Increased expression of interferon regulated and antiviral response genes in CD31+/CD102+ lung microvascular endothelial cells from systemic sclerosis patients with end-stage interstitial lung disease

S. Piera-Velazquez[1], F.A. Mendoza[2], S. Addya[3], D. Pomante[3], S.A. Jimenez[1]

[1]Jefferson Institute of Molecular Medicine and Scleroderma Center, [2]Division of Rheumatology, Department of Medicine, Thomas Jefferson University, Philadelphia, PA; [3]Kimmel Cancer Center, Sidney Kimmel Medical College of Thomas Jefferson University, Philadelphia, PA, USA.

Abstract

Objective

Systemic sclerosis (SSc) is characterised by severe fibroproliferative vasculopathy, fibrosis in skin and multiple internal organs, and humoral, cellular and innate immunity abnormalities. Vascular alterations are the earliest and most severe SSc manifestations, however, the mechanisms responsible have remained elusive. To investigate the molecular abnormalities involved in SSc-vasculopathy we examined global gene expression differences between highly purified lung microvascular endothelial cells (MVECs) from patients with SSc-interstitial lung disease (SSc-ILD) and normal lung MVECs.

Methods

MVECs were isolated from fresh transplanted lungs from patients with SSc-ILD. Sequential CD31 and CD102 immunopurification was performed to obtain highly purified CD31+/CD102+ lung MVECs. Global gene expression analysis was successfully performed in CD31+/CD102+ MVEC from two SSc-ILD patients and from two normal lungs. RT-PCR, Western blots, and indirect immunofluorescence validated the gene expression results.

Results

Numerous interferon-regulated genes (IRGs) including IFI44, IFI44L, IFI6, IFIH1, IFIT1, ISG-15, BST-2/Tetherin, and RSAD2/Viperin, genes encoding innate immunity antiviral responses (OAS1, OAS2, OAS3, OASL) and antiviral MX1 and MX2 proteins, and mesenchymal cell-specific genes were significantly overexpressed in CD31+/CD102+ SSc-ILD lung MVECs.

Conclusion

Highly purified CD31+/CD102+ MVECs from lungs from SSc patients with end stage SSc-ILD displayed remarkable overexpression of numerous IRGs and of genes encoding antiviral innate immune response and antiviral proteins. These observations suggest that interferon-induced and antiviral response proteins may participate in the pathogenesis of SSc vasculopathy and SSc-ILD. The CD31+/CD102+ lung MVECs from SSc-ILD also showed elevated expression of mesenchymal cell-specific genes confirming the presence of endothelial to mesenchymal transition in SSc-ILD.

Key words

systemic sclerosis, interstitial lung disease, fibroproliferative vasculopathy, lung microvascular endothelial cells, interferon-stimulated genes, innate immunity anti-viral response genes, myxovirus response genes, endothelial-mesenchymal transition

*Sonsoles Piera-Velazquez, PhD**
*Fabian A. Mendoza, MD**
Sankar Addya, MD
Danielle Pomante, BS
Sergio A. Jimenez, MD

**These authors contributed equally.*

Please address correspondence to:
Sergio A. Jimenez,
Jefferson Institute of Molecular Medicine,Thomas Jefferson University, 233 S. 10th Street, Room 509 BLSB, Philadelphia, PA 19107-5541, USA.
E-mail: sergio.jimenez@jefferson.edu

Received on July 29, 2020; accepted in revised form on October 21, 2020.

Funding: supported by NIH/NIAMS RO-1 grant AR019616 to S.A. Jimenez.

Competing interests: none declared.

Introduction

Systemic sclerosis (SSc) is characterised by cutaneous and internal organ fibrosis, severe fibroproliferative vasculopathy and multiple immunologic abnormalities (1, 2). The generalised microvascular involvement includes endothelial cell (EC) dysfunction and fibro-proliferative alterations causing progressive vessel lumen narrowing and eventual occlusion (3, 4). Owing to the high frequency, progressiveness, and severity of SSc vascular involvement it has been suggested that SSc should be considered a vascular disease (5-7). Extensive clinical and laboratory investigations have been performed to identify the mechanisms involved in these alterations (8-10). However, despite intensive investigations, these mechanisms have remained incompletely understood (11). Numerous studies have described EC morphological and structural abnormalities including increased vascular permeability allowing trans-endothelial migration of circulating inflammatory cells and deleterious macromolecules, impaired angiogenesis and increased production of pathogenetic molecules such as endothelin-1 (7-10). Furthermore, ECs in SSc may directly contribute to tissue fibrosis through their phenotypic conversion into activated mesenchymal cells (myofibroblasts), a process known as endothelial to mesenchymal transition or EndMT (12-16). Indeed, there is strong evidence that microvascular ECs (MVECs) from affected SSc dermis and from lung tissues from patients with SSc-associated interstitial lung disease (SSc-ILD) undergo EndMT as demonstrated by the co-expression of endothelial and mesenchymal macromolecules in MVECs (7, 14-16).

Given the prominent role of microvascular alterations in SSc, identification of molecular differences between normal and SSc MVECs should provide novel information about the pathogenesis of SSc-vasculopathy. However, previous studies with purified EC from SSc patients are scarce owing to the difficulties and limitations in obtaining sufficient number of highly purified EC from affected SSc tissues. Here, we performed extensive immunopurification of MVECs isolated from fresh lung tissues from patients with end-stage SSc-ILD undergoing lung transplantation. We obtained CD31+ cells which were further purified employing CD102 antibodies to yield CD31+/CD102+ lung MVEC. We then performed global gene expression studies with these cells to identify molecular differences in comparison with CD31+/CD102+ MVECs isolated from normal lungs. These studies revealed remarkable gene expression differences between MVEC isolated from fresh SSc-ILD human lungs compared with MVEC isolated from normal lungs. The most notable differences included a marked increase in the expression of numerous interferon-regulated genes (IRG) and genes encoding proteins involved in innate and acquired immune antiviral responses. These results suggest the participation of novel mechanisms in the pathogenesis of SSc vasculopathy.

Materials and methods

Lung tissue samples

Fresh lung tissues were obtained following the Institutional Review Board-approved protocols from three patients with end-stage SSc-ILD who underwent lung transplantation at the University of Pittsburgh Medical Centre, USA. The patients were two females aged 68 and 52 years old and one 64 years old male. The three patients had severe and progressive diffuse SSc with terminal SSc-ILD. None of the patients had pulmonary arterial hypertension (PAH) as assessed by right heart chamber catheterisation. These tissues were kindly provided to us by Dr Carol Feghali-Bostwick. Normal lung tissues were obtained from the National Disease Research Interchange from autopsies of two females aged 55 and 38 years of age who did not have pulmonary or autoimmune disease. All studies were conducted according to Institutional Review Board-approved protocols from Thomas Jefferson University.

