Thermo Fisher Scientific).

Supplementary Figure S1
Representative MS data and MS/MS spectra assigned to unique peptides of MED30 or TPRX1

MED30
Seuence: TMEIFQLLR, Charge: +2, Monoisotopic m/z: 575.81769 Da (-0.43 mmu/-0.74 ppm)
MH+: 1150.62810 Da, RT: 23.69 min, Identified with: Sequest HT (v1.3); XCorr: 1.33
Fragment match tolerance used for search: 0.8 Da



TPRX1
Seuence: GPSGILPAAEPTICSLHQAWGGPGCR, Charge: +3
Monoisotopic m/z: 897.10565 Da (+1.64 mmu/+1.83 ppm), MH+: 2689.30240 Da, RT: 17.91 min
Modification: C14-Carbamidomethyl (57.02146 Da), C25- Carbamidomethyl (57.0214 Da)
Identified with: Sequest HT (v1.3); XCorr: 3.04, Fragment match tolerance used for search: 0.8 Da