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THE PATHOGENIC ROLE OF IMMUNE COMPLEXES CONTAINING SCLERODERMA-SPECIFIC AUTOANTIBODIES

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Summary

AIM OF THE STUDY

Systemic sclerosis (SSc) is a chronic autoimmune condition characterized by excessive tissue fibrosis, microvascular alterations and immune dysfunction with the production of characteristic autoantibodies. These autoantibodies are highly specific for SSc diagnosis, and provide the most reliable tool to predict disease subset and the pattern of internal organ involvement. Despite such diagnostic and prognostic role, no evidence supporting the pathogenic potential of these autoantibodies has to date been raised. The working hypothesis of this study envisaged that immune complexes (ICs) containing scleroderma specific autoantibodies -rather than the mere antibody- might be able to elicit a proinflammatory and pro-fibrotic signaling cascade in target cells, thus contributing to SSc multifaceted etiopathogenesis. Since scleroderma autoantibodies bind –either directly or via bridge proteins- to nucleic acids, it was also postulated that the effects induced by SSc-ICs might be mediated by Toll-like Receptors (TLR).

MATERIALS AND METHODS

Fibroblasts have been isolated from skin biopsies from healthy controls and then cultured in adequate conditions. ICs have been purified from sera of scleroderma patients bearing different autoantibody specificities (antibodies against centromeric proteins [ACA], DNA topoisomerase I [ATA], RNA polymerase [ARA] and Th/To [anti-Th/To]) or of healthy controls using polyethylen glycol precipitation. Fibroblasts were transiently silenced for *tlr3* using a specific small interfering RNA (siRNA); silencing was confirmed by RT-PCR and Western Blotting. Naïve and tlr3-silenced cells have been incubated with pathologic and control ICs and with TLR3 [Poly(I:C)] and TLR4 (LPS) agonists. Several parameters of cell activation have been assessed in the different experimental conditions. In particular, mRNA levels of type I interferons (IFN-α and IFN-β) and TLR (TLR3 and TLR9) have been investigated by real-Time PCR; ICAM-1 expression has been evaluated by cell-ELISA and the secretion of IL-6 and IL-8 in culture supernatants has been

measured by commercial ELISA kits. Furthermore, the involvement of intracellular signaling pathways culminating with the activation of p38 MAPK and NFκB has been assessed.

RESULTS

Stimulation of normal skin fibroblasts with pathologic ICs induced a significant increase in the gene expression levels of both IFN-α and IFN-β; similar results have been reported in the presence of TLR agonists but not of control ICs and medium alone. In addition, ICAM-1 expression and IL-6 and IL-8 secretion were up-regulated by Poly(I:C), LPS and ICs from scleroderma patients but not healthy controls and medium alone. Further, pathologic ICs induced the activation of both p38 MAPK and NFκB. The expression levels of TLR3 and -to a greater extent- TLR9 were significantly increased in cells treated with TLR3 agonist and ICs from SSc patients but not healthy controls. The efficiency of *tlr3* silencing in skin fibroblasts was confirmed at both mRNA and protein levels. *tlr3* silencing significantly affected ICAM-1 expression and IL-6, but not IL-8, secretion in cells treated with both TLR agonists and ICs from SSc patients but not healthy controls and medium alone.

CONCLUSIONS

These data provide the first demonstration of the pathogenic role of ICs isolated from scleroderma patients with different autoantibody specificities in the inductor phase of SSc. Indeed, pathologic ICs can interact with normal skin fibroblasts, inducing a pro-inflammatory phenotype mediated by p38 MAPK and NFκB. In particular, ICs isolated from SSc patients have been shown to recruit TLR3 leading to fibroblast activation, with upregulation of adhesion molecules and secretion of pro-inflammatory interleukins. Other TLRs, such as TLR7, TLR8 and TLR9, might be implicated in the cell response to scleroderma ICs. These evidences fit well with the diagnostic and prognostic role of scleroderma-specific autoantibodies.

Introduction

Systemic sclerosis: clinical features

Systemic sclerosis (SSc) is a chronic autoimmune disease of unknown etiology. This condition was first described in 1753 by Carlo Curzio, a Neapolitan physician. It is also known as scleroderma, from the ancient greek words "σχλερος" (tight) and "δερμα" (skin); this term was coined in 1847 by Gintrac[1]. SSc is indeed characterized by an excessive deposition of collagen and other components of the extra-cellular matrix (ECM) in the skin and other target organs, which in turns leads to tissue fibrosis. Scleroderma is a systemic disease: besides the skin, it most commonly affects the gastrointestinal tract, the lungs and the kidneys.

- **Epidemiology**

SSc is a rare clinical condition, with an estimated incidence of 19 cases per 1.000.000 inhabitants per year; disease prevalence ranges between 4 and 286 cases per million. The highest prevalence of SSc has been reported in the Choctaw native American tribe in Oklahoma: 469 cases per 100.000 subjects[2].

Scleroderma mainly affects women, with a male/female ratio of 1:3-5; female preponderance is most marked during childbearing years, narrowing towards the fifth decade to 1:2-3. The disease onset occurs most commonly between 30 and 50 years of age, with an average of 42 years among Caucasian women[3].

The risk of developing SSc has been related to the occupational exposure to silica dust and organic solvents; in particular, a high final cumulative exposure to crystalline silica, trichlorethylene, chlorinated solvents, welding fumes, and any types of solvents was found to be associated with SSc. Occupational exposure-related SSc is more common among men, being characterized by a severe skin thickening and a mild organ involvement[4].

- **Survival**

SSc diagnosis carries an increased mortality, with a 4.6 fold risk of death compared to the general population. The average life expectancy in SSc patients is 16-34 years less than matched controls. In one study, the survival rates at 5 and 10 years were 86% and 69% respectively[5]. In addition, prognosis is worse in men compared to women, in older subjects compared to youngsters. Several predictors of survival have been identified: extensive skin involvement, cardiopulmonary disease, renal involvement and positivity for anti-DNA topoisomerase I antibodies (ATA). In particular, pulmonary artery hypertension (PAH) and interstitial lung disease (ILD) are the two leading causes of SSc-related mortality, with a pooled standardized mortality ratio of 3.5 and 2.6 respectively[6].

- **Classification criteria for systemic sclerosis**

A joint committee of the American College of Rheumatology (ACR)/ European League against Rheumatism (EULAR) has recently proposed new classification criteria for SSc[7].

The skin thickening of the fingers proximal to the metacarpophalangeal joints is regarded as a sufficient criterion for classifying the patient as having SSc. If this criterion is not present, seven additional items are then considered:

- Skin thickening of the fingers
    - Sub-items: Puffy fingers/Sclerodactily of the fingers
- Fingertip lesions
    - Sub-items: Digital tip ulcers/Fingertip pitting scars
- Teleangiectasias
- Abnormal nailfold capillaries
- ILD or PAH
- Raynaud's phenomenon
- SSc-related autoantibodies

A score ranging from 2 to 4 has been appointed to each additional item; patients with a total score above 9 are classified as having definite SSc. In the validation sample, this set of criteria displayed a sensitivity of 0.91 with a specificity of 0.92[7].

- **Disease subsets**

In 1988, Carwile LeRoy proposed to identify two disease subsets according to the extent of the skin thickening[8]. Diffuse cutaneous SSc (dcSSc, 35% of SSc patients) is characterized by a cutaneous involvement affecting the face, the trunk, the thighs and the arms. In the limited form of the disease (lcSSc, 60% of SSc patients), the skin of the trunk and the proximal portions of the limbs are spared. dcSSc and lcSSc differ also with regards to the pattern of organ involvement and autoantibody profile. Patients with dcSSc are at higher risk of developing clinically significant major internal organ dysfunction. In particular, ILD and scleroderma renal crisis (SRC) are more common in the diffuse subset, while patients with limited disease frequently develop PAH. dcSSc is associated with positivity for ATA, patients with the limited form are more likely to carry anti-centromere antibodies (ACA)[9]. Mortality is higher in dcSSc compared to lcSSc, with a median disease duration from onset of Raynaud's phenomenon to death of 7.1 years and 15.0 years respectively[5].

In a third variant of the disease (5% of patients with SSc), namely SSc sine scleroderma, patients develop vascular and fibrotic damage to internal organs associated with SSc-autoantibodies, in the absence of any skin thickening[10].

SSc can also be associated with manifestations of other connective tissue diseases (CTDs): these clinical entities are referred to as overlap syndromes (SSc/rheumatoid arthritis overlap syndrome, SSc/myositis overlap syndrome…)[11].

- **Clinical manifestations**

  - **Raynaud's phenomenon**

Raynaud's phenomenon is secondary to transient episodes of vasospasm of peripheral small vessels as arteries, arterioles, pre- and post-capillary venules. It can be clinically characterized by triphasic (pallor in the ischemic phase, cyanosis in the deoxygenation phase, and erythema in the reperfusion phase) or bifasic (pallor and cyanosis or cyanosis and erythema) colour changes. Typically, it involves fingers and toes and, much less commonly, the nose, the earlobes and the tongue. Episodes can be triggered by the exposure to cold conditions, emotional stress, pharmacological agents, and vibratory injuries. Raynaud's phenomenon represents the first manifestation of the disease in 95% of scleroderma patients. Together with PAH and SRC, it is a clinical expression of scleroderma vasculopathy: vessels display fibrotic intimal hyperplasia, adventitial fibrosis and reduced arterial lumen. In patients with limited scleroderma, Raynaud's phenomenon might precede by several years the onset of cutaneous and internal organ involvements. Conversely, dcSSc patients present an abrupt onset of Raynaud's phenomenon, shortly followed by other manifestations of the disease[12].

In scleroderma patients, attacks are usually asymmetric, intense, frequent and painful. Episodes might result in impaired oxygenation of the distal extremities leading to digital ulcers of both fingers and toes in 30% of patients. Ulcers can also manifest over the finger creases, the extensor surface of joints or over the site of calcium subcutaneous deposits. Acral skin ulcerations might resolve with hyperkeratosic pitting scars. Severe cases might also evolve to necrosis and dry gangrene; in these situations, acral bone resorption and self-amputation of the phalanges can occur. Chronic ulcers might become infected, then resulting in gangrene and osteomyelitis[13].

- **Skin involvement**

Cutaneous involvement is the clinical hallmark of SSc. It usually begins with an early phase of skin oedema, manifesting as swollen fingers and hands. Fibrosis appears later: skin becomes hardened, with the loss of its natural pliability; appendages degenerate leading to hair loss and anhidrosis. In the later stages, atrophy supervenes: the skin gets thin and dry.

Skin thickening usually starts from the fingers and evolves with a distal to proximal pattern. In the active phase of the disease, the skin darkens, is shiny and itchy. A "salt and pepper" appearance may result from patchy hypo and hyperpigmented area. The extent of skin thickening may vary widely: some patients present sclerodactyly only, whereas in most severe forms skin thickening might affect the whole integument, with joint mobility limitation and development of flexion contractures. Peri-oral fibrosis leads to microcheily, reduced mouth aperture and microglossy, the nose is pinched, the appearance is expressionless: the typical "scleroderma facies". Telangiectasias, which are dilated superficial blood vessels, can be observed on the fingers, palms, lips, face, tongue and in the mouth; telangiectasias are more commonly reported in the limited form of the disease[14].

An additional skin finding in SSc is calcinosis cutis, due to the deposition of calcium hydroxyhepatite crystals in the soft tissues. Usually it occurs at pressure points, typically the extensor surface of hand joints, but also of forearms, buttocks and other body areas. These lesions might extrude from the skin and ulcerate[14].

The modified Rodnan skin score (mRSS) is a semi-quantitative method to assess skin thickening. It considers 17 anatomic areas, and rates each area by clinical palpation using a 0–3 scale (0=normal skin; 1=mild thickness; 2=moderate thickness; 3=severe thickness with inability to pinch the skin into a fold)[15]. A mRSS over 20 has been shown to predict heart involvement, SRC and an increased mortality risk.

- **Gastrointestinal involvement**

Gastrointestinal symptoms usually occur early on disease course, often being the presenting event after Raynaud's phenomenon. The gastrointestinal tract is the most affected organ after the skin: 95% of SSc patients present gastrointestinal involvement. Approximately half of these patients complain of clinical symptoms, 10% display severe manifestations. Any tract, from the mouth to the anus, might be affected; consequently, gastrointestinal manifestations may vary greatly, ranging from bloating and heartburn to dysphagia and anorectal dysfunction to severe weight loss and malabsorption. Gastrointestinal involvement exerts a strong impact on patients' quality of life, even though it is rarely the cause of death[16].

Esophageal dysmotility can be detected in 90% of patients, with an alteration of peristaltic activity of the smooth muscle of the distal two thirds of the oesophagus and an impaired barrier function of the lower esophageal sphinctera. Clinically, it manifests with gastroesophageal reflux and dysphagia. Chronic reflux may be complicated by esophageal strictures and Barrett's metaplasia of the distal esophagus. Approximately half of patients complain of gastroparesis symptoms: nausea, vomiting, weight loss, early satiety, bloating or epigastric pain. In turn, delayed gastric emptying can worsen reflux symptoms. Gastric antral vascular ectasia (GAVE) represents an additional vascular complication of scleroderma. GAVE displays a unique endoscopic appearance, known as "watermelon stomach": longitudinal stripes of red vessels radiate from the pylorus to the antrum. Estimated prevalence of GAVE in SSc ranges from 1.8% to 22.3%; it is often a cause of significant bleeding. Positivity for anti-RNA polymerase III antibodies (ARA) has been reported as an independent risk factor for GAVE development[17].

Bloating and postprandial fullness may also indicate small bowel involvement, which has been described in 20-60% of scleroderma patients. Most commonly, patients notice new onset nutritional intolerances, typically lactose intolerance. Impaired motility leads to stasis of luminal contents, which in turn induces secondary bacterial overgrowth; the frequent use of acid suppressive

medication for reflux disease may contribute to the development of bacterial overgrowth. Bacterial overgrowth can cause malabsorption, which is considered a poor prognostic factor: mortality rate at 8 years is 50%. Small bowel involvement might be further complicated by pseudo-obstruction[18].

Colorectal involvement can be detected in 10-50% of patients; the anorectal tract is mostly affected. Clinical manifestations include constipation, diarrhea and/or fecal incontinence[16].

Approximately 8% of patients with limited SSc carry anti-mitochondria antibodies (AMA); on the other hand, ACA can be detected in 9-29% of patients with primary biliary cirrhosis. All scleroderma patients with positive AMA display a certain degree of cholestasis with damage of small intra-hepatic bile ducts[19].

- **Lung involvement**

Lung involvement is the second most common visceral complication among SSc patients, its prevalence ranges between 25 and 90%, depending on the detection method. Due to advances in the management of renal complications, lung disease including ILD and PAH has emerged as the leading cause of mortality in SSc: up to 30% of deaths are directly attributable to lung fibrosis. The most rapid decline in lung function occurs in the first three years from disease onset: 42% of patients with SSc-ILD die of disease progression within 10 years from diagnosis[20,21].

Some studies suggest that ILD is more common among patients with diffuse subset than limited cutaneous SSc: in a 2003 study, lung function tests demonstrated a restrictive pattern in 23% of patients with limited disease and in 40% of those with the diffuse subset[22]. The autoantibody profile is strongly predictive of the risk of developing ILD: ATA, anti-Th/To, anti-U_{11}/U_{12} and anti-Pm/Scl antibodies are associated with an increased hazard whereas ACA positivity confers relative protection from SSc-associated pulmonary fibrosis[23]. Noteworthy, neither the extent of cutaneous involvement nor the antibody profile can differentiate between moderate and severe restrictive

disease[24]. ILD manifests clinically with dyspnea, often associated with dry cough. Pulmonary function tests show a restrictive pattern, with reduced respiratory volumes and impaired diffusion capacity. The most frequently noted histopathological picture is non-specific interstitial pneumonitis (NSIP, 76% of patients, with isolated ground-glass pattern of opacification on high-resolution computed tomography [HRCT]); an histologic finding suggestive of usual interstitial pneumonia (UIP) is described in 13% of cases, generally associated with a reticular HRCT pattern[25]. Not all patients with deterioration in pulmonary function tests have a progressive disease: 30% of patients develop progressive lung disease and only 16% will develop severe lung fibrosis[26].

