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**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/39025> since

*Published version:*

DOI:10.1002/art.22266

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# A Novel Autoantigen to Differentiate Limited Cutaneous Systemic Sclerosis From Diffuse Cutaneous Systemic Sclerosis

## The Interferon-Inducible Gene IFI16

Michele Mondini,<sup>1</sup> Matteo Vidali,<sup>2</sup> Marco De Andrea,<sup>1</sup> Barbara Azzimonti,<sup>2</sup> Paolo Airò,<sup>3</sup> Roberta D'Ambrosio,<sup>4</sup> Piersandro Riboldi,<sup>5</sup> Pier Luigi Meroni,<sup>5</sup> Emanuele Albano,<sup>6</sup> Yehuda Shoenfeld,<sup>7</sup> Marisa Gariglio,<sup>6</sup> and Santo Landolfo<sup>8</sup>

**Objective.** To investigate the presence and clinical significance of autoantibodies against the interferon-inducible gene IFI16 in systemic sclerosis (SSc), systemic lupus erythematosus (SLE), and other autoimmune diseases.

**Methods.** Immunohistochemical analysis was used to evaluate the expression of IFI16 in skin biopsy specimens obtained from patients with SSc and patients with SLE. Levels of antibodies against IFI16 in sera from 82 patients with SSc and 100 patients with SLE were determined by enzyme-linked immunosorbent assay. Other autoimmune diseases such as primary Sjögren's syndrome (SS), rheumatoid arthritis (RA), chronic urticaria, and hepatitis C virus (HCV) infection were also examined.

**Results.** Expression of IFI16 was greatly increased and was ubiquitous in all layers of the epidermis and in the dermal inflammatory infiltrates of lesional skin from both patients with SLE and patients with SSc. Patients with SLE, those with primary SS, and those with SSc exhibited significantly higher anti-IFI16 IgG antibody levels compared with normal controls (for SLE,  $P < 0.002$ ; for primary SS,  $P < 0.001$ ; for SSc,  $P < 0.0005$ ). Anti-IFI16 titers above the ninety-fifth percentile for control subjects were observed in 26% of the patients with SLE, 50% of those with primary SS, and 21% of those with SSc (28% of patients with limited cutaneous SSc [lcSSc] versus 4% of patients with diffuse cutaneous SSc [dcSSc]). In contrast, the prevalence of anti-IFI16 was 4% in patients with RA, 5% in those with chronic urticaria, and 13% in those with HCV infection.

**Conclusion.** The results of this study provide evidence that an IFN-inducible gene, IFI16, may be involved in the pathophysiologic mechanisms of connective tissue disorders such as SSc. Moreover, a strict correlation with lcSSc was also demonstrated, thus providing a novel tool in the differential diagnosis of lcSSc from dcSSc.

Supported by grants from the Italian Ministry for Education, Universities and Research; Regione Piemonte "Ricerca Sanitaria Finalizzata e Applicata (CIPE)"; Fondazione Internazionale di Ricerca in Medicina Sperimentale (FIRMS) – Fondazione San Paolo, Turin; and Ricerca Corrente 2005 IRCCS Istituto Auxologica Italiano. Dr. Mondini is recipient of a research fellowship from FIRMS. Dr. Meroni is recipient of a research fellowship from FIRMS.

<sup>1</sup>Michele Mondini, PhD, Marco De Andrea, PhD: Medical School of Turin, Turin, and Medical School of Piemonte Orientale, Novara, Italy; <sup>2</sup>Matteo Vidali, MD, Barbara Azzimonti, PhD: Medical School of Piemonte Orientale, Novara, Italy; <sup>3</sup>Paolo Airò, MD: Spedali Civili and University, Brescia, Italy; <sup>4</sup>Roberta D'Ambrosio, MD: Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan, Italy; <sup>5</sup>Piersandro Riboldi, MD, Pier Luigi Meroni, MD: IRCCS Istituto Auxologico Italiano, and University of Milan, Milan, Italy; <sup>6</sup>Emanuele Albano, MD, Marisa Gariglio, MD: Medical School of Piemonte Orientale, and Interdisciplinary Center for Research of Autoimmune Diseases, Novara, Italy; <sup>7</sup>Yehuda Shoenfeld, MD: Sheba Medical Center, Tel Hashomer, Israel; <sup>8</sup>Santo Landolfo, MD: Medical School of Turin, Turin, Italy.