Isolation and purification of CD31+/CD102+ lung MVECs from SSc-ILD patients

The fresh lung tissues were carefully dissected to remove pleural lining and

large vessels and then they were immediately minced and enzymatically digested for cell isolation with clostridial collagenase as described previously (16). We performed the cell isolation in fresh unfrozen tissues to avoid any potential modification in their cellular phenotype. The resulting cell suspensions were purified employing a negative immunomagnetic isolation with CD45 microbeads (Miltenyi Biotec) to remove contaminating erythrocytes and inflammatory cells. The MVEC were isolated and purified by immunomagnetic selection with a rabbit anti-human CD31 antibody followed by magnetic bead separation using goat anti-rabbit IgG-conjugated microbeads (1:5, Miltenyi Biotec). The isolated CD31+ MVEC were cultured as described previously (16) in 2% gelatin pre-coated tissue culture dishes for 5–10 days. Following expansion, the cells were further purified employing a second immunologic separation using a rabbit anti-human CD102 antibody to obtain highly purified CD31+/CD102+ MVEC. The purified CD31+/CD102+ MVEC were extensively characterised as previously described (16) and were further expanded for 3-6 passages. Owing to the complexity of the sequential immunoprecipitation procedure one of the SSc-ILD MVEC cell lines was lost following CD102 antibody immunoprecipitation. Therefore, all subsequent studies were conducted with two different SSc-IDL CD31+/CD102+ MVEC. All studies were performed with passage 4-6 cells to assure preservation of their original phenotype.

Phase contrast microscopy and confocal microscopy

Confocal microscopy indirect immunofluorescence was performed to assess the EC morphology and to demonstrate the simultaneous expression of the CD31 EC-specific cell surface marker with the IRG-encoded protein IFI44. For these studies, the cells were seeded onto glass culture slides, fixed with 3.7% formaldehyde and permeabilised with 0.1% Triton X-100 in PBS for 3 min and examined employing co-staining with immuno-specific CD-31 (1:200 dilution) and IFI44 (1:100 dilution) antibodies. The slides were then incubated with secondary antibodies followed by the addition of DAPI for nuclear staining as described previously (16).

RNA Microarray analysis

Global gene expression evaluation was performed employing microarray analysis as previously described (17). We performed two separate microarrays each in duplicate from two different preparations of RNA from SSc-ILD lung CD31+/CD102+ MVECs isolated from lung tissues from two different SSc-ILD patients and from two different preparations of CD31+/CD102+ MVECs from lungs of the two normal individuals. Total RNA was extracted from normal and SSc-ILD lung MVECs employing RNeasy kit (Qiagen). Amplification of cDNA was performed using the Ovation Pico WTA-system V2 RNA amplification system (NuGen Technologies, Inc.) from 50 ng of total RNA. Amplified cDNA was purified with Qiagen MinElute reaction cleanup kit. A sample containing 5 μg cDNA was chemically labelled with biotin to generate biotinylated cDNA using FL-Ovation cDNA biotin module (NuGen Technologies, Inc.). Affymetrix gene chips, (Human Gene 2.0 ST Array), were hybridised with 5 μg fragmented and biotin-labelled cDNA in 200 μl of hybridisation cocktail. Target denaturation was performed at 99°C for 2 min and then at 45°C for 5 min followed by hybridisation for 18 h. Arrays were then washed and stained using Gene chip Fluidic Station 450, using Affymetrix GeneChip hybridisation wash and stain kit. Chips were scanned on an Affymetrix Gene Chip Scanner 3000 7G using Command Console Software.

Data analyses were performed using GeneSpring software 14.5 (Agilent Technologies, Inc.). The probe set signals were calculated with the Iterative Plier 16 summarisation algorithm, baseline to median of all samples was used as baseline option. Data were filtered by percentile and lower cut off was set at 25. The criteria for differentially expressed genes were set at ≥2-fold changes. Statistical analysis was performed using unpaired T test analysis with a *p*-value for statistical significance equal to or less than 0.05. Heat maps and a Volcano plot were generated from the differentially expressed gene list. The list of all differentially expressed genes was also examined by Ingenuity Pathway Analysis (IPA) (Qiagen Bioinformatics 8.0 software) and by Gene Ontology (GO) to perform biological network pathway analyses.

Reverse transcription-polymerase chain reaction

CD31+/CD102+ lung MVEC cultures obtained from each of the two patients with SSc-ILD and the two control individuals were used for RNA extraction (RNeasy kit; Qiagen), including a genomic DNA digestion step. Total RNA (1 μg) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) to generate first-strand complementary DNA. MVECs transcript levels were quantified using SYBR Green real-time PCR (Bimake). Triplicate PCR reactions were performed employing the primers shown in Supplementary Table 1. The specificity of the primers was established at the end of the PCR amplification employing melting curve analysis. The method of relative quantitation {ΔΔCt} was employed to determine the level of gene expression. ΔCt values were computed from the Ct (cycle threshold number) values of the target gene and the housekeeping control gene (18S) as described previously (18).

Western blot analysis

Cell extracts were prepared from the two normal control and the two SSc-ILD CD31+/CD102+ lung MVECs cell lines employing RIPA lysis buffer containing a mixture of protease inhibitors and were sonicated and then processed for Western blotting under denaturing conditions as described previously (18). The membranes were incubated overnight at 4°C with either a polyclonal anti-IFI44 antibody (Biorbyt), a polyclonal anti-IFIT1 antibody (Biorbyt), or a polyclonal anti-STAT1 antibody (Biorbyt), and a β-Actin antibody (Abcam) as a house keeping control protein. Thereafter, the membranes were incubated with the appropriate conjugated secondary antibodies cou-

pled to peroxidase and the ECL system (Thermo Scientific Pierce) was employed for detection of the identified proteins. Quantification of the protein bands was performed employing Image J software and the signals were normalised to those of β-actin as described previously (18).

Results

Clinical and histopathological features

The SSc-ILD patients whose lung transplanted tissues were studied were a 68 years old female and a 64 years old male. Both patients had severe end-stage SSc-ILD requiring lung transplantation and did not have hemodynamic evidence of PAH. Histopathologically, their lung tissues showed advanced interstitial fibrosis and scattered parenchymal infiltration with mononuclear inflammatory cells. Arterioles and small to medium-sized arteries demonstrated severe sub-intimal accumulation of fibrotic tissue resulting in marked vessel lumen narrowing. These alterations have been described in greater detail previously (16).