Approximately 60% of SSc subjects display bronchiectasis, which leads to an increased infective risk. Pleural effusion is an uncommon finding in scleroderma patients, being reported in 10% of patients[20].

- **Renal involvement**

At autoptic studies, 60-80% of SSc patients were found to have a renal involvement. Indeed, moderate clinical manifestations of kidney impairment are frequent among SSc patients: arterial hypertension, mild proteinuria, raised creatininemia[27].

Differently, SRC is a life-threatening complication of SSc, characterized by an abrupt onset of moderate to severe arterial hypertension associated with progressive renal failure[28]. SRC presents in 10-20% of patients with diffuse disease, much less commonly in the limited subset. It occurs early in the disease course, almost invariably within the first five years from diagnosis. Up to 25% of patients are diagnosed with SSc at SRC onset[29]. Medium-high dose corticosteroids have been linked to SRC, with 60% of patients having received steroids prior to presentation. ARA provide a well-described risk factor for SRC development: in one scleroderma cohort, approximately 60% of patients experiencing SRC harboured ARA. Conversely, ATA and ACA are considered to be

protective against the development of SRC. Additional SRC risk-factors include: female gender, a severe diffuse skin involvement, recent onset anaemia, pericardic effusion and joint contractures[30]. SRC is regarded as a vascular complication of SSc: intima walls of interlobular arteries present the typical onion-skin lesions, which lead to the narrowing of the lumen with the consequent reduction of blood flow. The introduction of angiotensin-converting-enzyme inhibitors (ACEIs) in the pharmacological management of SRC has dramatically improved disease history: survival at 1 year has increased from 15 to 76%[30]. However, SRC still carries a poor prognosis, with a mortality rate at 5 years ranging between 30 and 40%[29,31]. Haemodialysis is required in two thirds of patients, approximately half of subjects on renal replacement therapy then discontinue dialysis[32]. In a UK cohort, median time to dialysis discontinuation was 11 months, renal recovery is uncommon after 24 months[29].

- **Heart involvement**

Primary cardiac involvement is common in SSc although often clinically occult: its prevalence among scleroderma patients depends on the sensitivity of the diagnostic tools, ranging from 10 to 75%[33]. It is more prevalent in the diffuse subset than in the limited form (32% versus 23%)[34]; in particular, diffuse patients with a rapid skin thickening rate are at higher risk. To note, ATA positivity has been associated with conduction disturbances while anti-U_3RNP positive patients are at higher risk of myocardial involvement[23,35]. Cardiac involvement usually occurs early on disease course, most commonly within 3 years from the diagnosis[34]. Scleroderma heart disease is thought to be mediated by a chronic vasospasm of small coronary arteries and arterioles, with a secondary ischemia/reperfusion injury, which in turn leads to tissue fibrosis. Fibrotic changes follow a patchy distribution, with a mosaic pattern unrelated to large coronary artery distribution. Scleroderma disease can involve myocardium, coronary arteries, pericardium and the conduction system: the clinical spectrum of scleroderma heart disease is thus heterogeneous[36]. Diastolic dysfunction is the

most frequently described feature (17.4%), systolic dysfunction is a much rarer finding. Atrial and ventricular tachyarrhythmias result from myocardial fibrosis whereas conduction defects and bradyarrhythmias are a consequence of conduction system fibrosis. Supraventricular arrhythmias are more common than those of ventricular origin, and occur in two thirds of cases. Conduction system involvement is rather uncommon, and rarely clinically manifest. Valvular disease has been noted in SSc patients, mainly involving mitral and aortic valves; valvular vegetations provide a rare finding. Inflammation may also play a role in SSc cardiac disease: a myocarditis -an acutely deteriorating cardiac failure with persistent troponin leakage- has been occasionally reported in SSc patients with severe symptoms, and might evolve into restrictive cardiomyopathy and heart failure[33]. Endocarditis may develop in association with severe myocarditis. A pericardial disease is clinically evident in 5-16% of SSc patients, presenting as pericarditis, pericardial adhesions, pericardial effusion and –much rarely- pericardial tamponade and constrictive pericarditis. Reports about the prevalence of coronary artery atherosclerosis in SSc patients are conflicting: some studies observed a prevalence similar to that of the general population, other authors found an higher prevalence[37]. Heart involvement is recognized as a poor prognostic factor: the annual mortality rate attributable to cardiac disease is 1%; at ten year follow-up, disease mortality is 20%[38].

- **Pulmonary artery hypertension**

PAH is a haemodynamic condition characterized by a mean pulmonary artery pressure (mPAP) above 25 mmHg with a pulmonary capillary wedge pressure < 15 mmHg at right heart catheterism. PAH prevalence in SSc ranges between 10 and 12%; 57% of SSc-PAH patients have limited disease. It is a late complication of scleroderma, with a median time from SSc diagnosis to PAH onset of 10 years[39].

PAH ethiopathogenesis is mediated by the vascular remodeling of pulmonary vessels of small/medium size: inflammation induces cell proliferation and an unbalance between vasoconstrictor and vasodilator mediators, ultimately leading to increased pulmonary vasculature resistance. Patients with PAH complain of progressive dyspnea, fatigue and chest palpitation.

The gold standard for PAH diagnosis is provided by right heart catheterism, which allows an accurate measurement of pulmonary artery haemodynamics. Transthoracic echocardiography allows to indirectly estimate the systolic pulmonary artery pressure and is routinely used as a screening tool in the whole SSc population. Pulmonary function tests might suggest an underlying PAH when the diffusing capacity of the lung for carbon monoxide (DL_{CO}) is markedly reduced. Recently, the DETECT study has elaborated an algorithm to identify patients at risk of PAH who should undergo echocardiography. This algorithm includes the following variables: ACA positivity, right axis deviation at electrocardiogram, serum urate, N-terminal of the prohormone brain natriuretic peptide (N-TproBNP) levels, telangiectasias and forced vital capacity (FVC)/DL_{CO} ratio[40]. PAH provides the leading cause of mortality among scleroderma patients: a 50% mortality rate is registered at 3 years from diagnosis, with a median survival of 2.5 years[41].

- **Articular involvement**

Approximately 12-65% of patients complain of arthralgias at disease onset; the rate increases up to 46-97% during disease course. An inflammatory arthritis is rarely described. Articular involvement is more common among patients with diffuse disease compared to those with lcSSc; subjects with positivity for rheumatoid factor are at increased risk of developing articular involvement[42].

Skin thickening and tendon fibrosis may result in joint contractures; flexion contractures of fingers, wrists, elbows and shoulders have all been reported. Fibrosis of tendinous structures might become

apparent as tendon friction rubs, which were described by Rodnan and Medsger as a 'leathery, crepitus feel' perceived on palpation during active or passive articular motion.

Tendon friction rubs have been described to occur in the early stages of the disease, being associated with disease activity and systemic inflammation. They are regarded as a poor prognostic factor, being predictive of future development of renal, cardiac and gastrointestinal involvement[43].

- **Muscle involvement**

Scleroderma myopathy is a rather heterogeneous clinical entity, including both fibrotic and inflammatory cases. Muscle weakness of non-inflammatory nature is a common complaint in SSc, being reported by 60-80% of SSc patients. It typically affects the proximal muscles, being usually associated with a moderately elevated aldolase and, less frequently, creatine kinase. Histologic findings comprise interstitial and perivascular fibrosis, myofibril atrophy and necrosis. Clear cases of inflammatory myopathy, not distinguishable from polymyositis, develop in approximately 6 to 12% of cases. A strong association between scleroderma/myositis overlap syndrome and positivity for anti-Pm/Scl antibodies has been confirmed in many studies[44].

- **Autoantibodies**

Autoantibodies against a wide array of self-antigens have been described in SSc. Approximately 90% of scleroderma patients display anti-nuclear antibodies (ANA) reacting against various cellular components. ACA and ATA are the most frequent scleroderma-specific autoantibodies, providing half of the autoantibody specificities detected among SSc patients. Antibodies producing a nucleolar staining in indirect immunofluorescence on human epithelioma type 2 (HEp-2) cells – ARA, anti-Th/To, anti-U_1RNP and anti-U_3RNP antibodies- are described less commonly in SSc

patients[35]. These autoantibodies are highly specific for scleroderma, being rarely detected in healthy subjects and in patients with other systemic autoimmune conditions. Autoantibody fine specificity is usually exclusive. The diagnostic and prognostic role of scleroderma-specific autoantibodies is supported also by the recent incorporation of the serum positivity for ATA, ARA and ACA in the new criteria for SSc classification[7].

Interestingly, the prevalence of each specific SSc-associated autoantibody depends on the geographic regions, possibly because of the different genetic and environmental factors. Indeed, each autoantibody positivity has been associated with a characteristic HLA asset[45].

The detection of scleroderma-specific autoantibodies allows to risk-stratify scleroderma patients at disease presentation. Interestingly, it is increasingly recognized that antibody levels may vary over time, correlating with disease activity.

- **Anti-centromere antibodies**

ACA react against at least six centromeric polypeptides (CENP-A, CENP-B, CENP-C, CENP-D, CENP-E and CENP-F). The most common antigenic target is provided by CENP-B, an 80 kDa haploid protein that binds to highly repetitive α-satellite DNA through a highly conserved 17 base pair sequence known as CENP-B box[35]. The CENP-B box contains CpG-rich sequences, which, especially if demethylated, can stimulate the production of IFN-α. ACA display a very high specificity for SSc (99.9%), with a sensitivity of 33%. ACA positivity is detected in 20-30% of SSc patients. ACA are strongly associated with limited subset, even though a small proportion (5-7%) of ACA positive subjects develop a diffuse disease[46]. ACA have been linked to a greater lag time to overt clinical disease compared to other autoantibodies. ACA predict subcutaneous calcinosis, PAH and gastrointestinal involvement. Patients with ACA are at increased risk of developing primary

biliary cirrhosis. On the other hand, ACA have been found to be protective against the development of SRC, ILD, synovitis, tendon friction rubs, joint contractures and myopathy[23].

- **Anti-DNA topoisomerase I antibodies**

ATA target the enzyme DNA topoisomerase I, a 100 kDa chromatin-associated enzyme involved in supercoiled DNA relaxation. They were first named anti-Scl-70 as they were found to react against a 70 kDa protein by immunoblotting. It was later acknowledged that the 70 kDa protein was a breakdown product of a native full-length 100 kDa protein, topoisomerase I, an enzyme located in the nucleus and in the nucleolus[35]. ATA are very specific for SSc (97-100%), but sensitivity is rather low (9-20%). The prevalence of ATA in SSc cohort has been reported to be 9-39%; ATA are strongly associated with diffuse subset, being detected in approximately 40% of dcSSc patients. Less than 10% of ATA-positive patients develop the limited disease[46]. ATA positivity has been shown to correlate with the severity and the activity of the disease. ATA are also predictors of the development of ILD and digital ulcers, but appear to be protective against PAH[23].

- **Anti-RNA polymerase antibodies**

ARA target RNA polymerase I and III, enzymes that catalyze the replication of RNA from an RNA template. RNA polymerase I has a nuclear localization, whereas RNA polymerase III is a nucleolar enzyme[35]. ARA are extremely specific for SSc (97-100%). ARA frequency in SSc has been reported between 4 and 25% of the total cohort, and is described much less commonly in European SSc cohort as compared with North American populations[23]. A strong association of ARA with diffuse subset has been recognized: 67-93% of ARA positive patients develop dcSSc. In particular, ARA positivity has been linked to severe cutaneous involvement, with flexion contracture of hands and tendon friction rubs. ARA provide one of the strongest risk factor for SRC: 43% of patients

carrying ARA develop SRC, 59% of patients diagnosed with SRC display ARA. ARA confer also an increased hazard of GAVE[17]. On the other hand, ARA were found to protect against ILD and inflammatory myopathy[23]. ARA levels were reported to fluctuate over time, reflecting changes in the skin score[47].

An increasing burden of evidence points towards an association between ARA positive SSc and cancer. Indeed, malignancy rate is higher in patients with ARA compared to those without ARA (44% versus 9-11% in the work by Airo'[48] and 26% versus 13% in another study by Moinzadeh[49]). In particular, Shah and coworkers observed a close temporal relation between cancer and SSc onset in patients with ARA: the first SSc symptoms manifest shortly after cancer (mainly breast tumours) diagnosis[50].

- **Anti-Th/To antibodies**

Anti-Th/To antibodies target small ribonuclear proteins associated with RNA fragments (H1/8-2 and Th/7-2 RNA), which are components of the RNA processing enzymes RNase P and RNase MRP. This multi-protein-RNA complex consists of a catalytic RNA and several protein components[35]. Anti-Th/To are highly specific for SSc, and are detected in 2-5% of patients[23]. Cutaneous involvement is usually limited, positivity for anti-Th/To correlates with severe ILD and carries an increased mortality hazard[46].

- **Anti-RNP antibodies**

Anti-RNP antibodies are directed against components of the spliceosome, a mithocondrial ribonucleoprotein complex involved in pre-mRNA processing into mature RNA. The antigenic determinants of the complex are provided by ribonucleoproteins processed with small RNA

molecules of 80-350 nucleotides[35]. Antibodies against U_1RNP can be detected in 6% of scleroderma patients, whereas anti-U_3RNP antibodies (formerly known as anti-fibrillarin) can be recognized in 8% of SSc subjects. Anti-fibrillarin antibodies are more common in the diffuse subset; 50-60% of positive patients are of afro-american origin[46]. Antibodies against U_3RNP have been shown to predict the development of inflammatory myopathy: 23-33% of patients with anti-U_3RNP develop a muscle disease as compared to 14% of those with other autoantibody specificities[23]. Anti-U_3RNP antibodies have also been associated with gastrointestinal involvement and PAH[51].

- **Genetics**

The incidence of SSc is higher in families where a relative holds a scleroderma diagnosis (1.5-1.7% versus 0.026% in the general population). The relative risk of developing scleroderma is increased by 15 to 19-fold for siblings of SSc patients and by 13 to 15- fold for first-degree relatives. HLA genes have emerged as strongly associated with an increased susceptibility to SSc. A wide array of HLA genes has been linked with SSc across several studies conducted in different populations. Various associations between each HLA and autoantibody specificities or organ involvements have been reported. Genome wide association studies (GWAS) and candidate-gene studies have revealed an association of scleroderma with single nucleotide polymorphisms mapping in the following genes: STAT4, IRF3, IRF5, IRF7, IRF8, DNASE1L3[52], TLR2, CTGF, PTPN22, IL2RA, IL12RB2, CD247, PPAR-γ and caveolin 1[53].

Systemic sclerosis: etiopathogenic features

The pathogenesis of scleroderma is extremely complex and still not fully elucidated. It is characterized by three cardinal pathophysiologic processes, namely vascular dysfunction, inflammation and fibrosis. Fibrosis is the hallmark of scleroderma; however, endothelial dysfunction and immune cell infiltrates are thought to antedate the development of tissue fibrosis. Indeed, in tissue samples from scleroderma patients, rarefaction of capillaries and perivascular inflammatory infiltrates were observed to precede fibrotic changes. It is currently not clear which event provides the primary insult in scleroderma: different paradigms for SSc pathogenesis have been proposed, each envisaging a potential trigger for SSc: endothelial damage, viral infections, ECM injury… Surely, the three pathophysiologic processes are disparate, but yet strictly interrelated one with the other. The interplay between the pathophysiologic processes in scleroderma etiopathogenesis is illustrated in **Figure 1**.