Drs. Mondini, Gariglio, and Landolfo have a patent pending for the use of anti-IFI16 autoantibody detection.

Address correspondence and reprint requests to Santo Landolfo, MD, Laboratory of Viral Pathogenesis, Department of Public Health and Microbiology, Medical School of Turin, Via Santena 9, 10126 Turin, Italy. E-mail: santo.landolfo@unito.it.

Submitted for publication March 8, 2006; accepted in revised form September 5, 2006.

A family of interferon (IFN)-inducible genes, designated HIN-200 in humans and Ifi200 in murine species, encodes evolutionarily related human proteins (IFI16, IFIX, MND4, and AIM-2) and mouse proteins (p202, p203, p204, p205/D3) (1). The IFI16, p202, and p204 nuclear phosphoproteins participate in the inhibition of cell cycle progression, the modulation of differ-

entiation, and cell survival. Gene expression analyses in congenic mice have identified *Ifi202* as a candidate gene for lupus susceptibility (2).

Anti-IFI16 antibodies were reported in 29% of patients with systemic lupus erythematosus (SLE) (3) and in up to 70% of both patients with primary Sjögren's syndrome (SS) and those with secondary SS (4). A lower prevalence (0–13%) was observed in patients with rheumatoid arthritis (RA) and in those with scleroderma (systemic sclerosis; SSc)–polymyositis overlap syndrome (3%) (3,4). However, the studies on SS and RA enrolled small series of patients, and no data are available on the presence of these autoantibodies in SSc. The first aim of the current study was to evaluate the presence of anti-IFI16 autoantibodies in a larger number of patients with SS or RA as well as in patients with SSc and controls, and to partially characterize their antigenic specificity.

Moreover, the expression of IFI16 in target tissue involved in the autoimmune process (i.e., the salivary glands) has also been described (4). This finding raises the possibility that local tissue expression (or even up-regulation) can be pivotal in triggering an autoimmune response against this protein. Interestingly, physiologic expression of IFI16 was observed in vascular endothelial cells and in stratified squamous epithelia such as skin (5). Both of these tissues are targets for the main clinical manifestations of SLE and SSc. Accordingly, we evaluated the expression of IFI16 in lesional skin samples obtained from patients with SLE and patients with SSc, in order to investigate whether enhanced IFI16 expression might be associated with the occurrence of autoantibodies.

## PATIENTS AND METHODS

**Patients and controls.** The study group comprised 100 patients with SLE, 20 patients with primary SS, 82 patients with SSc, 50 patients with RA, and 38 patients with chronic urticaria. As controls, we investigated 80 sex- and age-matched healthy blood donors and 80 patients with chronic hepatitis C virus (HCV) infection. Informed consent was obtained from all participants.

All patients with SSc (73 women and 9 men, mean age 57 years [range 21–80 years]) were classified as having limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) according to the classification system described by LeRoy et al (6). Disease severity was assessed using the preliminary disease severity scale described by Mødsger et al (7). All of the patients with SLE (93 women and 7 men; mean age 40 years [range 18–65 years]) fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) re-

vised criteria for the classification of SLE (8). Among the patients with SLE, 48% had kidney involvement, and 17% had secondary antiphospholipid syndrome. Disease activity was evaluated according to the European Consensus Lupus Activity Measure (9). Patients with RA (39 women and 11 men; mean age 54 years [range 25–70 years]) fulfilled the ACR 1987 classification criteria for RA (10). Patients with primary SS (17 women and 3 men; mean age 48 years [range 27–64 years]) were classified according to the classification criteria described by Vitali (11). Patients with chronic urticaria (29 women and 9 men; mean age 38 years [range 22–50 years]) were selected as previously described (12).