Gene expression microarrays

Differential gene expression levels were observed in 134 transcripts (66 upregulated and 68 downregulated) at a significance of $p<0.05$ in the average of CD31+/CD102+ SSc-ILD lung MVECs compared to the average of the two normal lung CD31+/CD102+ MVEC. The top upregulated genes (greater than three-fold increased expression) in the CD31+/CD102+ lung SSc-ILD MVEC are listed in Table I and the complete lists of the up-regulated and down-regulated genes are shown in Supplementary Tables S2 and S3, respectively. The most relevant differentially expressed genes are shown as a heatmap in Figure 1A, and as a volcano plot in Figure 1B. The results showed that the expression levels of numerous IRGs were significantly and substantially (greater than three-fold) upregulated in SSc-ILD lung CD31+/CD102+ MVECs compared to normal lung CD31+/CD102+ MVECs (Table I). A remarkable observation was the greater than five-fold upregulation of BST-2, and the greater than four-fold upregulation of ISG15 and RSAD2 genes. The ISG15 gene encodes an interferon-induced ubiquitin-like molecule with potent antiviral effects (19). The BST-2 gene encodes BST-2/Tetherin, a type II transmembrane protein that was demonstrated to be a potent interferon-inducible antiviral factor (20-23). BST-2/Tetherin has a unique structure and cellular distribution topology that endow it with remarkable protein tethering and signalling activities (22). BST-2/Tetherin is a key effector of the antiretroviral activity of type I interferon *in vitro* and *in vivo* (21), inhibiting the release of nascent viral particles and preventing viral dissemination. Furthermore, through its signalling properties it can initiate and regulate host responses to viral infections, as well as play an important role in other immunologic and inflammatory reactions (22, 23). The RSAD2 gene encodes Viperin, a protein first identified as being a highly expressed product of CMV-infected fibroblasts with potent antiviral effects (24, 25). Numerous subsequent studies have demonstrated that Viperin is a potent interferon induced antiviral protein (26). The gene expression for STAT1, a key IFN pathway regulator was also upregulated but to a lesser extent (slightly less than two-fold).

The results also demonstrated that numerous transcripts corresponding to genes involved in the innate immunity antiviral response and in acquired antiviral responses were significantly overexpressed in the SSc-ILD lung CD31+/CD102+ MVECs. These genes encode members of the oligo-adenylate synthetase (OAS) family of proteins (OAS1, OAS2, OAS3, OASL) and the myxovirus resistance proteins 1 and 2 (MX1, MX2). One important observation was that the expression of several genes associated with mesenchymal cell extracellular matrix (ECM) mac-

Table I. List of the highest upregulated genes (greater than three-fold) in CD31+/CD102+ lung MVEC from patients with SSc-ILD.

Gene symbol	Gene description	Fold increase	*p*-value
CADM3	Cell adhesion molecule 3	11.18	<0.01
CADM3-AS1	CADM3 antisense RNA1	8.68	<0.01
MX1	MX dynamin-like GTP ase 1	6.28	0.03
IFI6	Interferon, alpha-inducible protein 6	6.23	0.02
ACKR1	Atypical chemokine receptor 1 (Duffy blood group)	6.05	<0.01
MX2	MX dynamin-like GTP ase 2	5.76	0.01
OAS2	2-5-oligo adenylate synthetase 2	5.48	0.03
OAS1	2-5-oligo adenylate synthetase 1	5.40	0.03
BST2	Bone marrow stromal cell antigen 2	5.15	0.04
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	4.52	0.05
IFITM1/IFITM2	Interferon-induced transmembrane protein 1/2	4.50	0.03
IFI44L	Interferon-induced protein 44-like	4.36	0.04
CEMIP	Cell migration inducing protein, hyaluronian binding	4.36	0.03
IGSF10	Immunoglobulin super family, member 10	4.29	0.03
RSAD2	Radical S-adenosylmethionine domain containing 2	4.24	0.02
ISG15	Interferon stimulated gene 15	4.18	0.01
INMT	Indolethylamine-N-methyltransferase	3.82	0.03
CTHRC1	Collagen triple helix repeat containing 1	3.68	0.01
CCNA1	Cyclin A1	3.66	<0.01
HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family	3.60	0.02
SNORA23	Small nucleolar RNA, H/ACA box 23	3.52	0.01
OASL	2-5-oligo adenylate synthetase-like	3.49	0.01
HTR2B	5-hydroxytryptamine (serotonin) receptor 2B, G protein-coupled	3..47	<0.01
C12orf60	Chromosome 12 open reading frame 60	3.45	<0.01
RARRES2	Retinoic acid receptor responder (tazarotene-induced) 2	3.45	0.01
CHI3LI	Chitinase 3-like 1 (cartilage glycoprotein-39)	3.43	0.03
LUM	Lumican	3.36	0.01
MGP	Matrix GIa protein	3.27	<0.01
COL3A1	Collagen, type III, alpha 1	3.27	0.04
IFI44	Interferon-induced protein 44	3.24	0.05
CENPW	Centromere protein W	3.15	<0.01
COL1A1	Collagen, type I, alpha 1	3.08	0.02