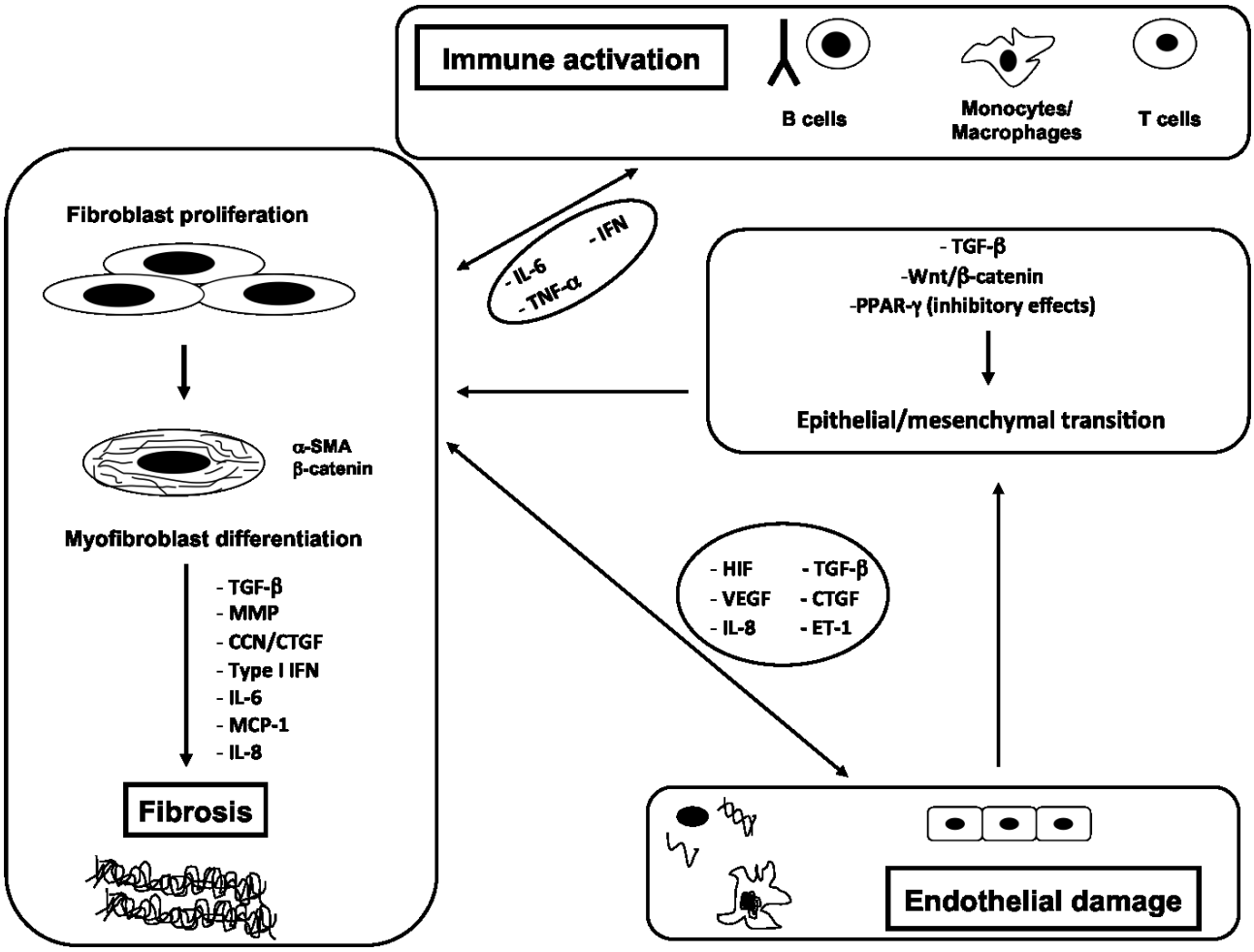


Figure 1. Schematic overview of the interplay between endothelial damage, immune activation and fibrosis in scleroderma pathogenesis (Original figure).

IL: interleukin; TGF-β: transforming growth factor –β; PPAR-γ: peroxisome proliferator-activated receptor -γ; MCP-1: monocyte chemotactic protein -1; MMP: matrix metalloproteinase; CTGF: connective tissue growth factor; IFN: interferon; VEGF: vascular endothelial growth factor; HIF: hypoxia-inducible factor; ET-1: endothelin-1.

- **Vascular dysfunction**

The prominent endothelial dysfunction characteristic of SSc is secondary to a dysregulation of vascular tone, with subsequent vascular spasm and decreased blood flow.

Endothelial cells have been appointed as the main player in vascular dysfunction. Indeed, the above-cited poor control of vascular tone is ascribable to the unbalanced production by endothelial cells of vasoconstrictor and vasodilator mediators. In particular, scleroderma endothelial cells have been shown to synthetize a markedly lower amount of nitric oxide, the most potent endothelial vasodilator agent. Interestingly, transforming growth factor –β (TGF-β) has been reported to inhibit the enzyme nitric oxide synthase. The resulting impaired production of nitric oxide was found to contribute to platelet activation and to oxidative stress and to promote intimal hyperplasia. In addition, an up-regulation of endothelin-1 (ET-1), the most potent vasoconstrictor agent, has been reported[54]. In SSc, endothelial cells have been shown to express higher levels of adhesion molecules as endothelial-leukocyte adhesion molecule (ELAM) -1, inter-cellular adhesion molecule (ICAM)-1 and CD34[55].

Endothelial cells have been proposed as the first target in SSc: an initial insult to the endothelium (cold exposure, viral infection, anti-endothelial antibodies [AECA]) might induce vasoconstriction leading to hypoxia, ultimately resulting in tissue fibrosis. Consistently, endothelial cell apoptosis has been described early on disease course; on the other hand, there is no evidence from animal models that an endothelial injury might result in tissue fibrosis[55].

SSc is also characterized by a defective neoangiogenesis, a complex process that requires mobilization of endothelial cell progenitor cells (EPCs) and proliferation and differentiation of resident cells. Reduced numbers and functionality of circulating $CD34^+$ and $CD133^+$ EPCs have been reported in scleroderma[55]. Neoangiogenesis is regulated by a fine-tuning of pro-angiogenic and anti-angiogenic mediators. Surprisingly, the vascular endothelial growth factor (VEGF), a potent angiogenic factor, was over-expressed in skin and serum samples from SSc patients; its serum levels have been found to correlate with the development of fingertip ulcers. This might be explained by the evidence that VEGF exerts a proangiogenic activity when over a concentration threshold; below this value, VEGF stimulates the formation of chaotic and instable blood vessels.

Similarly, TGF-β displays a pro- or anti-angiogenic action depending on its concentration[56]. Vascular rarefaction ultimately results in tissue hypoxia, which leads to the activation of the transcription factor hypoxia inducible factor -1 (HIF-1). Besides inducing VEGF expression, HIF-1 serves as a potent pro-fibrotic stimulus: it induces synthesis of ECM, expression of TGF-β and connective tissue growth factor (CTGF) and promotes epithelial-mesenchymal transition (EMT)[57]. From a clinical point of view, vascular dysfunction results in the reduced density and the irregular architecture of the capillary network, leading to a decreased blood flow and subsequent tissue hypoxia. Vascular manifestations of SSc comprise Raynaud's phenomenon, digital ulcers, PAH and SRC.

- **Immune activation**

An inflammatory infiltrate with perivascular localization is characteristic of the early phases of scleroderma. Such infiltrate consists mainly of CD4+ T cells and macrophages. Consistently, elevated levels of monocyte chemoattractant protein (MCP)-1/CCL2, a potent chemoattractant for both T cells and monocytes, have been reported in both sera and tissue samples from scleroderma subjects[58].

In scleroderma patients, circulating T cells as well as T cells infiltrating skin or lung tissues display a predominant Th2 profile. Th2 polarized response is characterized by the production of cytokines as interleukin (IL)-4, IL-5, IL-13, IL-21 and MCP-1/CCL2. These Th2 cytokines have been reported to directly favour collagen production by scleroderma fibroblasts and to inhibit matrix metalloproteinase (MMP) function; moreover, IL-4 has been reported to enhance fibroblast proliferation and to synergize with TGF-β. Activated T cells stimulate SSc fibroblasts via CD40/CD154, inducing inflammatory mediators.

A growing research interest has focused on an additional T cell subset in scleroderma, the Th17 cells. IL-6, IL-1α, IL-23 and TGF-β drive the differentiation towards Th17 subset. Th17 cells synthetize high amount of IL-17; in turn, IL-17A has been shown to induce IL-6 and IL-8 production and ICAM-1 expression in human fibroblasts. In endothelial cells, IL-17A enhances TGF-β production[59]. The frequency of circulating Th17 cells is markedly raised in SSc[60]. Increased levels of IL-17 and of IL-17 inducing cytokines (IL-1, IL-6, IL-23) have been reported in SSc patients[61].

Macrophages found in SSc skin biopsies appear to be alternatively activated (M2); this lineage of macrophages highly expresses several receptors such as hemoglobin scavenger receptor (CD163), class A scavenger receptor (CD204) and mannose receptor (CD206). M2 macrophages are activated by Th2 cytokines as IL-4 and IL-13. M2 macrophages are thought to play a role in tissue remodeling and profibrotic phenotypes, providing an important source of profibrotic cytokines as TGF-β. The role of macrophages in SSc is further highlighted by the fact that lipopolysaccharide (LPS)-mediated pro-inflammatory and pro-fibrotic effects were prevented in a macrophage-deficient mouse model[62]. To note, M2 macrophages are more numerous in scleroderma skin compared to control specimens; SSc patients have been shown to display higher serum soluble CD163 levels[63].

The pathogenic role of B cells in SSc has been progressively recognized. Indeed, in scleroderma skin B cell genes have been found to be highly up-regulated[64], and B cells from patients with scleroderma to over-express CD19, resulting in hyper-responsiveness and autoantibody formation[65]. Increased levels of B-cell activating factor (BAFF) have been described in in the serum and skin samples from SSc subjects, suggesting that BAFF might contribute to B cell abnormal function in

SSc[66]. B cells play a pivotal role in SSc-ILD: they are prominent in lymphocytic infiltrates seen in lung biopsy specimens from SSc-ILD patients, and ILD progression has been associated with an increased percentage of CD19-positive cells in bronchoalveolar lavage fluid[67]. *In vitro*, circulating B cells isolated from scleroderma patients induced IL-6, TGF-β_1, CCL2 and collagen secretion in cocultured dermal fibroblasts; collagen secretion was attenuated by anti-TGF-β_1 antibody[68]. Consistent evidence has been raised in vivo: CD19-deficient mice were resistant to fibrosis after bleomycin treatment, while CD19 over-expression exacerbated fibrosis[69].

The pathophysiologic mechanisms underlying the strong association of scleroderma-specific autoantibodies with both scleroderma subsets and the pattern of organ involvement have still to be unravelled. Available evidence in support of a direct pathogenic role of each scleroderma-specific antibody is hereby discussed.

Early studies showed that purified human *ATA* inhibit relaxation of supercoiled DNA. More recently, research attention has focused on ATA antigen. Indeed, topoisomerase I has been reported to bind to the surface of dermal fibroblast cell lines. Such binding appeared to be dose-dependent and saturable, and was mediated by G-protein coupled chemokine receptor (CCR) 7 and heparan sulfate proteoglycans[70,71]. Topoisomerase I itself was shown to engage intra-cellular signaling pathways with downstream recruitment of Cγ1, c-Raf, ERK-1/2 and p38 MAPK culminating in the stimulation of fibroblast migration in an *in vivo* wound healing assay[71]. In addition, surface-bound antigen provided a docking site for ATA: indeed, affinity purified ATA have been demonstrated to bind to fibroblasts at flow cytometry, immunofluorescence and confocal microscopy[72]. Moreover, ATA amplified topoisomerase I binding to fibroblast surface in a titer-dependent manner. Interestingly, binding of ATA to fibroblasts required the presence of topoisomerase I, a nuclear enzyme which is released by apoptotic cells. Once bound to the surface of fibroblasts, ATA

complexed with topoisomerase I induced adhesion and activation of cocultured monocytes[73]. This whole burden of data prompted the authors to envisage that the autoantigen topoisomerase I might allow to alert the immune system leading to in situ recruitment of inflammatory cells.

Researchers from another group observed that scleroderma sera obtained from ATA positive patients were shown to induce higher levels of IFN-α compared to ACA and antibodies against nucleolar proteins in peripheral blood mononuclear cells (PBMCs). Such effect was prevented by bafilomycin (an endosomal acidificator), by an antibody against Fcγ Receptor (FcγR) II (CD32) and by RNA digestion, suggesting that ATA-containing immune complexes induce IFN-α via Fcγ or via endosomal Toll-like Receptors (TLRs)[74].

In early reports, *ACA* were found to disrupt mitosis. More recently, it was shown that CENP-B bound to the surface of contractile pulmonary artery smooth muscle cells, whereas no binding was observed to fibroblasts and endothelial cells. Endothelial cells could provide a potential source of extracellular antigen: CENP-B from apoptotic endothelial cells was observed to bind to smooth muscle cells. In addition, CENP-B was reported to activate intra-cellular signaling cascade through the engagement of CCR3. Moreover, CENP-B binding induced a CCR3-epidermal growth factor receptor (EGF-R) crosstalk via a MMP-dependent mechanism[75]. CENP-B binding culminated with the downstream recruitment of focal adhesion kinase (Fak), Src, ERK-1/2, and p38 MAPK pathways. These signaling cascades culminated in the synthesis of IL-6 and IL-8 and in the stimulation of the migration of smooth muscle cells[76]. Antibodies against CENP-B were reported to prevent EGF-R transactivation and the subsequent secretion of IL-8[75].

In early studies, *ARA* were demonstrated to inhibit RNA transcription. Most recently, the striking association and the temporal clustering between cancer and scleroderma with ARA positivity has

gained attention. In cancer tissues from patients with ARA-positive scleroderma, genetic alterations in the PLOR3A locus coding for the enzyme RNA polymerase III were noted, whereas no alteration could be detected in tissue samples from patients with ARA-negative SSc. In addition, analyses of peripheral blood lymphocytes suggested that the mutant peptide could act as an immunogen and induce ARA production *in vivo*. ARA purified from SSc patients recognized the wild type and the mutant form to a similar extent, suggesting that antibody response does not discriminate between the two isoforms[77].

Several other autoantibodies have been described in SSc; however, most are not specific for scleroderma, being described in several other CTDs and in healthy subjects.

Antibodies to fibroblasts (AFA) can be detected in approximately 46-58% of scleroderma sera, with a higher prevalence among diffuse compared to limited SSc. In particular, AFA were reported to bind to the cell surface of fibroblasts but not of endothelial and smooth muscle cells. In addition, AFA can induce a pro-adhesive, pro-fibrotic and pro-inflammatory fibroblast phenotype[78]. AFA are internalized by fibroblasts via a caveolin-linked and Fc-independent pathway[79]. It has been suggested that anti-fibroblast activity might be mediated by ATA. Indeed, AFA purified from SSc patients strongly reacted with topoisomerase I at ELISA and immunoblotting. Consistently, positivity for AFA at high titers has been shown to correlate with pulmonary involvement and death[72].

AECA can be detected in 25-85% of patients with SSc, but can be found in patients with other autoimmune conditions as well. It has been proposed that AECA could mediate endothelial cell activation through activation of the caspase 3 pathway, antibody-dependent cell-mediated cytotoxicity and endothelial cell apoptosis[80].

Stimulatory autoantibodies against the platelet derived growth factor receptor (PDGF-R) have been reported in sera from SSc patients. These antibodies were shown to induce phosphorylation of PDGF-R, with up-regulation of α-SMA and collagen expression in fibroblasts[81]. However, confirmation of these results in a larger cohort is still warranted. Recently, it has emerged that positivity for anti-PDGF-R antibodies is not restricted to SSc, but could also be detected in healthy individuals[82-84].

Autoantibodies against angiotensin and endothelin receptors (anti-AT_1R and anti-ET_AR antibodies) have been recently described in scleroderma patients, with elevated antibody levels correlating with major disease manifestations[85]. These autoantibodies activated human microvascular endothelial cells (HMEC), inducing IL-8 and vascular cell adhesion molecule (VCAM) -1. Stimulation of HMEC with whole IgG from SSc patients resulted in increased neutrophil migration and collagen production[86].

Lastly, IgG fractions purified from scleroderma patients have been shown to bind also to *vascular smooth muscle cells*, culminating in the induction of cell contraction[87] and in a pro-fibrotic response, with a greater stimulation of ERK1/2 and Akt phosphorylation compared to control IgG. Such effect was mediated by EGF-R but not by PDGF-R[88].