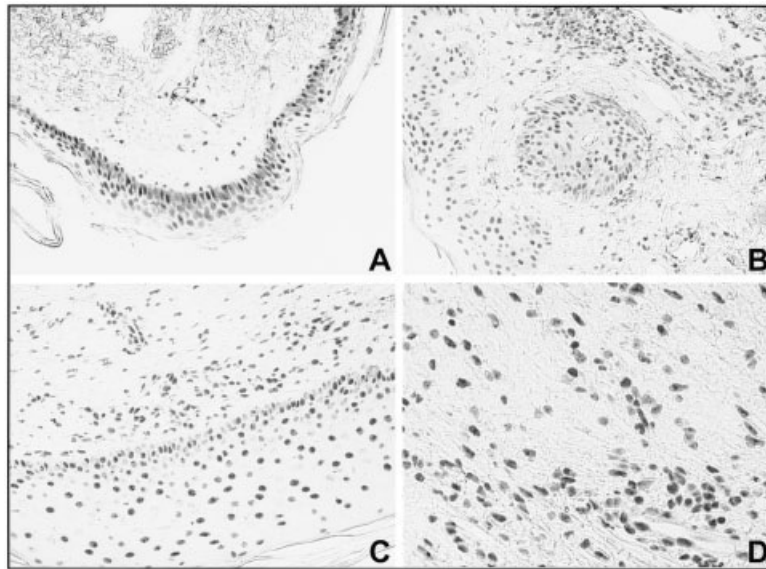
Keratoconjunctivitis was evaluated by ophthalmoscopic evaluation, Schirmer's test, tear break-up time, and rose bengal staining. Keratoconjunctivitis was diagnosed in 26% of patients with SSc, 10% of patients with SLE, 19% of patients with RA, and 6% of patients with HCV infection.

Skin biopsy specimens from 6 patients with SSc (4 with lcSSc and 2 with dcSSc) and from 8 patients with SLE, all of which were obtained for diagnostic purposes during a period of active skin disease, were available for immunohistochemical analysis. Control biopsy samples were obtained from the unaffected skin of patients undergoing surgery for unrelated diseases.

**Recombinant proteins.** The entire coding sequence of the b isoform of human IFI16 was subcloned in the pET30a expression vector (Novagen, Madison, WI), containing an N-terminal histidine tag. The sequences encoding the N-terminal (IFI16 N-term [amino acid residues 1–205]) or C-terminal (IFI16 C-term [amino acid residues 525–726]) fragments of IFI16 were amplified by polymerase chain reaction and cloned in frame in the pET30a vector. Expression and affinity purification were performed according to standard procedures. The purity of the proteins was assessed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. As negative controls for enzyme-linked immunosorbent assay (ELISA) and immunoblotting, the polypeptide encoded by the pET30a empty vector (control peptide) was expressed and purified according to the same protocol.

**Immunoblotting and immunohistology.** Immunoblotting for recombinant IFI16, IFI16 N-term, IFI16 C-term, or control peptide (pET30a) was carried out as previously described (13). Immunohistochemical analysis for IFI16 expression was performed on paraffin-embedded tissue sections, as previously described (5).

**Determination of antibody titers toward human recombinant IFI16, by ELISA.** Polystyrene microwell plates (Nunc-Immuno MaxiSorp; Nunc, Roskilde, Denmark) were coated for 16 hours at 4°C with 2 µg/ml of either recombinant IFI16 or control peptide in phosphate buffered saline (PBS). After blocking with 3% bovine serum albumin–PBS, sera (1:100) were added in duplicate and incubated for 1 hour at 37°C. After washing, horseradish peroxidase–conjugated rabbit anti-human IgG (Dako Cytomation, Carpinteria, CA) was added and incubated for 1 hour at 37°C. After the addition of substrate, absorbance was measured at 490 nm, using a microplate reader (Bio-Rad, Hercules, CA). The background reactivity of the reference mixture was subtracted to calculate the results. A standard curve was constructed by serially diluting anti-IFI16–positive patient sera.



**Figure 1.** Expression of IFI16 in **A**, the skin of a healthy control subject, **B**, lesional skin from a patient with systemic lupus erythematosus, and **C**, lesional skin from a patient with limited cutaneous systemic sclerosis (original magnification  $\times 10$ ). **D**, Higher-magnification view of **C**, showing IFI16 staining in inflammatory cells.

**Statistical analysis.** Statistical analysis was performed with SPSS software (SPSS, Chicago, IL), using one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. Either Fisher's exact test or the chi-square test was used to measure association. The independent effect of significant variables was assessed using forward conditional logistic regression analysis. Positivity cutoff values were calculated as the ninety-fifth percentiles for the control population. In the event of substantial deviation from normality, data were natural log-transformed before parametric analysis was performed.