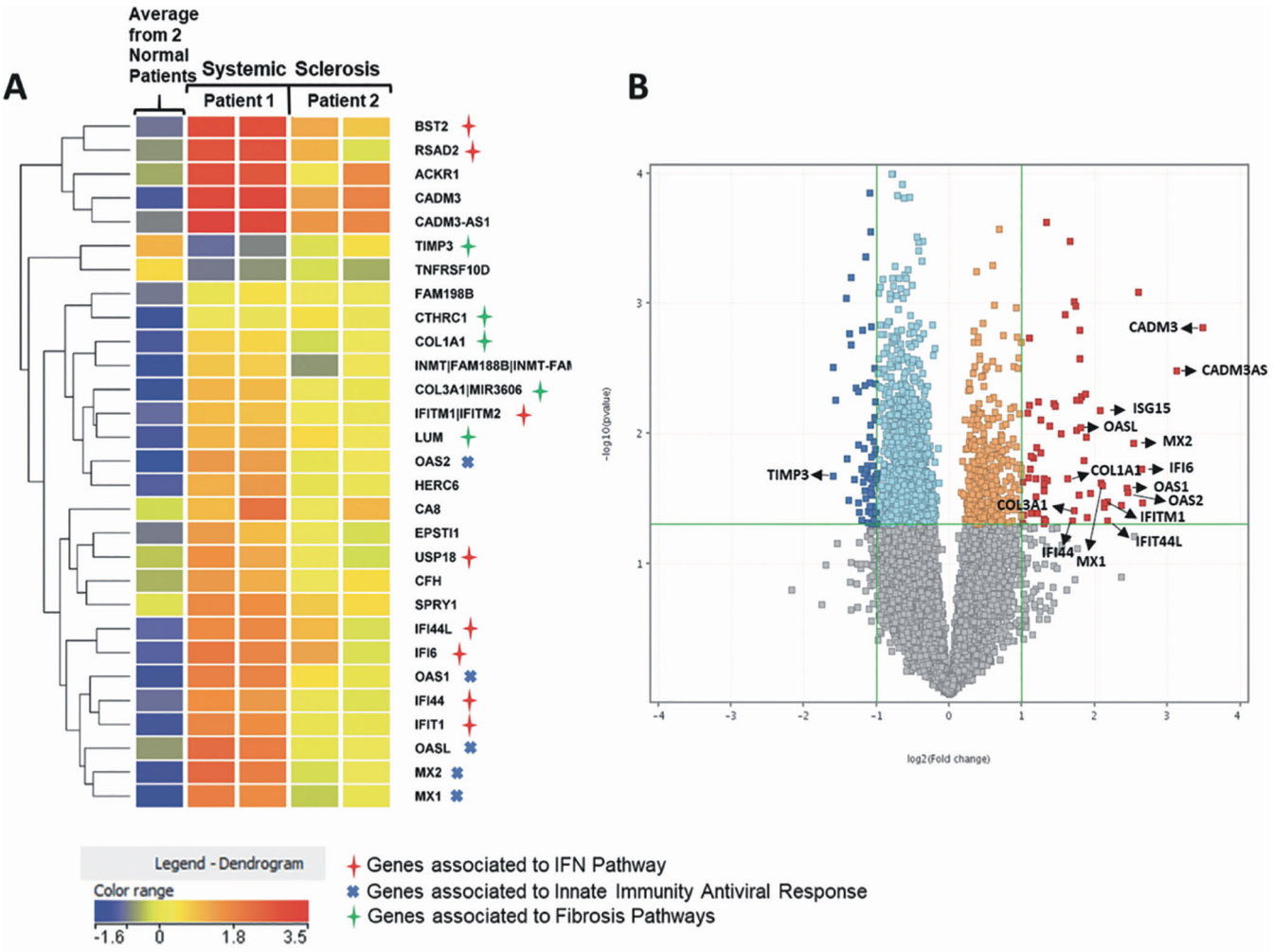

Fig. 1. Global gene expression analysis of CD31+/CD102+ MVEC isolated from lungs from patients with SSc-ILD and from normal control lungs. **A:** Heat map and dendrogram (hierarchically clustered) of genes with the highest differential expression reveals groups of genes with high expression levels. Interferon related genes are marked with a red cross. Innate antiviral immunity and antiviral genes are marked with a blue X, and fibrosis-associated pathway genes are marked with a green cross. **B:** Volcano Plot showing differentially expressed transcripts with 2-fold or greater difference (p<0.05) in expression between SSc-ILD and normal control lung CD31+/CD102+ MVECs.

romolecules including COL3A1 and COL1A1 were significantly increased in the SSc-ILD lung CD31+/CD102+ MVECs. Ingenuity pathway analysis showed that type I IFN played a central role in the biological network of the overexpressed IRGs (Fig. 2A). The GO pathway analysis showed that the most significantly elevated pathways were those related to interferon activation and tissue fibrosis (Fig. 2B).

Validation of increased expression of relevant genes by Real Time PCR

Quantitative PCR was employed to validate the increased expression of 6 selected IRG (IFIT1, IFI44L, IFI44, IFI6, IFIH1, and IFIT3) in CD31+/CD102+ SSc-ILD lung MVECs. The results demonstrated that the transcript levels corresponding to IFIT1, IFI44, IFI44L, and STAT1 displayed the most differentially increased expression in the SSc-ILD lung MVECs samples (Fig. 3A). Quantitative PCR also validated increased expression levels of OAS1, OAS2, OAS3, OASL and MX1 and MX2 transcripts in CD31+/CD102+ lung SSc-ILD MVEC (Fig. 3B).

Increased IFI44, IFIT1, and STAT1 protein levels

Protein levels of IFI44, IFIT1, and STAT1 present in CD31+/CD102+ lung MVECs from SSc-ILD patients were analysed by Western blotting and the results demonstrated a substantial increase compared to cell extracts from control lung CD31+/CD102+ MVECs as shown in Figure 4A. Confocal laser microscopy was employed to confirm the increased expression of the interferon-induced protein IFI44 in the purified CD31+/CD102+ lung SSc-ILD MVECs. These studies demonstrated high levels of IFI44 protein and the co-localisation of the EC-specific marker CD31 with IFI44 in cultured lung CD31+/CD102+ MVECs from SSc-ILD patients (Fig. 4B). In contrast, CD31+/CD102+ MVECs isolated from the normal lung tissues examined in parallel and utilising the same antibodies did not show significant levels of IFI44 protein and there was no co-expression of CD31 with IFI44.