- **Fibrosis**

It was 1972 when Carwile LeRoy observed that scleroderma skin fibroblasts secrete higher amounts of collagen compared to healthy control cells[89]. Fibroblasts are currently regarded as the main effector cells in scleroderma. Besides their role in ECM deposition, SSc fibroblasts can synthetize pro-inflammatory cytokines and chemokines (IL-1, IL-6, IL-8, MCP-1)[90]. Through production of chemokines, fibroblasts recruit and stimulate T cells and monocytes, and attract and retain B

lymphocytes. In addition, scleroderma fibroblasts display an altered expression of cell surface integrins and receptors for TGF-β and platelet-derived growth factor (PDGF)[57].

Scleroderma fibroblasts can differentiate into myofibroblats, contractile α-smooth muscle actin (α-SMA) positive cells that secrete high amount of collagen and other ECM components. Myofibroblasts physiologically accumulate at wound site in response to tissue injury. However, in scleroderma myofibroblasts remain in situs even once the wound has been repaired, and their number is typically expanded[90]. Besides fibroblasts, several other cells -of mesenchymal as well non-mesenchymal origin- can differentiate into myofibroblasts: pericytes, fibrocytes, smooth muscle, epithelial and endothelial cells (**Figure 2**). The differentiation of resident and circulating cells into myofibroblasts has been appointed as an important mechanism contributing to SSc fibrosis. The process where epithelial cells transdifferentiate into mesenchymal cells is referred to as EMT[91]. TGF-β, Wnt and hypoxia are the most important mediators promoting EMT, whereas peroxisome proliferator-activated receptor (PPAR-γ) plays an inhibitory role[57].

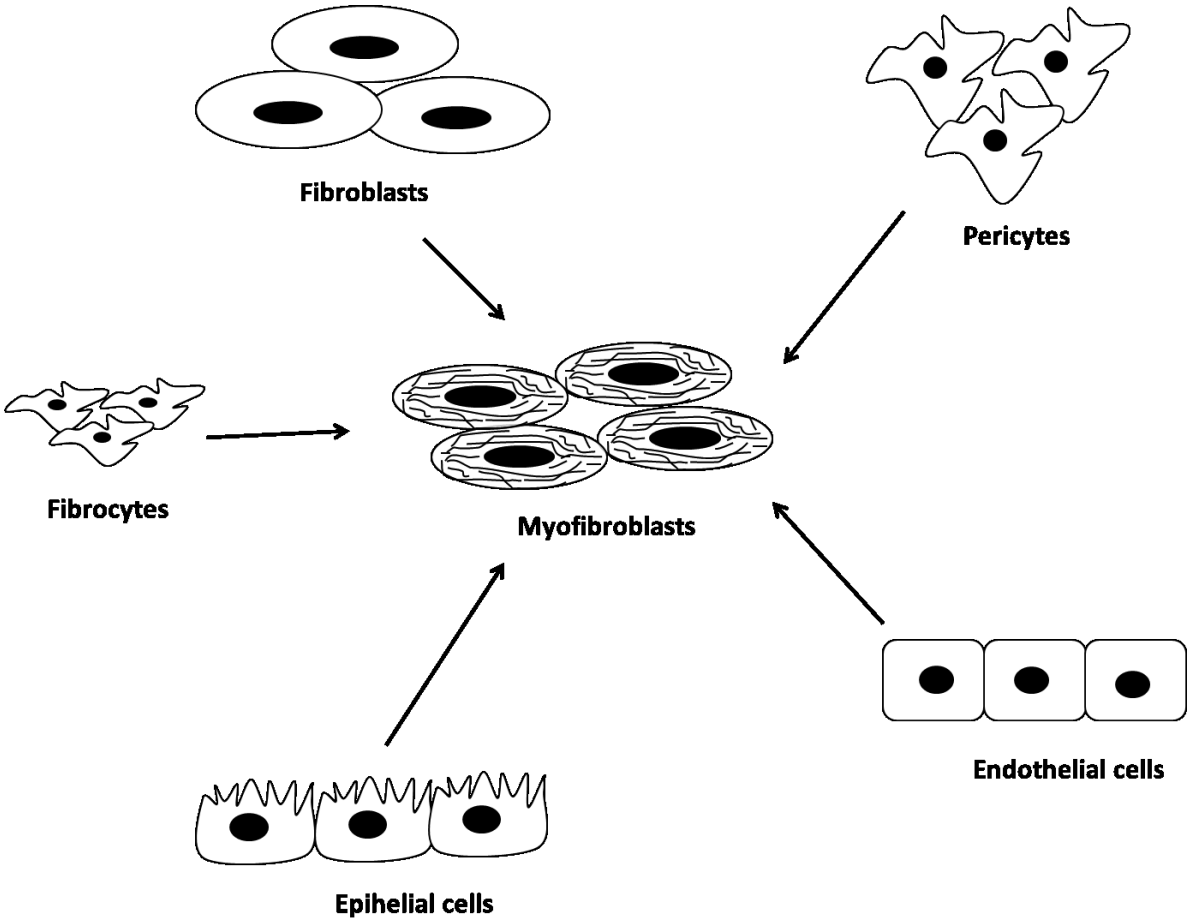


Figure 2. Cells potentially transdifferentiating into myofibroblasts (Original Figure).

The complex interplay between endothelial damage, immune activation and fibrosis involves several mediators, whose role in scleroderma etiopathogenesis is below discussed in details.

- **Transforming growth factor -β**

TGF-β belongs to the TGF-β superfamily, which enlists among the others bone morphogenic protein (BPM), nodals and activin. Three TGF-β isoforms have been to date identified: TGF-β_1, TGF-β_2 and TGF-β_3. The three isoforms share an almost identical structure comprising six cysteine residues joined by three disulfide bonds. Several cells synthetize TGF-β: fibroblasts, megakaryocytes, endothelial cells and T cells, to name some. TGF-β is secreted in a biologically

inactive molecule, which is sequestered in the extracellular matrix in a latent form. In the ECM, TGF-β binds to latent TGF-β binding protein (LTBP) to form a large latent complex (LLC). Great amounts of TGF-β are conserved in the connective tissues, providing a reservoir for easily available TGF-β. In turn, LLC binds to fibronectin and fibrillin, and these matrix interactions play an important role in activation of latent TGF-β. Indeed, upon injury, TGF-β is activated via interaction with thrombospondins and integrins. Interestingly, thrombospondin-1 and $\alpha v\beta_5$ and $\alpha v\beta_3$ integrins have been found to be significantly over-expressed in SSc fibroblasts. TGF-β activation also requires cellular tension: cells with α_v integrins on the cell membrane can exert traction on latent TGF-β, thus increasing its activation. Once activated, TGF-β binds to a TGF-β receptor (TGF-β-R), which is a heterodimer composed of two transmembrane serine/threonine kinase proteins. A TGF-β_1 pseudoreceptor, the bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI), has also been described. TGF-β recruits a Smad-dependent and a Smad-independent pathway. Smad2/3 is the main mediator of the Smad-dependent signaling cascade, which culminates with collagen transcription. Conversely, Smad7 exerts an inhibitory function[92,93]. Through the Smad-independent pathways, TGF-β activation leads to the engagement of several mediators, as phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, c-Abl (a Src family non-receptor tyrosine kinase) and Egr-1 (a DNA-binding protein zinc finger transcription factor)[94]. In addition, TGF-β interaction with integrin results in the phosphorylation of Fak, a non-receptor protein tyrosine kinase. Fak is recruited to focal adhesion upon integrin clustering and, when phosphorylated, increases its catalytic activity, leading to the activation of PI3K. Thus, Fak activation has been reported to lead to myofibroblast differentiation and to the expression of pro-fibrotic genes via MEKK/JNK pathway[95].

TGF-β is the most potent pro-fibrotic mediator in scleroderma pathogenesis. It favours the secretion of collagen and other components of the ECM, such as fibronectin and thrombospondin-1. TGF-β

has also been shown to induce several secondary mediators such as CTGF, platelet-derived growth factor (PDGF) and PDGF-R, plasminogen activator inhibitor (PAI) -1 and cartilage oligomeric matrix protein (COMP). In addition, TGF-β stimulates cell differentiation into myofibroblasts in the context of EMT. TGF−β also exerts multiple effects on endothelial cells: it was reported to up-regulate the expression of VEGF, ET-1, endothelial nitric oxide synthase and to down-regulate the vasodilator inducible nitric oxide synthase, MMP-1 and tumour suppressor, phosphatase and tensin homolog (PTEN)[93]. PTEN encodes a lipid phosphatase, which inhibits the PI3K/Akt signaling pathway. In addition, PTEN acts as cofactor for Smad3 phosphatase, with subsequent inhibition of Smads[96].

Unfortunately, the large reservoir of latent TGF-β prevents the evaluation of TGF-β *in vivo*. Indeed, TGF-β mRNA levels were reported to be elevated in skin samples from diffuse patients; however, serum active TGF-β_1 was reduced in patients with dcSSc and inversely correlated with mRSS[97]. At microarray analysis, a TGF-β-responsive signature was demonstrated in a subset of patients with dcSSc[98].

- **Connective tissue growth factor**

CTGF (also known as CCN2) is a 36 to 38 kDa cysteine-rich peptide containing 349 amino acids which belongs to the CCN family of growth factors. It is expressed in endothelial cells, fibroblasts, chondrocytes and smooth muscle cells. CTGF is induced by TGF-β, but also by PDGF, EGF and fibroblast growth factor (FGF). It has been shown to modulate fibroblast cell growth and ECM secretion, being required for TGF-β mediated effects. In particular, CTGF was required for the activation of Smad1 and Smad3, even though on its own CTGF could not trigger collagen expression. It is plausible that both CCN2 and TGF-β, bound to each other, are necessary for activation of Smad1/Erk1/2 signaling cascade[99].

- **Endothelin-1**

ET-1 is a 21-amino acid peptide, which provides the predominant of the three human isoforms. ET-1 binds to two G protein receptors, named ET_AR and ET_BR. ET-1 is produced mainly by endothelial cells, but also by fibroblasts and smooth muscle cells. ET-1 is a vasoactive peptide: it mediates vasoconstriction and the stimulation of cell proliferation in tissues both within and outside of the cardiovascular system. It activates adhesion molecules as ICAM-1 on endothelial cells and stimulates smooth muscle cell vasoconstriction. In addition, ET-1 increases the production of collagen and fibronectin by fibroblasts, and down-regulates MMP-1 expression[100]. The pro-fibrotic effects are mediated by a wide array of pro-fibrotic mediators: indeed, ET-1 has been shown to induce TGF-β and CTGF via ERK. It enhances the conversion to myofibroblasts, with the up-regulation of α-SMA mediated by ET_AR and the Akt/PI3K/rac signaling pathway[101]. In turn, TGF-β has been shown to induce ET-1 expression through a Smad-independent pathway involving JNK. Mice over-expressing ET-1 display progressive lung fibrosis, vascular dysregulation, remodeling, and increased macrophage infiltration of tissue and markers of vascular activation[102]. Consistently, ET-1 has been reported to be over-expressed in SSc skin, lung, and kidney; its serum levels were increased in the sera of scleroderma patients.

- **Interleukin-6**

IL-6 is a pleiotropic proinflammatory cytokine produced by lymphocytes, fibroblasts and monocytes. Its physiologic functions include regulation of cellular proliferation, activation and differentiation of different cell types. The expression of IL-6 receptor (IL-6R) is restricted to hepatocytes and leucocytes; however, IL-6 can stimulate cells not expressing IL-6R via the soluble receptor (sIL-6R). Engagement of IL-6R leads to cell proliferation and mitogenesis. Cell stimulation through sIL-6R is named trans-signaling, which activates an intra-cellular transduction

cascade through Jak, STAT and MAPK. Scleroderma dermal fibroblasts produce higher levels of IL-6 compared to cells obtained from healthy controls. In *in vitro* fibroblast coltures, IL-6 trans-signaling elicited a potent pro-fibrotic effects with enhanced synthesis of collagen, α-SMA and CTGF; these effects were mediated by JAK2/STAT3 and Erk pathways[103]. In fibroblasts from IL-6 knockout mice, IL-6 induced α-SMA expression via JAK1 kinase[104]. IL-6 serum levels have been shown to be increased among SSc patients compared to controls, in particular in those with early dcSSc[103,105,106]. IL-6 serum levels have been found to correlate with thrombocytosis and acute phase reactants and to be associated with severe cutaneous involvement and poor long-term survival. Expression of IL-6 in the skin is prominent in patients with early diffuse disease; at immunostaining, IL-6 expression appeared to be localized to dermal fibroblasts, mononuclear cells and endothelial cells[103].

- **Interleukin-8**

IL-8 is a CXC chemokine that acts as a chemoattractant factor for neutrophils. IL-8 and its receptors CXCR1 and CXCR2 play a central role in regulating angiogenesis by enhancing proliferation and survival and inhibiting apoptosis of endothelial cells expressing CXCR1 and CXCR2[107]. IL-8 is produced by endothelial and epithelial cells as well as by scleroderma fibroblasts and macrophages. IL-8 has been found to stimulate the production of MMPs; its secretion is induced by hypoxic stimuli via NFκB. Dermal fibroblasts obtained from SSc patients were reported to secrete higher quantities of IL-8 compared to cells from healthy controls. IL-8 levels have been found to be increased in skin samples from SSc subjects, in particular in those patients with early disease[108]. SSc patients present higher –even though not significantly- IL-8 serum levels compared to healthy subjects[106]. Three polymorphisms in IL-8 gene have been associated with the development of fibrosing alveolitis among SSc patients[109].

- **Matrix metalloproteinases**

MMPs are a family of at least 23 calcium-activated and zinc-dependent enzymes; their activity is inhibited by the tissue inhibitors of metalloproteinases (TIMP). These endopeptidases are deputed to the degradation of ECM components; they also favor neovascularization as are implicated in the degradation of vessel basal membrane. The expression of MMPs can be induced by several mediators: IL-1, IL-12, EGF, PDGF and TNF, while TGF-β down-regulates their levels. Dermal fibroblasts from SSc subjects have been observed to produce higher amounts of MMP-9 and MMP-12 compared to cells from healthy controls in response to IL-1β and tumour necrosis factor (TNF) -α[110] In scleroderma patients, levels of MMP-9, MMP-7 and MMP-12 have been found to be raised, in particular among those with diffuse disease[111-113], whereas TIMP-1 levels were decreased. The subsequent disequilibrium between MMPs and TIMP-1 in scleroderma is believed to promote the accumulation of ECM[114].

- **Interferon**

Interferons (IFNs) provide a heterogeneous family of cytokines originally identified as inductors of resistance to viral infections. Type I IFNs enlist IFN-α, IFN-β and IFN-ω. IFN-α and –β share the same structure, cellular receptors and biologic effects, such as anti-viral, anti-proliferative and immune modulatory functions[63]. In particular, IFN has been demonstrated to enhance B cell response by inducing autoantibody production in patients; it also contributes to T cell priming[115]. It has recently emerged that IFNs might exert an anti-fibrotic effect. IFN-γ suppresses collagen transcription through recruitment of collagen transcription start site of a multiprotein repressor complex containing PPAR-γ, class II trans-activator (CIIA) and regulatory factor for X-box 5 (RFX5). In cultured human dermal fibroblasts IFN–α attenuated CTGF and collagen up-regulation

by TGF-β[116]. Moreover, in fibroblasts incubation with IFN-β abrogated collagen and α-SMA expression[117].