## RESULTS

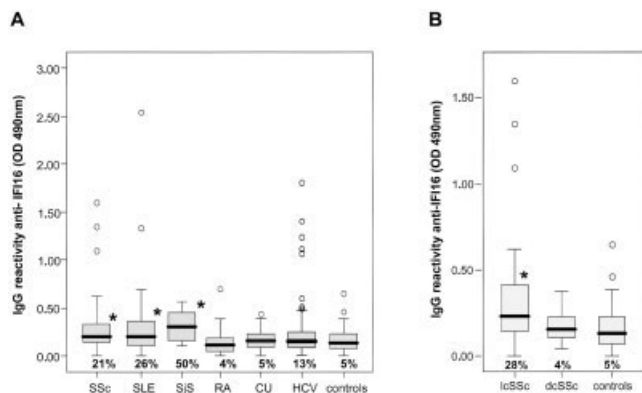
**Elevated levels of anti-IFI16 autoantibody by ELISA, and IFI16 immunoreactivity in SSc and SLE skin.** In normal epidermis obtained from healthy control subjects, the expression of IFI16 was restricted to the basal layer (Figure 1A). Notably, the expression of IFI16 was greatly increased and was ubiquitous in all layers of the epidermis in lesional skin from both patients with SLE (Figure 1B) and those with SSc (either lcSSc or dcSSc) (Figure 1C). Furthermore, the dermal inflammatory infiltrate showed IFI16-positive staining, indicating that IFI16 is expressed at a high level in lymphocytes, fibroblasts, and endothelial cells.

The presence of anti-IFI16 autoantibodies in both patient and control sera was assessed by ELISA. All absorbance levels were in the range of assay linear-

ity. Values higher than the ninety-fifth percentile for control subjects were considered positive. Patients with SSc, patients with SLE, and patients with SS exhibited significantly higher anti-IFI16 IgG antibody levels compared with control subjects (for SSc,  $P < 0.0005$ ; for SLE,  $P < 0.002$ ; for primary SS,  $P < 0.001$ ) (Figure 2). In contrast, the prevalence of anti-IFI16 IgG was not statistically significantly different in patients with RA, patients with chronic urticaria, and those with HCV infection, in comparison with normal control subjects. Interestingly, although the mean anti-IFI16 autoantibody titer in the whole group of patients with HCV infection was not statistically significantly different from that in control subjects, the subgroup of patients who received treatment with type I IFN ( $n = 20$ ) displayed considerably higher levels (mean optical density [OD] 0.346) compared with the untreated subgroup (mean OD 0.206). This finding indirectly supports a role for type I IFN in the up-regulation of IFI16 expression and the eventual generation of specific autoantibodies.

**Association of anti-IFI16 antibodies with clinical parameters.** Univariate analysis showed that anti-IFI16 autoreactivity in patients with SSc was not associated with the duration or severity of disease, organ involvement, and positivity to other autoantibodies (data not shown). In contrast, a strict association between anti-IFI16 reactivity and the limited cutaneous form of SSc





**Figure 2.** IgG titers against human recombinant IFI16 in **A**, patients with systemic sclerosis (SSc) (n = 82), systemic lupus erythematosus (SLE) (n = 100), primary Sjögren's syndrome (SjS) (n = 20), rheumatoid arthritis (RA) (n = 50), chronic urticaria (CU) (n = 38), and hepatitis C virus (HCV) infection (n = 80), and healthy control subjects (n = 80), and **B**, patients with limited cutaneous SSc (lcSSc) (n = 57) and diffuse cutaneous SSc (dcSSc) (n = 25), and healthy control subjects (n = 80). Data are presented as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent the median. Circles indicate outliers. Differences between the groups were evaluated by one-way analysis of variance with Bonferroni adjustment for multiple comparisons, after logarithmic transformation. Values beneath the boxes represent the percentage of subjects with IgG titers above the cutoff value (0.360), calculated as the 95th percentile for the control population. OD = optical density. \* =  $P < 0.002$  versus controls in **A**; \* =  $P < 0.02$  versus patients with dcSSc and controls in **B**.

was observed, with patients with lcSSc displaying higher anti-IFI16 IgG titers compared with patients with dcSSc ( $P = 0.017$ ). Indeed, anti-IFI16 titers above the ninety-fifth percentile for control subjects were observed in 28% of patients with lcSSc but in only 4% of those with dcSSc (95% confidence interval 5–37%) (Figure 2B).

Consistent with previous reports, the presence or titers of anti-IFI16 antibodies did not correlate with clinical manifestations or disease activity in either patients with SLE or those with primary SS (data not shown).