Discussion

In this study, we compared the global gene expression profile of immunopu-

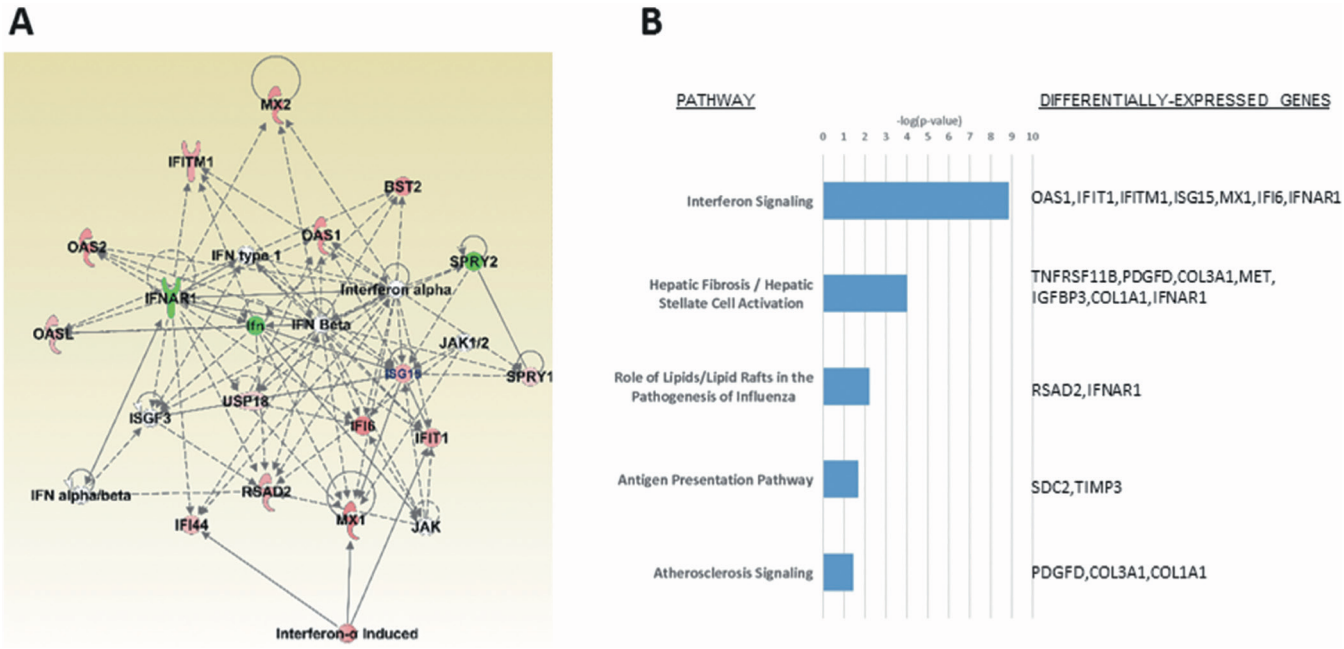

Fig. 2. Pathway analysis of differentially expressed Interferon Regulated Genes (IRGs).
A: Ingenuity Pathway Analysis (IPA) of differentially expressed IRGs. The proteins encoded by genes showing a 2-fold change or greater were selected and processed by IPA software to display potential interactions. The most upregulated proteins are shown in red. Solid lines indicate direct interactions experimentally proven in the literature whereas dashed lines indicate potential indirect interactions.
B: Canonical Gene Ontology Pathways enriched in the MVECs from SSc-ILD compared with normal control lung MVECs.

rified CD31+/CD102+ MVECs isolated from lung tissues from two patients with end-stage SSc-ILD undergoing lung transplantation to that of CD31+/CD102+ MVECs isolated from two normal lungs. A remarkable observation was that numerous IRG including IFI44, IFI44L, IFI6, IFIH1, IFIT3, IFIT1, ISG15, BST-2 and RSAD2, several genes involved in the innate immunity antiviral response including OAS1, OAS2, OAS3, and OASL, and the genes encoding MX1 and MX2 (myxovirus antiviral proteins) displayed highly significant differential upregulation in CD31+/CD102+ lung MVECs from SSc-ILD patients compared to normal CD31+/CD102+ lung MVECs.

The demonstration of a very strong IFN signature in the highly purified CD31+/CD102+ lung MVECs from SSc-ILD patients indicates an important role of IFN pathways in the induction and maintenance of the EC abnormalities and endothelial dysfunction characteristic of SSc. These observations are in agreement with results from several prior studies implicating dysregulation of type I IFN and IFN-inducible genes in SSc pathophysiology (27, 28). One of these studies examined peripheral blood cells from SSc patients and demonstrated that these cells displayed differential expression of 18 interferon-inducible genes compared with cells from control individuals (29). Other studies obtained similar results in whole peripheral blood samples (30, 31) and in peripheral blood mononuclear cells (32). A related investigation determined the expression of a panel of 11 type I IFN inducible genes in whole-blood samples from SSc patients at various stages of disease evolution that included SSc patients with very early disease (33). The results showed a strong expression of an IFN type I signature in all SSc clinical groups with significantly higher IFN scores in patients at the earliest stages of the disease even prior to the development of clinically detectable cutaneous fibrosis (33). Of relevance to the studies reported here, Christmann et al. demonstrated increased expression of many interferon-responsive genes in whole lung tissues from SSc patients with SSc-ILD (34). However, the presence in the affected tissues of various IFN-producing cells including resident dendritic cells and macrophages, may have been responsible for their observations. Collectively, numerous studies have provided substantial evidence for the participation of IFN-mediated mechanisms and IFN-activated pathways in SSc (Reviewed in 27,28,30). The results we describe here strongly indicate that type I IFN pathway activation occurs in highly purified MVECs from affected SSc-ILD lung tissues and support the notion that type I IFN may play a crucial role in the pathogenesis of SSc-associated vasculopathy and SSc-associated ILD.

Another important finding was the demonstration that several genes encoding innate immunity antiviral response proteins (OAS1, OAS2, OASL) and other antiviral proteins (MX1, MX2) were significantly overexpressed in the CD31+/CD102+ SSc-ILD MVECs. The OAS proteins are members of a large family of intracellular enzymes capable of recognising cytoplasmic double stranded RNA (dsRNA) indicative of a viral infection and inducing in response the synthesis and production of 2'–5'-linked oligoadenylate which then mediates the degradation of the viral RNAs (35). The MX proteins are members of the dynamin-like large GTPases also endowed of potent antiviral activity (36). Although increased ex-