There is an increasing evidence of an "interferon signature" in SSc, similarly to what reported in other autoimmune diseases, such as systemic lupus erythematosus. IFN-α as well as IFN-regulated genes have been described as increased in SSc dermis, with an over-expression of allograft inflammatory factor -1 (AIF-1), an IFN-γ inducible gene. The IFN-regulated gene expression in the skin was found to correlate with mRSS. In addition, peripheral blood cells and PBMCs from SSc patients were reported to express increased levels of IFN-regulated genes [118]. Circulating monocytes and tissue macrophages from scleroderma patients were reported to highly express the sialoadhesin Siglec-1, an IFN-induced gene. In addition, upon stimulation with sera from SSc patients and necrotic cell material, healthy PBMCs and purified plasmacytoid dendritic cells produced increased amounts of IFN-α. IFN-α production by stimulated dendritic cells was decreased by antibodies against FcγRII or by chloroquine: IFN-α production by plasmacytoid dendritic cells might then be modulated by immune complexes containing RNA via a FCγRII-dependent and endosome-dependent pathway. Sera with ACA or ATA induced low levels of IFN-α, whereas sera with multiple autoantibodies to RNP and/or SSA induced high levels of IFN-α[119]. In particular, ATA-positive sera were shown to induce higher levels of IFN-α compared to ACA and antibodies against nucleolar proteins[74].

It has been proposed that activation of the innate immune receptors TLRs might account for the increased levels of IFN in SSc[57]. Consistently, agonists of TLR3, TLR7 and TLR9 – but not TLR2 and TLR4 ligands- were reported to induce Siglec-1 expression in dermal fibroblasts[120]. In turn, type I IFNs were shown to up-regulate TLR3 in dermal fibroblasts, and such IFN-induced increase in TLR3 expression was augmented by TGF-β coincubation[116].

- **Peroxisome proliferator-activated receptor γ**

PPAR-γ belongs to the nuclear hormone receptor superfamily; it acts as a ligand-activated intracellular transcription factor providing a master regulator of glucose and lipid homeostasis. PPAR-γ is targeted by the insulin-sensitizing drugs thiazolidinediones**,** while rosiglitazone is a potent PPAR-γ agonist. PPAR-γ is increasingly recognized as a regulator of connective tissue homeostasis, since it exerts potent anti-fibrotic effects. Consistently, PPAR-γ expression was inhibited by several pro-fibrotic mediators: TGF-β, Wnt and CTGF[57]. It has been shown to antagonize TGF-β effects: indeed, PPAR-γ inhibited TGF-β induced myofibroblast activation and abrogated TGF-β induced collagen production. In particular, TGF-β was reported to attenuate Smad-3 mediated response. This activity is mediated by the inhibition of Fak, a kinase involved in the activation of Akt. Thus, inhibition of Fak by PPAR-γ results in the inhibition of PI3K/Akt pathway[121]. Moreover, PPAR-γ can modulate EMT, providing the most potent negative regulator[57]. The role for PPAR-γ as an effective inhibitor of dermal fibrosis has been confirmed in the bleomycin-induced mouse model of SSc. Administration of rosiglitazone significantly diminished inflammation and fibrosis, with reduced monocyte infiltration and prevention of collagen up-regulation and myofibroblast accumulation[122]. Consistently, triterpenoid, a PPAR-γ agonist, was recently proved to ameliorate fibrosis in two animal models of scleroderma by abrogating TGF-β Smad-dependent and Akt signaling pathways[123]. In lesional tissue samples from scleroderma patients, PPAR-γ expression was impaired, being inversely correlated with TGF-β activity.

- **Wnt/β-catenin**

Wnts are a family of 19 secreted signaling glycoproteins playing a key role in embryonic development and organogenesis. Wnt signaling is mainly mediated by the canonical β-catenin

pathway. In the absence of Wnt ligand, cellular β-catenin is phosphorylated and then ubiquitinated undergoing proteasomal degradation. Upon binding of Wnt ligand to Frizzled (FZD) cell surface receptors, β−catenin is stabilized, accumulates in the cytosol and translocates into the nucleus where it interacts with DNA-binding factors such as TCF/LEF thus regulating the transcription of several target genes. The canonical Wnt pathway has been shown to stimulate fibroblast activation; a cross-talk between Wnt and TGF-β signaling pathways has also been identified. TGF-β has been shown to increase the responsiveness of scleroderma fibroblasts to Wnt proteins; in turn, Wnt3a induced TGF-β_1 with phosphorylation of Smad2 and Smad3. In addition, Wnt signaling cascade was proved to induce adipocyte and mesenchymal progenitor cell differentiation *in vitro*. *In vivo* the transgenic Wnt expression has been demonstrated to induce a scleroderma-like skin fibrosis in the mouse[124]. A constitutive activation of Wnt-β-catenin cascade has been reported using skin and lung specimens from patients with SSc-associated pulmonary fibrosis. The source of Wnt ligand in SSc are unknown, but gene-expression profiling of scleroderma skin has demonstrated an over-expression of Wnt ligands and β-catenin regulated genes[125].

- **PI3K/Akt/mTOR**

Akt activity has been shown to be increased in scleroderma fibroblasts; PI3K/Akt signaling has been implicated in collagen production and cell proliferation. Akt activation can lead to the recruitment of the mammalian target of rapamycin (mTOR). mTOR is a 289-kDa serine-threonine kinase that regulates cell functionality; its expression can be modulated by TGF-β in dermal fibroblasts from healthy controls[126]. mTOR has been shown to positively regulate collagen production in dermal fibroblasts via a PI3K-independent pathway. Interestingly, in human oral keratinocytes mTOR has been shown to modulate the expression of pro-inflammatory cytokines induced by Poly(I:C), the TLR3 agonist[127]. Its role in mediating fibrosis has been demonstrated using the bleomycin-induced and the tight-skin scleroderma mouse models. Rapamycin, a mTOR

inhibitor, was proved to reduce skin fibrosis in both models, attenuating the production of IL-4, IL-6 and TGF-β_1[128]. In another study, vertical inhibition of PI3K/Akt/mTOR pathway resulted in a protective effect against skin fibrosis in two animal models[126].

- **PTEN**

PTEN is a phosphatase whose main substrate is provided by PI3K, the Akt activator. PTEN expression is suppressed by TGF-β and is stimulated by PPAR-γ. *In vitro*, PTEN-deficient fibroblasts were reported to over-express collagen and CTGF, an effect that was mediated by Akt/PI3K. *In vivo*, PTEN deletion appeared to be sufficient to induce fibrogenesis. PTEN expression has been described as reduced in skin fibroblasts from subjects with diffuse disease[96].

The interplay between PI3K, Akt, mTOR, Fak and PTEN is schematically presented in **Figure 3**.

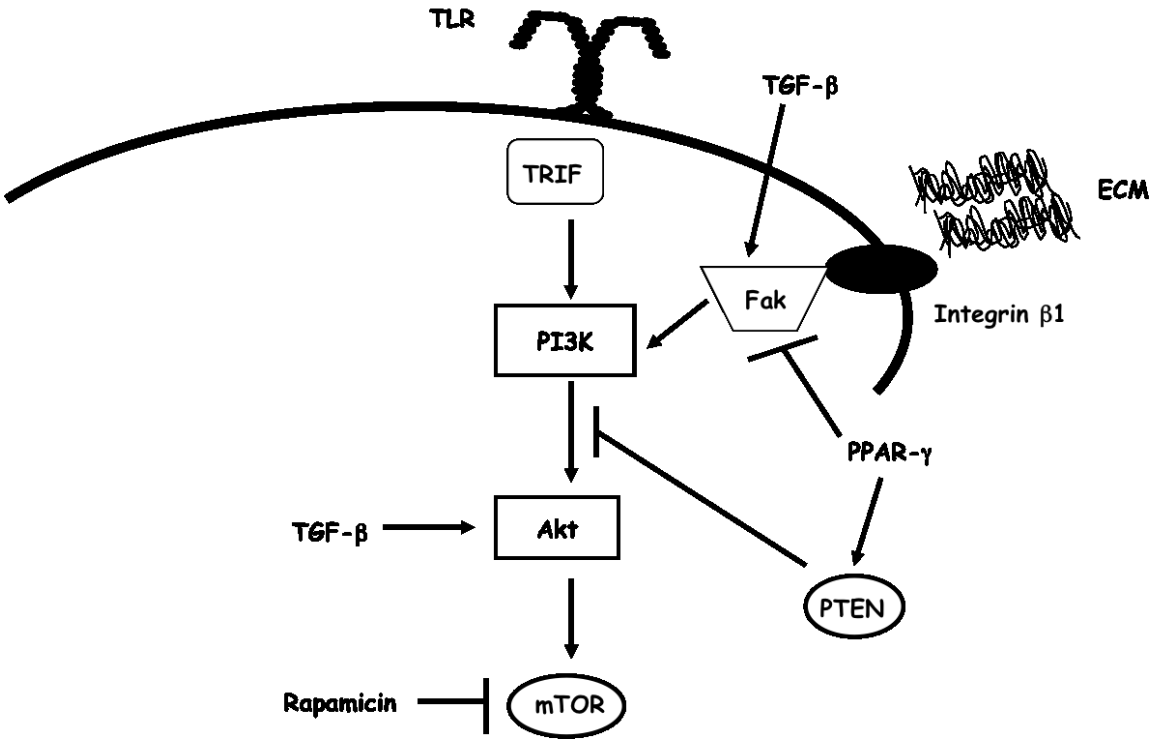


Figure 3. Schematical representation of the interplay between TLR, PI3K, Akt, mTOR, Fak and PTEN (Original figure).

TLR: Toll-like Receptor; TRIF: TIR-domain containing adaptor-inducing interferon; PI3K: phosphatidylinositide 3-kinases; mTOR: mammalian target of rapamycin; PTEN: tumour suppressor, phosphatase and tensin homolog; PPAR-γ: Peroxisome proliferator-activated receptor-γ; Fak: focal adhesion kinase.

- **Toll-like Receptors**

TLRs are a family of highly conserved pattern recognition receptors (PRR), which play a critical role in innate immunity, They are crucial in sensing pathogen-associated molecular patterns (PAMPs) of both microbial and viral origins; they also binds endogenous damage associated molecular patterns (DAMPs), which are produced upon cellular stress or mechanical trauma and act

as danger signals[129]. TLRs are trans membrane proteins consisting of three main domains: i) a leucin-rich repeat (LRR) extracellular domain; ii) a trans-membrane domain and iii) a cytoplasmic horseshoe-like TIR (Toll-IL-1R resistance) domain. In human, ten TLRs (TLR1 to TLR10) have been described; the respective ligands are detailed in **Table 1**.

TLR	**Exogenous ligands**	**Endogenous ligands**	**Synthetic ligands**
TLR1	Bacterial triacylated lipoproteins		
TLR2	Bacterial peptidoglycan and lipoproteins, zymogen yeast particles	HMGB-1, hsp60, hsp70 and hsp90, serum amyloid	LPS
TLR3	Viral dsRNA		Poly(I:C)
TLR4	Gram-negative bacterial LPS	Hyaluronan fragments, HMGB-1, hsp-20, hsp60, hsp70, hsp96, Fibrinogen, Extra domain A of fibronectin, Tenascin C, Surfactant protein-A	LPS
TLR5	Bacterial flagellins		
TLR6	Lipotechoic acid, yeast zymosan particles	Soluble tuberculosis factor, porins, hsp60, hsp70, hsp96, MALP-2	
TLR7	Viral ssRNA	ICs containing self RNA	R-848, Bropirimine, Imidazoquinoline
TLR8	Viral ssRNA	ICs containing self RNA	Imidazoquinoline
TLR9	Unmethylated viral and bacterial CpG DNA	ICs containing self DNA	CpG ODN
TLR10	Unknown	Unknown	

Table 1. Exogenous, endogenous and synthetic ligands of human Toll-like Receptors.

TLR: Toll-like receptors; ICs: immune complexes; hsp: heath shock protein; ss: single stranded; ds: double stranded.

TLRs are widely expressed by many immune and non-immune cells, such as fibroblasts, epithelial and endothelial cells[130]. Some TLRs – such as TLR2, TLR4, TLR5 and TLR6- are expressed on the

cell surface. Other TLRs display an intra-cellular localization: lysosomal or endosomal compartments and the endoplasmic reticulum; this is the case of TLR3, TLR7, TLR8 and TLR9. TLRs form hetero- or homo-dimers which are thought to facilitate dimerization to engage intra-cellular signaling[131]. Upon engagement by ligands, TLR activation results in the production of pro-inflammatory mediators. All TLRs but TLR3 recruit as cytoplasmatic adaptor protein the myeloid differentiation primary response gene (88) (MyD88) and serine/threonine kinases of the IL-1R-associated kinase family. This pathway culminates with NFκB translocation into the nucleus and the transcription of various pro-inflammatory cytokines. TLR3 and TLR4 recruit a MyD88-independent pathway with TIR-domain containing adaptor-inducing interferon (TRIF) as adaptor protein. This signaling cascade ultimately leads to the induction of type I IFNs and IFN-inducible gene production. In particular, type I IFN production is mediated by two tumour necrosis factor – receptor-associated factor (TRAF) proteins, downstream of TRIF: TRAF3 and TRAF6. The most critically role is played by TRAF3: it activates interferon regulatory factor (IRF) 3, which in turn leads to the expression of IFN-β. Conversely, IFN-α production is mainly induced by TLR7 and TLR9 and does not require TRIF but MyD88 and IRF7[129].

The intra-cellular signaling mediators engaged by TLRs are presented in **Figure 4**.

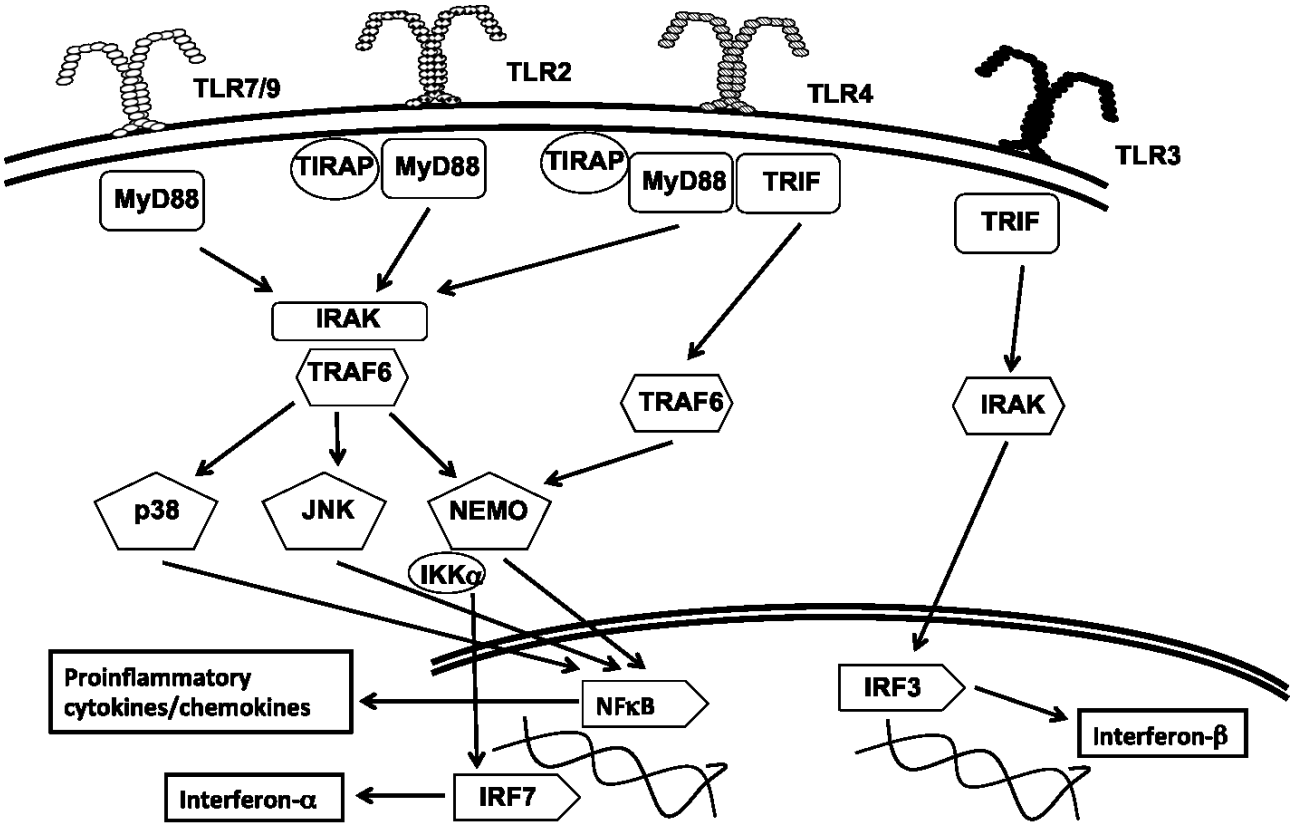


Figure 4. Intra-cellular signaling pathways engaged upon TLR activation. (original figure)

TRIF: TIR-domain containing adapter-inducing interferon; TIRAP: Toll/interleukin-1 receptor- domain containing adaptor protein; MyD88: myeloid differentiation primary response gene (88); IRAK: interleukin-1-receptor-associated kinase; TRAF: tumour necrosis factor –receptor-associated factor; IRF: interferon regulatory factor.