**Logistic regression model and sensitivity-specificity analysis.** Consistent with data in the literature, the prevalence of anticentromere antibodies (ACAs) and the prevalence of anti-Scl-70 antibodies in patients with lcSSc were 65% and 9%, respectively, compared with 8% and 75%, respectively, in patients with dcSSc. Logistic regression analysis showed that anti-Scl-70 antibodies, ACAs, and anti-IFI16 antibodies were independent predictors of the cutaneous form of SSc, and their combination was able to explain 62% of

the associated variability. This model was able to correctly predict 89% of the clinical variants. Moreover, anti-IFI16 reactivity displayed lower sensitivity (28%) and higher specificity (96%) than were observed for ACAs (65% and 92%, respectively). The combined use of anti-IFI16 and ACA markers gave rise to the highest sensitivity and specificity scores (79% and 92%, respectively). Interestingly, in the subgroup of patients with SSc who were negative for both ACAs and anti-Scl-70 antibodies (n = 24), all those with anti-IFI16 positivity (n = 6) displayed the limited cutaneous form (100% specificity and 100% positive predictive value). None of these patients presented with an overlap syndrome or were positive for anti-RNP autoantibodies, and keratoconjunctivitis sicca was observed in 3 of these 6 patients. No association was observed between anti-IFI16 antibodies and keratoconjunctivitis sicca in patients with SSc or in those with SLE, RA, or HCV infection.

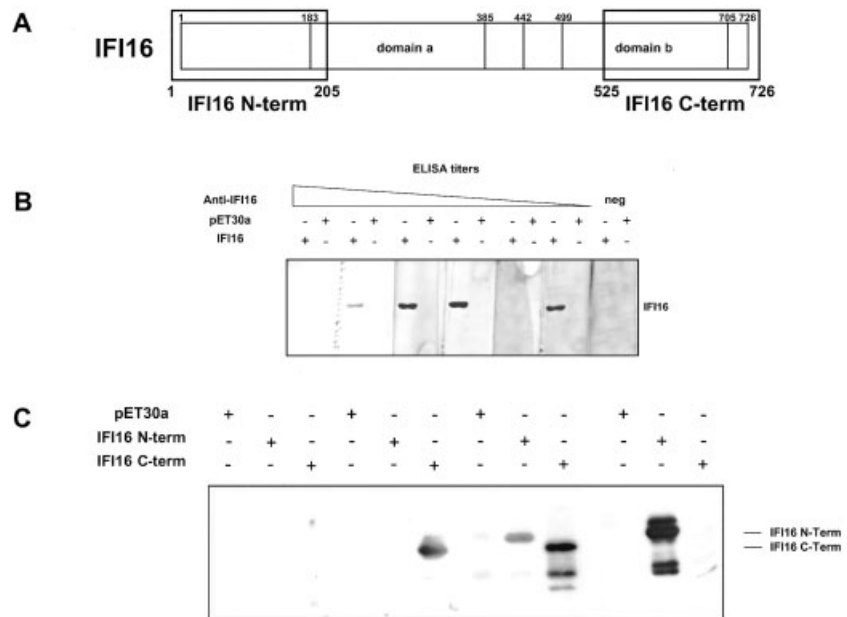
#### Immunoblot analysis for anti-IFI16 antibodies.

Immunoblot analysis using recombinant IFI16 protein, either full-length or deleted fragments (Figure 3A), was also performed. Western blotting was performed on 25 SSc sera, 17 of which had anti-IFI16 titers above the ninety-fifth percentile for control subjects and 8 of which had titers below the ninety-fifth percentile for control subjects. Low-titer sera did not exhibit reactivity with IFI16, thus confirming specificity of the ELISA technique (Figure 3B). In contrast, only 10 of 17 sera with high titers of IFI16 were positive, very likely because of the ability of immunoblotting to detect antibodies against linear epitopes, while ELISA recognizes autoantibodies against both linear and conformational epitopes. A correlation between anti-IFI16 autoantibody titers and the intensity of immunoreactive bands was not observed.

To further characterize the antigenic specificity of the IFI16-positive sera, 10 sera recognizing linear epitopes were analyzed for their reactivity against the N-terminal (IFI16 N-term) and C-terminal (IFI16 C-term) fragments of IFI16, respectively. Four sera displayed reactivity against the N-terminal fragment, 3 sera displayed reactivity against the C-terminal fragment, 2 sera recognized both fragments, and 1 serum sample displayed no reaction (Figure 3C). Taken together, these data suggest a polyclonal nature of the immune response against IFI16 in patients with SSc.