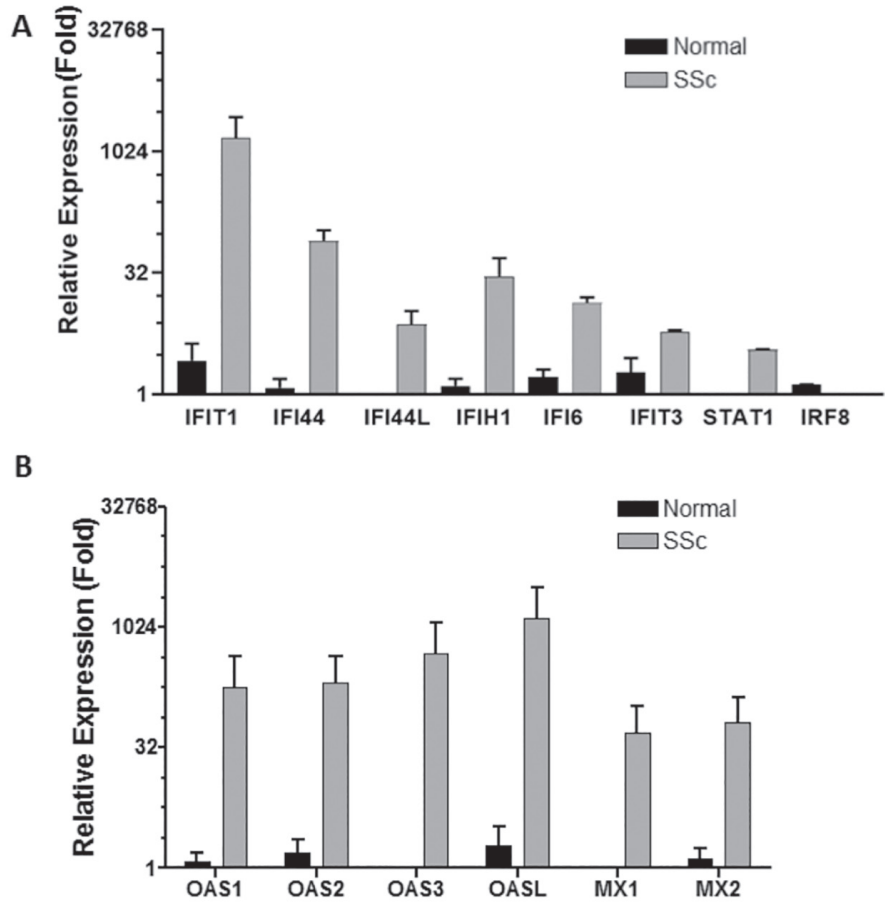

Fig. 3. Real time PCR validation of specific IRGs.
A. Semiquantitative RT-PCR showing the average of the relative fold gene expression of selected IRGs (IFI44, IFI44L, IFIH1, IFI6, IFIT1, IFIT3, and STAT1) in CD31+/CD102+ SSc-ILD lung MVECs compared with the average levels of expression of the same genes from normal control CD31+/CD102+ lung MVECs. The values for IRF8, an IRG that did not show a significant difference between the SSc-ILD and normal lung MVECs samples, were used to calculate the relative level of expression for the other IRGs. **B.** Semiquantitative RT-PCR showing the average of the relative fold gene expression for OAS1, OAS2, OAS3, OASL, MX1 and MX2 in SSc-ILD lung MVECs compared with their expression levels in normal lung MVECs. All values are shown in a logarithmic scale.

patients (34, 37, 38), the results described here are the first to demonstrate a marked elevation of expression of these genes in purified CD31+/CD102+ MVECs from SSc-ILD lung tissues. Along similar lines are our results demonstrating a marked upregulation of the gene ISG15 that codes for a small ubiquitin-like molecule that is powerfully stimulated by IFN-1 and that among its multiple intracellular functions has been implicated as a crucial member of that host antiviral response (19). Collectively, these results provide strong support to the hypothesis that viral-mediated injury, through either direct viral effects to the endothelium, or through indirect alterations caused by activated cytotoxic T cells or by antibody-dependent responses, may be a key and early event in the pathogenesis of SSc (39-41) and of SSc-associated vascular alterations (42, 43).

Our studies further showed that expression of CADM3, a gene encoding Nectin-like molecule 1, a protein associated with cell adhesion, migration and proliferation (44) was the most elevated (greater than 11-fold) in SSc-ILD lung MVECs. Remarkably, the CADM3 antisense RNA (CADM3-AS1) was the second most increased gene (greater than 8-fold). We also found substantial elevation of the genes encoding the interstitial collagens COL1A1 and COL3A1, indicating that SSc lung MVECs

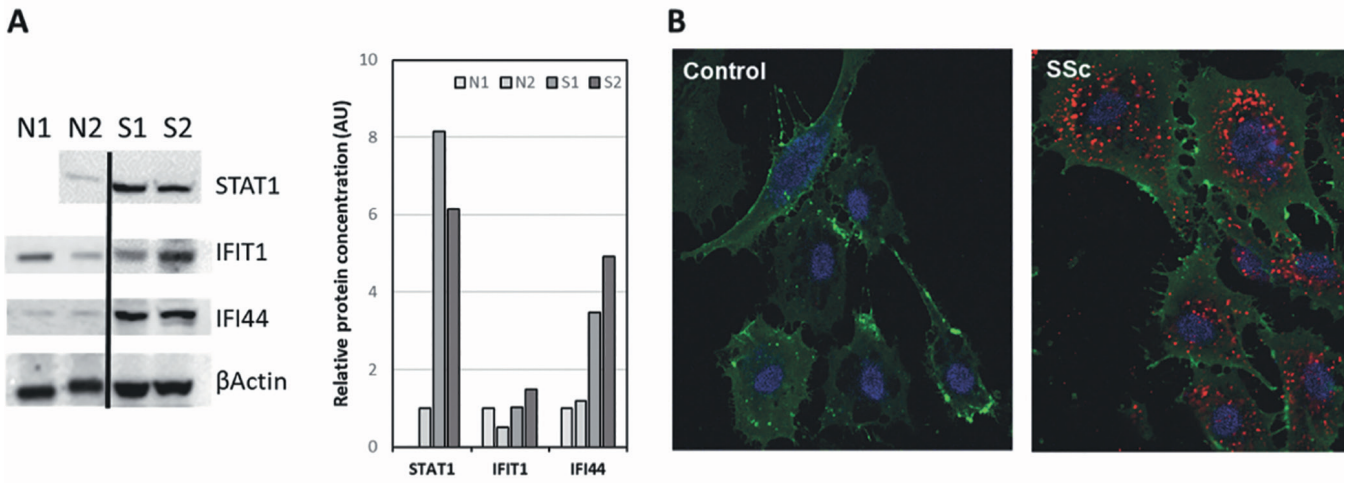

Fig. 4. Western blot analysis of IFI44, IFIT1 and STAT1 in cell extracts from normal control and SSc-ILD CD31+/CD102+ lung MVECs and confocal microscopy for co-expression of CD31 and IFI44.
A: Western blots showing protein levels of selected IRG protein products (IFI44, IFIT1, and STAT1) in two different CD31+/CD102+ SSc lung MVECs cell lines and one (for STAT1) or two (for IFI44, IFIT) normal control lung MVECs cell lines. **B:** Confocal microscopy showing the expression of CD31 (green fluorescence) and IFI44 (red fluorescence) in CD31+/CD102+ immunopurified lung MVECs isolated from one control subject and one SSc-ILD patient.

have undergone a phenotypic transition to mesenchymal cells, thus confirming the occurrence of the EndMT process previously suggested to play an important role in SSc tissue fibrosis (12-16).