TLR involvement in scleroderma is suggested by a consistent burden of experimental data[132]. In monocytes from scleroderma patients, a MyD88 inhibitor prevented the up-regulation of TIMP mediated by scleroderma sera[133]. Dendritic cells from patients with early SSc responded to TLR2, TLR3 and TLR4 ligands with the secretion of higher levels of IL-6, IL-10 and TNF-α compared to dendritic cells from patients with late disease and healthy controls. It has been proposed that the

altered activation of dendritic cells in SSc subjects might lead to a Th2-skewed T-cell activation which might in turn induce the production of fibrogenic T cell cytokines[134].

To date, most of available evidence points towards TLR3 and TLR4 involvement in SSc pathogenesis.

TLR3 binds to dsRNA oligonucleotides of at least 45 bp in length, which is the minimal length required for signal transduction[135]. Polyinosinic:polycytidylic acid [Poly(I:C)], a synthetic analogue of double stranded RNA (dsRNA), is the synthetic TLR3 agonist. TLR3 expression in dermal fibroblasts was demonstrated to be increased, resulting in a raise in IL-6 and MCP-1 synthesis upon incubation with Poly(I:C). TGF-β pretreatment augmented TLR up-regulation induced by IFN-α[116]. In skin fibroblasts obtained from SSc patients, Poly(I:C) was shown to induce an IFN-γ signature and to up-regulate TGF-β responsive genes (COMP and PAI-1)[136]. In primary human dermal microvascular endothelial cells, human pulmonary endothelial cells and human dermal fibroblasts, Poly(I:C) was the only TLR agonist to induce ET-1 and MX2 (an IFN-regulated gene) mRNA expression, whereas both TLR3 and LPS up-regulated ICAM-1. Stimulating cells with ligands of TLR2, TLR7, TLR8 and TLR9 elicited no effect[137]. Most recently, Fang and colleagues confirmed that stimulation with Poly(I:C) pursued in skin and lung fibroblasts the synthesis of IFN-β as well as an IFN-induced gene expression signature. However, they evinced that Poly(I:C) treatment elicited an anti-fibrotic response. Indeed, this dsRNA analogue induced a marked deregulation in the expression of collagen, ECM molecules (fibrillin 2, laminin, periostin) and α-SMA, with a dramatic increase in pro-inflammatory cytokines (IL-6, IL-8, chemokines) and in IFN-β –but not IFN-α- mRNA levels[117]. In addition, Poly(I:C) abrogated TGF-β modulated response; these effects were attributable to the disruption of canonical Smad signaling, with the attenuation of TGF-β induced phosphorylation of Smad3 via the up-regulation of Smad7, an endogenous Smad inhibitor. Notably, these effects appeared to be not mediated by TLR3 but by two Poly(I:C) cytosolic

receptors, namely the cytosolic RNA helicase retinoic acid inducible gene 1 (RIG-1) and the melanoma differentiation-associated gene 5 (MDA5)[117]. Cytoplasmic dsRNA receptors enlist dsRNA-dependent protein kinase R (PKR), TNA helicases, RIG-1 and MDA5.

In vivo, chronic Poly(I:C) infusion resulted in the development of a scleroderma-like disease with up-regulation of ET-1, ICAM-1 and IFN- and TGF-β-modulated genes. The induction of IFN- and TGF-β-induced genes was partially attenuated by TRIF deletion, suggesting that additional Poly(I:C) receptors other than TLR3 might be implicated[136,137]. In TICAM1$^{-/-}$ mice, Poly(I: C) failed to induce ET-1 but not ICAM-1, suggesting that the latter mediator might be regulated by TLR3-independent mechanisms[137].

TLR3 expression can be detected in both lesional and non lesional scleroderma skin specimens[116,136].

In monocytes isolated from patients with SSc-ILD, LPS – TLR4 agonist- was shown to induce a pro-fibrotic phenotype and to enhance differentiation into fibrocytes[138,139]. Healthy fibroblasts stimulated with LPS presented an increased expression of genes involved in ECM synthesis (multiple collagens) and fibrogenesis (plasminogen activator inhibitor-1, Wnt2, Wnt5a, CTGF, insulin-like growth factor binding protein 3), whereas the effect on pro-inflammatory genes is modest. Furthermore, TLR4 was shown to synergize with TGF-β in stimulating the synthesis of collagen, an effect that was mediated by a decrease in BAMBI, the TGF-β1 pseudoreceptor. To note, incubation with LPS induced a marked reduction in the levels of miRNA29a, which is a negative regulator of collagen expression that is down-regulated in scleroderma fibroblasts[140].

The pivotal role played by TLR4 in the pathogenesis of scleroderma has been recently confirmed *in vivo*. TLR4-mutant C3H/HeJ mice were protected from the development of bleomycin-induced skin fibrosis, despite an important accumulation of endogenous ligands[140]. In wild type mice chronically

receiving LPS by a subcutaneous osmotic pump, a marked up-regulation of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and pro-fibrotic TGF-β regulated (PAI-1, TIMP, SPP1, collagen type 5, MMP-3, MMP-13, thrombospondin) genes was observed at one week. A less striking increase in pro-inflammatory mediators was reported after four weeks of LPS stimulation. In both TLR4- and MyD88-deficient mouse models, pro-inflammatory and pro-fibrotic effects elicited by chronic LPS stimulus were abrogated. In addition, exposure to LPS induced activation of macrophages, of both M1 and M2 phenotypes; macrophage recruitment was not reported in TLR4- and MyD88-deficient mice treated with LPS[62].

In two other mouse models of scleroderma (bleomycin induced fibrosis model and tight skin mice model), the abrogation of TLR4 resulted in an attenuation of dermal and lung fibrosis. In bleomycin-treated TLR4$^{-/-}$ mice, inflammatory cell infiltrates and neovascularization could not be detected. Upon bleomycin treatment, TLR4 knock out attenuated the production of IL-6, the levels of total serum IgG and ATA, B cell activation and the Th2/Th17 polarization[141].

The expression of TLR4 and its coreceptors CD14 and MD2 resulted to be markedly elevated compared to controls in both lesional skin and lungs obtained from scleroderma subjects, as well as in explanted fibroblasts. In particular, immunostaining of skin specimens revealed TLR4 expression by α-SMA positive myofibroblasts and microvascular endothelial cells[62].

Given the role played by TLR4, it has been proposed that upon injury several DAMPs might be produced. These molecules might induce TLR4 engagement, which in turns leads to the uncontrolled synthesis of ECM components. Indeed, besides LPS, TLR4 recognizes several endogenous ligands. In scleroderma, potential TLR4 ligands include three categories of molecules: i) matrix-derived molecules (hyaluronan, tenascin, byglican, alternatively spliced fibronectin extradomain [Fn-EDA]) ii) cellular stress proteins (Hsp60, Hsp70, HMGB1) iii) nucleic acids

released from apoptotic or necrotic cells, immune complexes[57]. Several endogenous ligands have been evaluated in scleroderma patients. Hsp70 serum levels were found to be increased in scleroderma patients compared to controls[142]. HMGB-1 is a 215-aminoacid soluble protein composed of two DNA binding binding domains; upon injury or inflammation, it is released in the extracellular space and it interacts with TLR2 and TLR4 and the soluble receptor RAGE. SSc patients were reported to have higher HMGB-1 serum levels compared to healthy controls; in addition, serum HMGB-1 correlated with skin score[143]. In scleroderma bioptic specimens, Fn-EDA, tenascin C and hyaluronan were all over-expressed. S100A8/A9 is an additional endogenous ligand for TLR4 since it acts as an alarmin. Interestingly, S100A8/A9 serum levels have been demonstrated to be markedly elevated in scleroderma patients compared to controls[144].

The TLR8 agonist ssRNA40 induced the secretion of TIMP in scleroderma monocytes[133].

In skin fibroblasts from healthy controls, the stimulation with TLR7 and TLR9 agonists induced IFN-regulated genes. Among the candidate ligands for TLR7 and TLR9, there is Epstein-Barr virus (EBV). Indeed, it has been shown to persistently infect scleroderma fibroblasts cultured *in vitro*. In addition, EBV was able to induce a pro-fibrotic phenotype in SSc fibroblasts and to up-regulate both TLR7 and TLR9 mRNA expression[145].

Aim of the study

Scleroderma autoantibodies are highly disease-specific, being rarely detected in healthy subjects or disease controls. In particular, serum antibodies reacting against SSc antigenic targets have been shown to precede disease onset, suggesting that they are not a mere reflection of disease process. Noteworthy, autoantibody positivity provides not only a strong harbinger of SSc diagnosis, but also predicts disease subset and the pattern of visceral involvement. Interestingly, antibody titers have been found to correlate with disease activity and mortality. Despite the precise diagnostic accuracy and the strong prognostic role played by scleroderma-specific autoantibodies, scarce evidence has to date been raised in support of their pathogenic potential.

The pathogenicity of scleroderma-specific autoantibodies is further suggested by some of their immunoglobulinic features: as pathogenic antibodies, they target highly specific and conserved functional domains, and are synthesized at high titers upon an antigen-driven and T cell-dependent response. In addition, SSc autoantibodies undergo IgM to IgG isotype switch and maturation, leading to high affinity IgG that go through intra-molecular and inter-molecular epitope spreading[146].

The working hypothesis of this study envisaged that immune complexes (ICs) containing scleroderma specific autoantibodies -rather than the mere antibody- might be able to elicit a pro-inflammatory and pro-fibrotic signaling cascade in target cells, thus contributing to SSc multifaceted etiopathogenesis. To test this innovative hypothesis, this study evaluated the effects elicited by scleroderma IC incubation on skin fibroblasts and endothelial cells obtained from healthy subjects. These cells, two key effectors in SSc etiopathogenesis, were used to reproduce *in vitro* the induction phase of the disease.

Since scleroderma autoantibodies bind –either directly or via bridge proteins- to nucleic acids, it was also postulated that the effects induced by ICs from SSc patients bearing different antigenic specificities might be mediated by TLRs. To test such hypothesis, cell response to IC stimulation was investigated in *tlr3*-silenced fibroblasts.

As a whole, the results emerging from this study might allow to shed light into the pathogenic role of scleroderma-associated autoantibodies, an insight that would revolutionize our understanding of scleroderma etiology potentially affecting the clinical approach to the disease.

Materials and Methods

- **Patients and healthy donors**

Four patients with a diagnosis of SSc fulfilling 2013 ACR/EULAR criteria were included in this study[7]. Patients were selected upon the autoantibody profile: in particular, one patient carried ATA, one subject harboured ACA, one patient presented ARA and another one displayed anti-Th/To antibodies. Two patients were diagnosed with dcSSc whereas the two other subjects presented a limited involvement according to LeRoy[8]. All subjects were female. The median age was of 58 years (interquartile range [IQR] 52-64). Median disease duration from the onset of first non-Raynaud's phenomenon symptom and inclusion in the study was 119 months (IQR 48-190). Serological and clinical features of the recruited scleroderma patients are summarized in **Table 2**. None of the patients presented severe gastrointestinal involvement nor SRC.

	ANA	**ENA**	**mRSS**	**RP**	**ILD**	**PAH**	**Heart Involvement**
Patient 1	+	ATA	26/51	+	+		+
Patient 2	+	ACA	7/51	+		+	
Patient 3	+	ARA	30/51	+			
Patient 4	+	anti-Th/To	9/51	+	+	+	

Table 2. Serological and clinical features of the recruited scleroderma patients.

ANA: anti-nuclear antibodies; ENA: antibodies against extractable nuclear antigens; ATA: anti-DNA topoisomerase I antibodies; ACA: anti-centromere antibodies; anti-Th/To: antibodies against Th/To; mRSS: modified Rodnan skin score; RP: Raynaud's phenomenon; ILD: interstitial lung disease; PAH: pulmonary artery hypertension.

Twelve healthy subjects were also enrolled. Healthy subjects had no history of autoimmune disease; the detection of serum autoantibodies tested negative in all cases.

- **Blood withdrawal**

Blood was withdrawn from 4 scleroderma patients and 2 healthy subjects. Serum samples were stored as frozen aliquots at -20°C.

- **Autoantibody profile**

Antibodies against nuclear antigens (ANA) were detected by indirect immunofluorescence on human epithelial type 2 (HEp-2) cells. Antibodies targeting extractable nuclear antigens (ENA) were detected using the commercial kit "EUROLINE - SSc profile" (Euroimmun, Lubeck, Germany). This is a qualitative *in vitro* immunoassay where membrane strips are coated with thin parallel lines of purified antigens. In particular, the SSc profile include 12 autoantigens: DNA topoisomerase I, CENP-A, CENP-B, RNA polymerase III, U_3RNP (fibrillarin), NOR90, Th/To, PM/Scl, Ku, Ro52, PDGF-R (**Figure 5**).

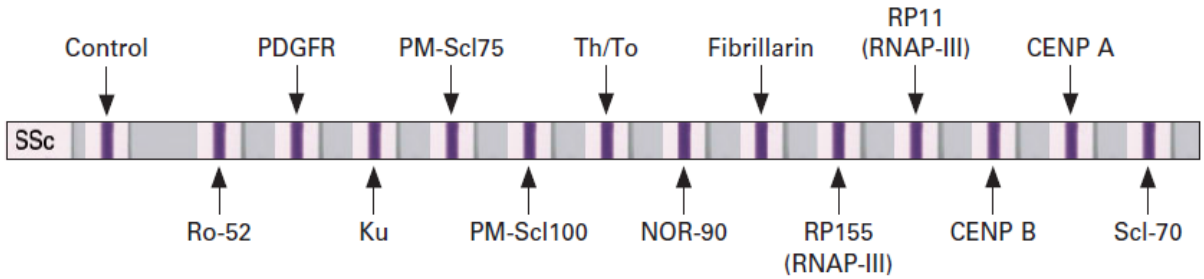


Figure 5. Scleroderma-specific antigens included in the "EUROLINE - SSc profile" (Euroimmun, Lubeck, Germany) (from: www.euroimmun.com).

- **Punch skin biopsy**

Punch skin biopsy specimens were obtained from 10 healthy donors. All subjects provided written consent to the procedure. Ethics committee approval for skin biopsy procedure was obtained at Istituto Ortopedico Gaetano Pini, Milan, Italy.

Punch skin biopsy was performed on the dorsal surface of the proximal forearm. The cutaneous area to be biopsied was cleansed with povidone-iodine solution, and then anaesthetized with 2% lidocaine. A disposable 4-mm skin biopsy puncher (Stiefel Laboratories Inc, Research Triangle Park, NC, USA) was used to perform the biopsy (**Figure 6**). The bioptic specimen was then wrapped in sterile gauzes moistened with physiological saline solution and processed within 3 hours.

Figure 6. A disposable 4-mm skin biopsy puncher (Stiefel Laboratories Inc, NC, USA) (from: www.stiefel.com).

- **Isolation of primary skin fibroblasts**

Human dermal fibroblasts were isolated from 4 mm punch skin biopsies obtained from healthy subjects[147]. The skin biopsies were put in a 6-cm sterile Petri dish (Corning Incorporated, Corning,

NY, USA), the adipose tissue was removed and the dermal layer was dissected by the means of a sterile needle and a skalpel. The specimens were then minced into small chops, these fragments were then transferred into a 15-ml tube (VWR International, Radnor, PA, USA) containing a sterile solution of 1 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, Missouri, USA) in Dulbecco's modified Eagle's medium (D-MEM, Gibco-Life Technologies, Gronigen, Netherlands). The bioptic samples were incubated for 2 hours at 37°C in a CO_2 incubator (Heracell 150i, Thermo Scientific Vantaa, Finland).