## DISCUSSION

To our knowledge, this is the first demonstration (by immunohistochemical analysis) of enhanced expression of IFN-inducible protein IFI16 in the epidermis and



**Figure 3.** A, Schematic representation of IFI16 and a series of recombinant IFI16 fragments. Domain a and domain b of HIN-200 are shown. Boxed areas indicate recombinant peptides corresponding to IFI16 amino acids 1–205 (IFI16 N-term) and amino acids 525–726 (IFI16 C-term), respectively. B, Immunoblot analysis showing reactivity with full-length IFI16. Recombinant IFI16 or control peptide (pET30a) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and then incubated with a 1:100 dilution of patient serum that was positive (lanes 1–12, with decreasing titers) or negative (lanes 13 and 14) using enzyme-linked immunosorbent assay (ELISA). C, Reactivity with IFI16 N-terminal (IFI16 N-term) and C-terminal (IFI16 C-term) fragments or control peptide (pET30a). Nitrocellulose membranes with transferred recombinant proteins were incubated with patient sera that were reactive (by immunoblotting) with full-length IFI16. After stripping, the membranes in B and C were reprobbed with anti-IFI16 C-terminal or anti-IFI16 N-terminal antibodies to confirm the presence of the recombinant proteins.

dermal inflammatory infiltrate from both SLE and SSc lesions. Additionally, we confirmed that anti-IFI16 autoantibody titers are significantly elevated in patients with SLE and those with primary SS. Interestingly, we observed that the prevalence of these autoantibodies was comparable in SSc as well. The results obtained demonstrated that anti-IFI16 autoreactivity was not associated with either disease duration or disease severity but was associated with the limited cutaneous form of SSc.

The most striking data came from logistic regression analysis of the 3 serologic markers of autoimmunity (anti-IFI16 antibodies, ACAs, and anti-Scl-70 autoantibodies). We actually observed that all 3 serologic markers were independent predictors of the cutaneous subsets of SSc, and their combination was able to explain 62% of the associated variability. Although we failed to

detect an association with specific clinical features, their presence seems to be an important clue to the development of lcSSc in ACA- and anti-Scl-70-negative patients. In addition, the finding that anti-IFI16 positivity distinguishes patients with lcSSc from among the subgroup of patients negative for both ACA and anti-Scl-70 reactivity strongly indicates that anti-IFI16 can be a useful diagnostic tool.

Prominent expression of IFI16 has been observed in endothelial cells and in stratified squamous epithelia such as skin. Its expression normally is restricted to the basal proliferative layer, suggesting a possible role in the control of skin homeostasis. Transduction of IFI16 into human umbilical vein endothelial cells by recombinant viruses efficiently suppressed the formation of capillary-like structures in vitro and cell cycle progression associated with cell death (14). In the current study, we

showed that IFI16 is expressed to a higher level in SSc (regardless of the clinical subset) and SLE lesions in both epithelial and inflammatory cells, and autoantibody titers against it are significantly elevated in both diseases. However, because the number of skin biopsy specimens available was low and corresponding sera were not available for all of them, we cannot make any statistical correlation between anti-IFI16 antibodies and the abnormal expression of IFI16 in skin.

Thus, the disease model we propose is as follows:

1) IFI16 expression in lesional skin may be enhanced by local production of type I IFN or by other proinflammatory stimuli; 2) IFI16 release, as a consequence of increased cell death, leads to the breakdown in tolerance to this self antigen, as confirmed by the generation of specific anti-IFI16 autoantibodies; 3) an additional pathogenic role of IFI16 is suggested by the observation that its endothelial expression triggers apoptosis, up-regulates the expression of genes encoding adhesion molecules (intercellular adhesion molecule 1, E-selectin) and chemokines (interleukin-8, monocyte chemoattractant protein 1) (Landolfo S: unpublished observations), and efficiently suppresses formation of capillary-like structures in vitro.

Because the etiopathogenesis of SLE, primary SS, and SSc is multifactorial, we can only speculate that such events can be shared in common and that additional and completely different downstream mechanisms may be responsible for the (clinical/histologic) differences between these diseases. Alternatively, regardless of the cause of overexpression of IFI16, increased exposure of the protein to immune effectors prone to autoimmunity—such as in SLE, primary SS, and SSc—may explain the production of specific autoantibodies.

#### ACKNOWLEDGMENT

We thank Dr. Mario Pirisi (University of Piemonte Orientale) for kindly providing part of the HCV samples and for performing a critical reading of the manuscript.

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