Numerous studies have examined the global gene expression of various cells and tissues in SSc (45-48) including an extensive and detailed analysis of purified dermal EC from patients with diffuse SSc (49), however, our study is the first to describe the global gene expression of highly purified MVECs isolated from SSc-ILD fibrotic lungs. Of relevance to our results, a very recent study analysed single cell gene expression profiles of SSc affected skin (50). These studies compared the single cell gene expression of an EC cluster that contained 9 cells from one sample of healthy control skin compared with an EC cluster of 8 cells from skin from one SSc patient. The results identified upregulated genes in SSc skin EC and included genes that were established markers of endothelial injury and activation, as well as previously identified markers of vascular dysfunction in SSc (50). However, this study did not demonstrate IFN pathway activation or elevated expression of genes encoding innate immunity antiviral response in the endothelial cells examined.

In conclusion, the results described here demonstrate that highly purified CD31+/CD102+ lung MVECs from patients with advanced end-stage SSc-ILD display a pattern of global gene expression consistent with the activation of IFN pathways as well as elevated expression of antiviral genes and of genes encoding innate immunity antiviral responses. These observations support the hypothesis that an endothelial cell injury caused by a viral pathogen may be involved in the initiation and development of SSc-associated EC dysfunction and SSc vasculopathy. Among the viral agents that have been suggested, cytomegalovirus (CMV) has been considered one of the most likely agents owing to the demonstration of increased levels of anti-CMV antibodies in the serum of SSc patients, the demonstration of molecular mimicry between certain autoantibodies and CMV proteins, as well as remarkable similarities between CMV vascular clinical manifestations and SSc vasculopathy (42, 43). The results described here do not provide information as to whether the pattern of gene expression of lung MVEC from SSc-ILD patients is triggered by direct viral infection, by direct contact with IFN producing cells, or by paracrine effects of cells present in the surrounding perivascular tissues. However, our observations suggest that IFN pathway activation and an innate immunity response to a viral infection may play a role in the pathogenesis of SSc vasculopathy in SSc-ILD.

We certainly acknowledge that a substantial limitation of this investigation is the small number of samples studied. However, it should be emphasised that the extreme difficulties in obtaining fresh lung tissues from SSc-ILD patients in sufficient amounts for the successful isolation of a large number of highly purified CD31+/CD102+ MVECs pose a highly serious limitation and is a strong barrier for the performance of extensive studies as the one described here. On the other hand, subsequent confirmation of our results may be highly relevant to the elucidation of the mechanisms of SSc vasculopathy and SSc-ILD and may lead to the development of novel therapeutic approaches for this serious SSc manifestation that currently lacks approved disease-modifying therapy.

Acknowledgements

We wish to acknowledge Dr Carol Feghali-Bostwick for providing the lung tissues utilised for this study. The tissues were obtained with informed consent from SSc patients undergoing lung transplantation at the University of Pittsburg Medical Center under a protocol approved by the University of Pittsburg Institutional Review Board.

The expert assistance of Alana Pagano in the preparation of this manuscript is greatly acknowledged.

References

1. VARGA J, ABRAHAM D: Systemic sclerosis: a prototypic multisystem fibrotic disorder. *J Clin Invest* 2007; 117: 557-67.
2. DENTON CP, KHANNA D: Systemic sclerosis. *Lancet* 2017; 390: 1685-99.
3. NORTON WL, NARDO JM: Vascular disease in progressive systemic sclerosis (scleroderma). *Ann Intern Med* 1970; 73: 317-24.
4. WIGLEY FM: Vascular disease in scleroderma. *Clin Rev Allergy Immunol* 2009; 36: 150-75.
5. CAMPBELL PM, LEROY EC: Pathogenesis of systemic sclerosis: a vascular hypothesis. *Semin Arthritis Rheum* 1975; 4: 351-68.
6. MATUCCI-CERINIC M, KAHALEH B, WIGLEY FM: Review: evidence that systemic sclerosis is a vascular disease. *Arthritis Rheum* 2013; 65: 1953-62.
7. MOSTMANS Y, CUTOLO M, GIDDELO C *et al*.: The role of endothelial cells in the vasculopathy of systemic sclerosis: A systematic review. *Autoimmun Rev* 2017; 16: 774-86.
8. KAHALEH B: Vascular disease in scleroderma: mechanisms of vascular injury. *Rheum Dis Clin North Am* 2008; 34: 57-71.
9. MULLER-LADNER U, DISTLER O, IBBA-MANNESCHI L, NEUMANN E, GAY S: Mechanisms of vascular damage in systemic sclerosis. *Autoimmunity* 2009; 42: 587-95.
10. TROJANOWSKA M: Cellular and molecular aspects of vascular dysfunction in systemic sclerosis. *Nat Rev Rheumatol* 2010; 6: 453-60.
11. ORLANDI M, LEPRI G, DAMIANI A *et al*.: One year in review 2020: systemic sclerosis. *Clin Exp Rheumatol* 2020; 38 (Suppl. 125): S3-17.
12. PIERA-VELAZQUEZ S, JIMENEZ SA: Endothelial to mesenchymal transition (EndMT): role in physiology and in the pathogenesis of human diseases. *Physiol Rev* 2019; 99: 1281-324.
13. JIMENEZ SA: Role of endothelial to mesenchymal transition in the pathogenesis of the vascular alterations in systemic sclerosis. *ISRN Rheumatol* 2013; 2013: 835948.
14. MANETTI M, ROMANO E, ROSA I *et al*.: Endothelial-to-mesenchymal transition contributes to endothelial dysfunction and dermal fibrosis in systemic sclerosis. *Ann Rheum Dis* 2017; 76: 924-34.
15. GOOD RB, GILBANE AJ, TRINDER SL *et al*.: Endothelial to mesenchymal transition contributes to endothelial dysfunction in pulmonary arterial hypertension. *Am J Pathol* 2015; 185: 1850-8.
16. MENDOZA FA, PIERA-VELAZQUEZ S, FARBER JL, FEGHALI-BOSTWICK C, JIMÉNEZ SA: Endothelial cells expressing endothelial and mesenchymal cell gene products in lung tissue from patients with systemic sclerosis-associated interstitial lung disease. *Arthritis Rheumatol* 2016; 68: 210-7.
17. WERMUTH PJ, ADDYA S, JIMENEZ SA: Effect of protein kinase C delta (PKC-δ) inhibition on the transcriptome of normal and systemic sclerosis human dermal fibroblasts in vitro. *PLoS One* 2011; 6: e27110.
18. PIERA-VELAZQUEZ S, MAKUL A, JIMÉNEZ SA: Increased expression of NAPDH oxidase 4 in systemic sclerosis dermal fibroblasts: regulation by transforming growth factor β. *Arthritis Rheumatol* 2015; 67: 2749-58.
19. PERNG YC, LENSCHOW DJ: ISG15 in antiviral immunity and beyond. *Nat Rev Microbiol* 2018; 16: 423-39.
20. NEIL SJ, ZANG T, BIENIASZ PD: Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 2008; 451: 425-30.