After a centrifugation at 1200 rpm for 10 minutes, pellets were washed twice with Dulbecco's phosphate buffered saline (D-PBS, Sigma-Aldrich) and then resuspended in 1 ml D-MEM (Gibco-Life Technologies) supplemented with 20% fetal bovine serum (FBS, previously decomplemented at 56°C, PAA Laboratories-GE Healthcare, Buckinghamshire, UK), 2 mM glutamine (Sigma-Aldrich), 5000 U/ml penicillin and 500 μg/ml of streptomycin (Sigma-Aldrich). The resuspended pellets were then transferred into a 25 cm^2 flask (T25, Corning Incorporated) by a sterile pipette. Cultures were maintained at 37°C in a 5% CO_2 humidified incubator. The day after, 1 ml of fresh culture medium was added. After approximately two weeks, fibroblasts were observed to migrate from the skin fragments to T25 surface. When cells reached confluence, non-adherent cells and dermal tissue were then removed by washing and the established fibroblasts were passaged with a 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco-Life Technologies).

- **Fibroblast cell culture**

Fibroblast cultures were maintained in D-MEM with 10% FBS, 2 mM glutamine, penicillin (100 U/ml) - streptomycin (100 μg/ml). Passages 4 to 8 skin fibroblasts were used for experiments. Confluent fibroblasts were passaged with a 0.25% trypsin/EDTA (Gibco-Life Technologies).

- **Isolation of primary human umbilical vein endothelial cells**

Human umbilical vein endothelial cells (HUVEC) were isolated from normal term umbilical cord vein by type 2 collagenase perfusion (Worthington, Lakewood, NJ, USA)[148]. Briefly, the cord was laid on a sterile pad under flow hood, excess blood was removed. A fresh cut with sterile scissors was performed on one end of the cord. A 21.5 G butterfly needle with the plastic needle sheath on was inserted into the cord vein, which is the larger vessel compared to the two smaller arteries. The needle was then clamped in place with a haemostat, a 20 cc syringe containing Hank's balanced salt solution (HBBS) was inserted into the needle. HBBS was pushed through the vein with moderate pressure to wash the vein and check for leaks along the cord. The 20 cc syringe was then replaced with a 30 cc syringe containing 20 ml collagenase warmed at 37°C. The collagenase solution was pushed into cord vein, the other end of the cord was clamped once the first leak passed through. The cord was filled with collagenase until a moderate distention of vein was observed, and incubated for 10 minutes. The cord was then massaged gently, and the second end of the vein was cut with sterile scissors. The collagenase solution containing HUVEC was collected in a 50 ml tube, and centrifuged at 1500 rpm for 10 minutes at 4°C. The cellular pellet was resuspended in a complete E199 medium (Flow Labs, Irvine Scotland, UK) supplemented with 20% heat inactivated FBS (PAA Laboratories -GE Healthcare,), 1% L-glutamine (MP Biomedicals, Santa Ana, CA, USA), 100 U/ml penicillin-streptomycin (MP Biomedicals) and 250 ng/ml Amphotericin B (MP Biomedicals). Cells were then seeded into a gelatinized T25 (Corning Incorporated). In all the experiments a pool of cells from at least three donors has been used at the first passage.

When cells reached a confluence, HUVEC were passaged with a 0.25% trypsin/EDTA (Gibco-Life Technologies).

- **Human umbilical vein endothelial cell culture**

HUVEC cell cultures were maintained in complete E199 medium (Flow Labs) supplemented with 20% heat inactivated FBS (PAA Laboratories-GE Healthcare), 1% L-glutamine (MP Biomedicals Inc.), 100 U/ml penicillin-streptomycin (MP Biomedicals) and 250 ng/ml Amphotericin B (MP Biomedicals). Confluent cells were passaged with a 0.25% trypsin/EDTA (Gibco-Life Technologies).

- **Polyethylene glycol-precipitation of immune complexes**

ICs were precipitated from healthy donors' and patients' sera following the procedure reported by Pontes-de-Carvalho[149]. Samples and reagents were kept on ice at all time. One and a half ml tubes (Greiner Bio-one, Frickenhausen, Germany) and 5 ml pipette tips (Thermo Scientific) were autoclaved the day before use. Briefly, 200 µl of serum samples were mixed with 200 µl of ice-cold 5% polyethylene glycol (PEG) 6000 (Sigma-Aldrich) - 0.1M EDTA (Bioscience Inc, La Jolla, CA, USA) and incubated overnight at 4°C. The following day, samples were diluted 1:3 with ice-cold 2.5% PEG 6000 in RPMI to a total volume of 1200 µl (Euroclone SpA, Pero, Italy). Five ml pipette tubes were cut 2.5 cm from the top; caps of 1.5 ml tubes were removed. A 1000 µl solution of 2.5% PEG 6000 supplemented with 5% human serum albumin (HAS) (Sigma-Aldrich) was then added in each tube, the modified 5 ml pipette tips were then introduced into each tube. Ice-cold diluted serum samples (1200 µl) were then pipetted against the wall of the 5 ml pipette tip, thus creating two layers, with the diluted serum sample on the top of 2.5% PEG-RPMI solution (**Figure 7, Panel A**). Tubes containing the pipette tips were then placed in 15 ml tubes (Falcon, VWR International) and centrifuged at 3500 rpm, + 4°C for 20 minutes. After centrifugation, the pipette tips were carefully removed from the tubes blocking the upper end with the thumb, a procedure that allows withdrawing the tip and its content (**Figure 7, Panel B**). The remaining pellets, which contained

precipitated ICs, were dissolved in 200 μl of cold sterile D-PBS (Sigma-Aldrich) to the initial serum volume and used fresh at 1:2 dilution factor.

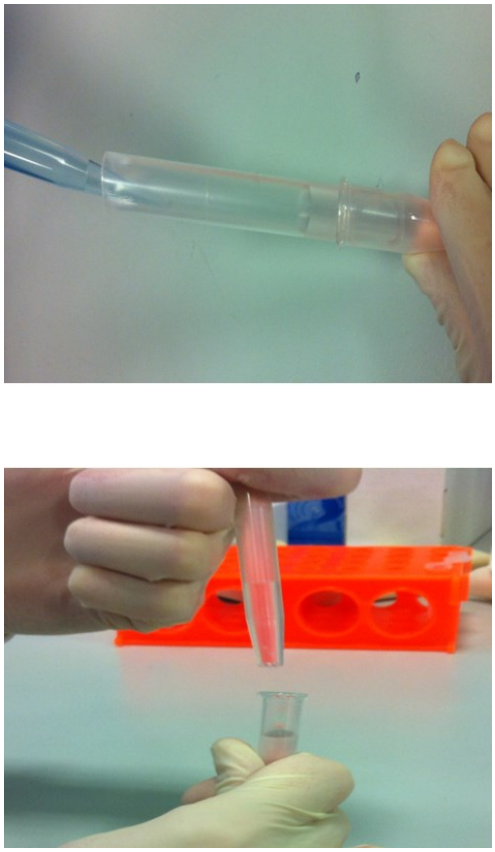


Figure 7. Panel A: Ice-cold diluted serum sample was pipetted against the wall of the 5 ml pipette tip inserted into a 1.5 ml tube. Panel B: Pipette tip was carefully removed from the tubes blocking the upper end with the thumb, in order to withdraw the tip and its content. (Original figure)

- **Flow cytometry analysis**

TLR3 surface expression on the intact non-permealized fibroblasts and HUVEC was investigated by flow cytometry analysis. Briefly, cells were grown to confluence, then detached by

trypsin/EDTA and incubated for 30 minutes at 4°C with mouse monoclonal anti-human TLR3 antibody (5 μg/10^6 cells, Abcam, Cambridge, UK) or with isotype control (20 μl/10^6 cells, BioLegend, San Diego, CA, USA), both conjugated with phycoerythrin. After two washes with D-PBS, 10.000 events were acquired for the analysis by a four-colour cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA). The results were expressed as percentage of gated events, using CELLQuest software (BD Biosciences).

- **ICAM-1 expression**

ICAM-1 surface levels were evaluated by home-made cell-ELISA. Confluent fibroblast monolayers were rested in D-MEM with 1% FBS overnight in 96-well plate; confluent HUVEC monolayers were rested in E199 with 1% FBS overnight in 96-well plate.

Naïve or *tlr3*-silenced fibroblasts and naïve HUVEC were then stimulated with 100 μl/well of: i) pathologic ICs (ATA, ACA, ARA and anti-Th/To; dilution 1:2); ii) control ICs (dilution 1:2); iii) LPS (1 μg/ml); iv) Poly(I:C) (1 μg/ml) and v) culture medium. After 48 hours of cell incubation, cells were washed twice with HBSS (Sigma-Aldrich), and incubated for 60 minutes at room temperature with 100 μl/well of murine monoclonal IgG specific for human ICAM-1 (CD54, R&D Systems, Minneapolis, MN, USA). The antibody was used at a final dilution of 1:500 in HBSS-FBS 2.5%. After two additional washes, cells were incubated for 90 minutes at room temperature with 100 μl of phosphatase-conjugated goat anti-mouse IgG (Cappel, Cochranville, PA, USA). The secondary antibody was used at a dilution of 1:1000 in HBSS-FBS 10%. After two washes with HBSS, 100 μl of the enzymatic substrate (p-nitrophenylphosphate in 0.05 M Mg-carbonate buffer pH 9.8, Sigma-Aldrich) were added. The optical density (OD) values were evaluated at 405 nm after 30 minutes of incubation by a semiautomatic reader (Titertek Multiskan MCC/340, Titertek Instruments Inc, Pforzheim, Germany).

In *tlr3*-silenced fibroblasts, ICAM-1 levels were expressed as percentage of Lipofectamine 2000-transfected cell response.

- **Interleukin-6 and interleukin-8 measurement**

Confluent fibroblast monolayers, either naïve or *tlr3*-silenced, were rested in D-MEM with 1% FBS overnight in 96-well plate. Confluent naïve HUVEC monolayers were rested in E199 with 1% FBS overnight in 96-well plate.

Naïve or *tlr3*-silenced fibroblasts and naïve HUVEC were then stimulated with 100 μl/well of: i) pathologic ICs (ATA, ACA, ARA and anti-Th/To; dilution 1:2); ii) control ICs (dilution 1:2); iii) LPS (1 μg/ml); iv) Poly(I:C) (1 μg/ml) and v) culture medium. After 48 hours of cell incubation, supernatants were collected by centrifugation and frozen at -80°C. IL-6 and IL-8 release was evaluated by commercial ELISA kit, according to manufacturer's protocol (R&D Systems). The OD values were evaluated at 450 nm after 30 minutes of incubation by a semiautomatic reader (Titertek Multiskan). IL-6 and IL-8 levels (pg/ml) in cell supernatants were calculated from a standard curve obtained using a specific reference preparation.

In *tlr3*-silenced fibroblasts, IL-6 and IL-8 levels were expressed as percentage of Lipofectamine 2000-transfected cell response.

- **Matrix metalloproteinase 9 measurement**

Confluent fibroblast monolayers were rested in D-MEM with 1% FBS overnight in 96-well plate. Naïve fibroblasts were then stimulated with 100 μl/well of: i) pathologic ICs (ATA, ACA, ARA and anti-Th/To; dilution 1:2); ii) control ICs (dilution 1:2); iii) LPS (1 μg/ml); iv) Poly(I:C) (1

μg/ml) and v) culture medium. After 48 hours of cell incubation, supernatants were collected by centrifugation and frozen at -80°C. MMP-9 release was evaluated by commercial ELISA kit, according to manufacturer's protocol (R&D Systems). The OD values were evaluated at 450 nm after 30 minutes of incubation by a semiautomatic reader (Titertek Multiskan). MMP-9 levels (pg/ml) in cell supernatants were calculated from a standard curve obtained using a specific reference preparation.

- **siRNA transfection**

Stealth™ silencing interference RNAs (siRNAs) targeting human *tlr3* were synthesized by Life Technologies. Three siRNA duplex oligoribonucleotides were designed to target human *tlr3* (GenBank Accession No. NM_003265.2): i) HSS110815; ii) HSS110816 and iii) HSS110817. Scrambled siRNA, with the same nucleotide composition but not the same sequence as *tlr3* siRNAs, was used as negative control (Life Technologies, Carlsbad, CA, USA); BLOCK-iT™ Fluorescent Oligo (Life Technologies) was employed to qualitatively assess Oligo uptake in cells by fluorescence microscopy with a standard FITC filter (λex=494 nm, λem=519 nm green).

To set siRNA transfection experimental conditions, 90.000 dermal fibroblasts were seeded in 6-well polystyrene plates (Corning) and cultered in D-MEM with 10% FBS overnight. Antibiotics were not added to culture medium in order to avoid interference with the formation of liposome-RNA complexes. Then, cells were transfected with siRNAs (40 nM) complexed with Lipofectamine™ 2000 (2.5 μg/ml, Life Technologies), according to manufacturer's instructions. Briefly, siRNAs and Lipofectamine 2000 pre-diluted in serum free Opti-MEM (Life Technologies) were mixed and incubated for 20 minutes at room temperature to allow complex formation. Successively the Lipofectamine 2000/siRNA mix was added to Opti-MEM in each well and 5 hours later the

medium was replaced by fresh antibiotic-free medium. The efficiency of RNA interference was evaluated both as mRNA expression and protein levels at different incubation times.

For experiments, 5625 fibroblasts per well were seeded in a 96-well plate and cultured in D-MEM-FBS (10%). The day after, fibroblasts were transfected with the selected *tlr3* siRNA for 72 hours following the procedure above described. *tlr3*-silenced skin fibroblasts cultured in a 96-well plate were incubated with different stimuli: naïve or *tlr3*-silenced fibroblasts and naïve HUVEC were then stimulated with 100 µl/well of: i) pathologic ICs (ATA, ACA, ARA and anti-Th/To; dilution 1:2); ii) control ICs (dilution 1:2); iii) LPS (1 µg/ml); iv) Poly(I:C) (1 µg/ml) and v) culture medium. Data were expressed as percentage (mean + standard deviation [SD]) of Lipofectamine 2000-transfected cell response, which was considered as 100%.

- **RNA isolation**

Confluent fibroblast monolayers, either naïve or *tlr3*-silenced, were rested in D-MEM with 1% FBS overnight in 96-well plate. Cells were then stimulated with 100 µl/well of: i) pathologic ICs (ATA, ACA, ARA and anti-Th/To; dilution 1:2); ii) control ICs (dilution 1:2); iii) LPS (1 µg/ml); iv) Poly(I:C) (1 µg/ml) and v) culture medium. Fibroblasts were then lysed with Trizol Reagent (Life Technologies) and maintained at -80°C. Total RNA from fibroblasts cultured in different experimental conditions was purified using Pure Link^{TM} RNA Mini kit (Life Technologies), following the manufacturer's instructions. Amplification Grade DNase I (Life Technologies) was used to eliminate residual genomic DNA from RNA samples. RNA concentration was evaluated by the nanophotometer P-300 (Implen, Westlake Village, CA, USA). The absorbance of a diluted RNA sample was measured at 260 and 280 nm. The A260/A280 ratio was used to assess RNA purity; only samples with a A260/A280 ratio between 1.8 and 2.1 were considered for experiments.