21. LIBERATORE RA, BIENIASZ PD: Tetherin is a key effector of the antiretroviral activity of type I interferon *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 2011; 108: 18097-101.
22. SWIECKI M, OMATTAGE NS, BRETT TJ: BST-2/tetherin: structural biology, viral antagonism, and immunobiology of a potent host antiviral factor. *Mol Immunol* 2013; 54: 132-9.
23. MAHAUAD-FERNANDEZ WD, OKEOMA CM: The role of BST-2/Tetherin in host protection and disease manifestation. *Immun Inflamm Dis* 2015; 4: 4-23.
24. ZHU H, CONG JP, SHENK T: Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: induction of interferon-responsive RNAs. *Proc Natl Acad Sci USA* 1997; 94: 13985-90.
25. CHIN KC, CRESSWELL P: Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. *Proc Natl Acad Sci USA* 2001; 98: 15125-30.
26. MATTIJSSEN S, PRUIJN GJ: Viperin, a key player in the antiviral response. *Microbes Infect* 2012; 14: 419-26.
27. WU M, ASSASSI S: The role of type 1 interferon in systemic sclerosis. *Front Immunol* 2013; 4: 266.
28. SKAUG B, ASSASSI S: Type I interferon dysregulation in systemic sclerosis. *Cytokine* 2020; 132: 154635.
29. TAN FK, ZHOU X, MAYES MD *et al*.: Signatures of differentially regulated interferon gene expression and vasculotrophism in the peripheral blood cells of systemic sclerosis patients. *Rheumatology* (Oxford) 2006; 45: 694-702.
30. ASSASSI S, MAYES MD, ARNETT FC *et al*.: Systemic sclerosis and lupus: points in an interferon-mediated continuum. *Arthritis Rheum* 2010; 62: 589-98.
31. ASSASSI S, WANG X, CHEN G *et al*.: Myeloablation followed by autologous stem cell transplantation normalizes systemic sclerosis molecular signatures. *Ann Rheum Dis* 2019; 78: 1371-8.
32. YORK MR, NAGAI T, MANGINI AJ, LEMAIRE R, VAN SEVENTER JM, LAFYATIS R: A macrophage marker, Siglec-1, is increased on circulating monocytes in patients with systemic sclerosis and induced by type interferons and toll-like receptor agonists. *Arthritis Rheum* 2007; 56: 1010-20.
33. BRKIC Z, VAN BON L, COSSU M *et al*.: The interferon type I signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high BAFF gene expression and high collagen synthesis. *Ann Rheum Dis* 2016; 75: 1567-73.
34. CHRISTMANN RB, SAMPAIO-BARROS P, STIFANO G *et al*.: Association of Interferon- and transforming growth factor beta-regulated genes and macrophage activation with systemic sclerosis-related progressive lung fibrosis. *Arthritis Rheumatol* 2014; 66: 714-25.
35. CHOI UY, KANG JS, HWANG YS, KIM YJ: Oligoadenylate synthase-like (OASL) proteins: dual functions and associations with diseases. *Exp Mol Med* 2015; 47: e144.
36. VERHELST J, HULPIAU P, SAELENS X: Mx proteins: antiviral gatekeepers that restrain the uninvited. *Microbiol Mol Biol Rev* 2013; 77: 551-66.
37. DE FREITAS ALMEIDA GM, OLIVEIRA DB, BOTELHO LM *et al*.: Differential upregulation of human 2'5'OAS genes on systemic sclerosis: Detection of increased basal levels of OASL and OAS2 genes through a qPCR based assay. *Autoimmunity* 2014; 47: 119-26.
38. COELHO LF, DE OLIVEIRA JG, DE OLIVEIRA DB *et al*.: Increased expression of 2'5'oligoadenylate synthetase and double-stranded RNA dependent protein kinase messenger RNAs on affected skin of systemic sclerosis patients. *Arch Dermatol Res* 2007; 299: 259-62.
39. HAMAMDZIC D, KASMAN LM, LEROY EC: The role of infectious agents in the pathogenesis of systemic sclerosis. *Curr Opin Rheumatol* 2002; 14: 694-8.
40. MORONCINI G, MORI S, TONNINI C, GABRIELLI A: Role of viral infections in the etiopathogenesis of systemic sclerosis. *Clin Exp Rheumatol* 2013; 31 (Suppl. 76): S3-7.
41. FARINA A, FARINA GA: Fresh insights into disease etiology and the role of microbial pathogens. *Curr Rheumatol Rep* 2016; 18: 1.
42. DOLCINO M, PUCCETTI A, BARBIERI A *et al*.: Infections and autoimmunity: role of human cytomegalovirus in autoimmune endothelial cell damage. *Lupus* 2015; 24: 419-32.
43. ARCANGELETTI MC, MACCARI C, VESCOVINI R *et al*.: A paradigmatic interplay between human cytomegalovirus and host immune system: possible involvement of viral antigen-driven $CD8^+$ T cell responses in systemic sclerosis. *Viruses* 2018; 10: 508.
44. TAKAI Y, MIYOSHI J, IKEDA W, OGITA H: Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nat Rev Mol Cell Biol* 2008; 9: 603-15.
45. WHITFIELD ML, FINLAY DR, MURAY JI *et al*.: Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci USA* 2003; 100: 12319-24.
46. SARGENT JL, MILANO A, CONNOLLY MK, WHITFIELD ML: Scleroderma gene expression and pathway signatures. *Curr Rheumatol Rep* 2008; 10: 205-11.
47. ASSASSI S, SWINDELL WR, WU M *et al*.: Dissecting the heterogeneity of skin gene expression patterns in systemic sclerosis. *Arthritis Rheumatol* 2015; 67: 3016-26.
48. STIFANO G, SORNASSE T, RICE LM *et al*.: Skin gene expression is prognostic for the trajectory of skin disease in patients with diffuse cutaneous systemic sclerosis. *Arthritis Rheumatol* 2018; 70: 912-9.
49. GIUSTI B, FIBBI G, MARGHERI F *et al*.: A model of anti-angiogenesis: differential transcriptosome profiling of microvascular endothelial cells from diffuse systemic sclerosis patients. *Arthritis Res Ther* 2006; 8: R115.
50. APOSTOLIDIS SA, STIFANO G, TABIB T *et al*.: Single cell RNA sequencing identifies HSPG2 and APLNR as markers of endothelial cell injury in systemic sclerosis skin. *Front Immunol* 2018; 9: 2191.