- **Reverse transcription reaction**

A reverse transcription reaction was performed with 1 μg RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Life Technologies). Universal PCR Master Mix No AmpErase UNG (Life Technologies) was used for Quantitative Real-Time PCR, performed using ABIPRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

- **Real-Time PCR**

Quantification of TLR3, TLR9, IFN-α and IFN-β mRNA expression in fibroblasts in different experimental conditions, was performed with TaqMan Gene Expression Assay (Life Technologies) for each target gene (TLR3 Hs01551078_m1, TLR9 Hs00370913_s1, IFN-α Hs00855471_g1 and IFN-β Hs01077958_s1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, HS99999905_m1) was used as endogenous reference gene to normalize transcription levels. The expression level of target genes was determined by the comparative Ct method normalizing the target to the endogenous gene (GAPDH). The PCR conditions were 94°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds and 72°c for 30 seconds. For experiments in naïve fibroblasts, the ΔCt was determined by subtracting the Ct of GAPDH control from the Ct of target gene and the ΔΔCt was calculated as the difference between the ΔCt of stimulated cells and the ΔCt of cells treated with medium only. For experiments in *tlr3*-silenced fibroblasts, the ΔCt was determined by subtracting the Ct of GAPDH control from the Ct of target gene and the ΔΔCt was calculated as the difference between the ΔCt of silenced cells and the ΔCt of cells treated with Lipofectamine 2000 only. The relative value of target to endogenous reference was described as the fold change (RQ) = $2^{-\Delta\Delta Ct}$. mRNA expression levels are shown as percentage of Lipofectamine 2000 transfected cell response.

- **SDS-PAGE and immunoblotting**

Confluent naïve fibroblast monolayers were rested in D-MEM with 1% FBS overnight in 96-well plate. Confluent naïve HUVEC monolayers were rested in E199 with 1% FBS overnight in 96-well plate. Cells were then stimulated with 100 μl/well of: i) pathologic ICs (ATA, ACA, ARA and anti-Th/To; dilution 1:2); ii) control ICs (dilution 1:2); iii) LPS (1 μg/ml); iv) Poly(I:C) (1 μg/ml) and v) culture medium. Total proteins were isolated from fibroblasts and HUVEC in different experimental conditions using RIPA Lysis Buffer (Sigma-Aldrich) added with Protease and Phosphatase inhibitor cocktail (Sigma-Aldrich) as needed.

Proteins (10 μg) were fractionated by NuPAGE BIS-TRIS 4-12% SDS-polyacrylamide pre-cast gel electrophoresis (Life Technologies) and then transferred to nitrocellulose using iBlot Transfer Stacks Nitrocellulose (Life Technologies) for 7 minutes. The membranes were blocked for two hours at room temperature in PBS/0.05% Tween 20 (Bio-Rad Laboratories, Hercules, CA, USA) (PT) containing 5% non fat milk powder (Mellin, Milan, Italy) and incubated with the primary antibody overnight at 4°C in PT/5% BSA (Sigma-Aldrich). Specific anti-human antibodies included: anti-TLR3 (R&D Systems, dilution 1:250); anti-NFκB p65 or anti-phosphorylated NFκB p65 (pNFκB p65), anti-p38MAPK or anti-phosphorylated p38MAPK (pp38MAPK) (dilution 1:1000), anti-p46SAPK-JNK or anti-phosphorylated p46SAPK-JNK (pp46SAPK-JNK) and anti-p54SAPK-JNK or anti-phosphorylated p54SAPK-JNK (pp54SAPK-JNK) (dilution 1:1000) (Cell Signaling Technology, Danvers, MA, USA).

After washes, the membranes were further incubated for 60 minutes at room temperature with the secondary antibody, a goat anti-murine IgG conjugated horseradish peroxidase (HRP, MP Biomedicals) diluted 1:2000 in PT/5% non-fat milk powder. After three washes, the antigen-antibody reactions were detected using the enhanced chemiluminescence (ECL) Plus Western Blotting Detection System (Amersham, Uppsala, Sweden). A sheet of autoradiography film

(Amersham Hyperfilm ECL 18x24cm, GE Healthcare Bio-Science, PA, USA) was exposed to the membrane in a dark room.

The detected blots of NFκB and p38 were acquired by a scanner (CanoScan 9000 F, Canon, Tokyo, Japan), the images were analyzed using the software ImageJ (Java). The relative density of each lane was calculated as the ratio of the percent value of the tested sample and the percent value of the control sample (medium). Data are expressed as the ratios of the relative density of pNFκB p65/NFκB p65, pp38MAPK/p38MAPK, pp46SAPK-JNK/p46SAPK-JNK and pp54SAPK-JNK/p54SAPK-JNK.

- **Statistical Analysis**

All experiments were run in triplicates.

The Kolmogorov–Smirnov test of normality was used to verify whether the distribution of variables followed a Gaussian pattern. Data with a normal distribution were given as mean ± standard deviation [SD], whereas variables with a skewed distribution were be expressed as median and IQR. For continuous and normally distributed variables, differences between experimental conditions were computed by one way analysis of variance (ANOVA) followed by an appropriate post-hoc test. ANOVA for repeated measurement followed by Bonferroni post-hoc test was used to compare repeated measurements among different experimental conditions. For continuous and skewed distributed variables, differences between different experimental conditions were assessed using Kruskal-Wallis test. A p-value less than 0.05 will be considered statistically significant. Statistical analysis will be performed using SPSS version 20.

Results

Experiments were first aimed at assessing the effects elicited by scleroderma ICs on naïve fibroblasts from healthy fibroblasts. This model allowed reproducing *in vitro* the induction phase of the disease. Several mediators involved in scleroderma pathogenesis have been investigated: ICAM-1, IL-6, IL-8, MMP-9, IFN-α and IFN-β.

- **ICAM-1 expression in fibroblasts stimulated with scleroderma and control immune complexes**

Fibroblast monolayers (96-well plate) were incubated with ICs purified from sera of SSc patients with different autoantibody specificities and of healthy subjects (dilution 1:2), TLR3 (Poly(I:C), 1 μg/ml) and TLR4 synthetic agonists (LPS, 1 μg/ml). All stimuli were prolonged for 48 hours. As shown in **Figure 8**, scleroderma ICs up-regulated ICAM-1 compared to the medium ($p<0.01$), all to a similar extent regardless of the antigenic specificity. To note, no increase in ICAM-1 expression was observed after stimulation with ICs from healthy donors. Both TLR ligands induced a significant increase in the protein levels of ICAM-1 as compared to the medium ($p<0.01$).

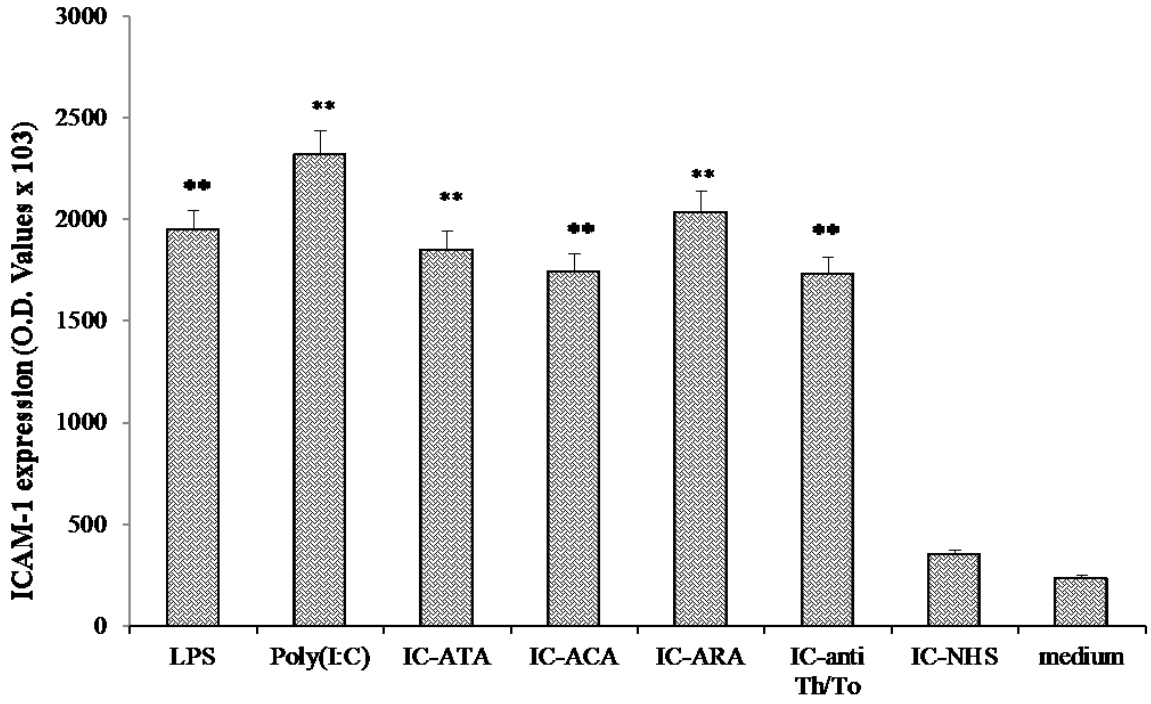


Figure 8. ICAM-1 expression levels (Optical density [OD] values) in skin fibroblasts from healthy subjects incubated for 48 hours with LPS (1 μg/ml), Poly(I:C) (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) as compared to culture medium. Experiments were run in triplicates.

- **IL-6 levels in fibroblasts stimulated with scleroderma and control immune complexes**

Fibroblast monolayers (96 well plate) were stimulated with ICs purified from SSc sera targeting different antigenic specificities and from control sera (dilution 1:2), TLR3 (Poly(I:C) [1 μg/ml]) and TLR4 synthetic agonists (LPS [1 μg/ml]). Culture supernatants were collected after 48 hour activation for IL-6 measurement. As shown in **Figure 9**, ICs from scleroderma patients up-regulated IL-6 levels compared to the culture medium ($p<0.01$). In particular, the strongest up-regulation was elicited by anti-Th/To ICs. Conversely, ICs isolated from healthy donors did not trigger any significant effect on IL-6. Poly(I:C) as well as LPS induced a significant increase in IL-6 protein levels as compared to the medium ($p<0.01$).

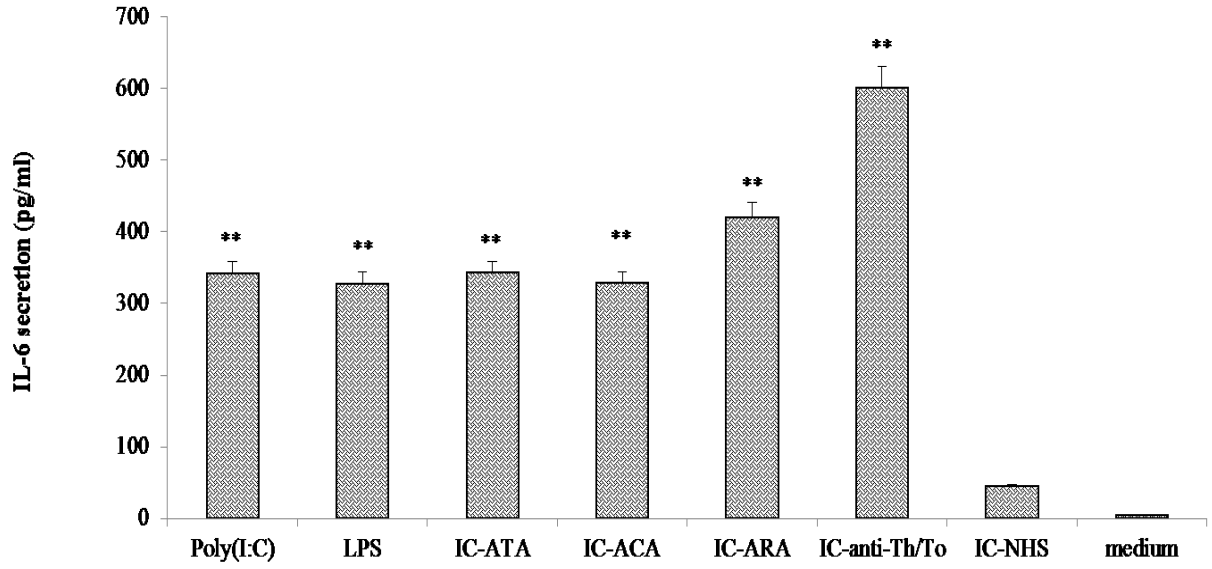


Figure 9. IL-6 protein levels (pg/ml) in the supernatants of skin fibroblasts from healthy subjects incubated for 48 hours with LPS (1 μg/ml), Poly(I:C) (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) as compared to culture medium. Experiments were run in triplicates.

- **IL-8 levels in fibroblasts stimulated with scleroderma and control immune complexes**

To determine IL-8 levels in the supernatants of fibroblasts in different experimental conditions, skin fibroblasts from healthy donors were incubated with ICs containing scleroderma-specific autoantibodies (dilution 1:2), ICs purified from serum samples obtained from healthy subjects and TLR synthetic ligands (Poly(I:C) [1 μg/ml] and LPS [1 μg/ml]). After 48 hours of stimulation, culture supernatants were collected to assess IL-8 levels by ELISA commercial kit. As shown in **Figure 10**, SSc-ICs significantly up-regulated IL-8 levels as compared to the culture medium ($p<0.01$). After incubation with ICs from healthy donors, IL-8 levels were similar to those of cells treated with medium alone. The two TLR agonists Poly(I:C) and LPS induced a significant increase in IL-8 protein levels compared to the medium ($p<0.01$).

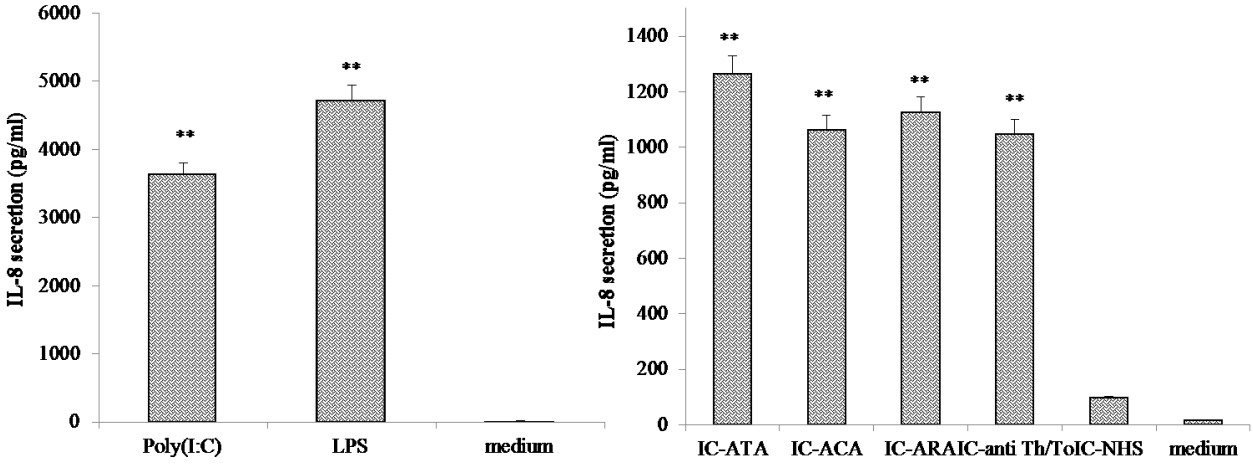


Figure 10. IL-8 protein levels (pg/ml) in the supernatants of skin fibroblasts from healthy subjects incubated for 48 hours with LPS (1 μg/ml), Poly(I:C) (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) as compared to culture medium. Experiments were run in triplicates.

- **MMP-9 levels in fibroblasts stimulated with scleroderma and control immune complexes**

MMP-9 levels were measured in the supernatants of skin fibroblasts from healthy donors incubated with ICs containing scleroderma-specific autoantibodies (dilution 1:2), ICs purified from serum samples obtained from healthy subjects and TLR synthetic ligands (Poly(I:C) [1 μg/ml] and LPS [1 μg/ml]). After 48 hours of stimulation, culture supernatants were collected to assess MMP-9 levels by ELISA commercial kit. As shown in **Figure 11**, SSc-ICs significantly up-regulated MMP-9 levels as compared to the culture medium ($p<0.05$). After incubation with ICs from healthy donors, MMP-9 levels were similar to those of cells treated with medium alone. Both TLR agonists, Poly(I:C) as well as LPS, induced a significant increase in MMP-9 protein levels compared to the medium ($p<0.05$).

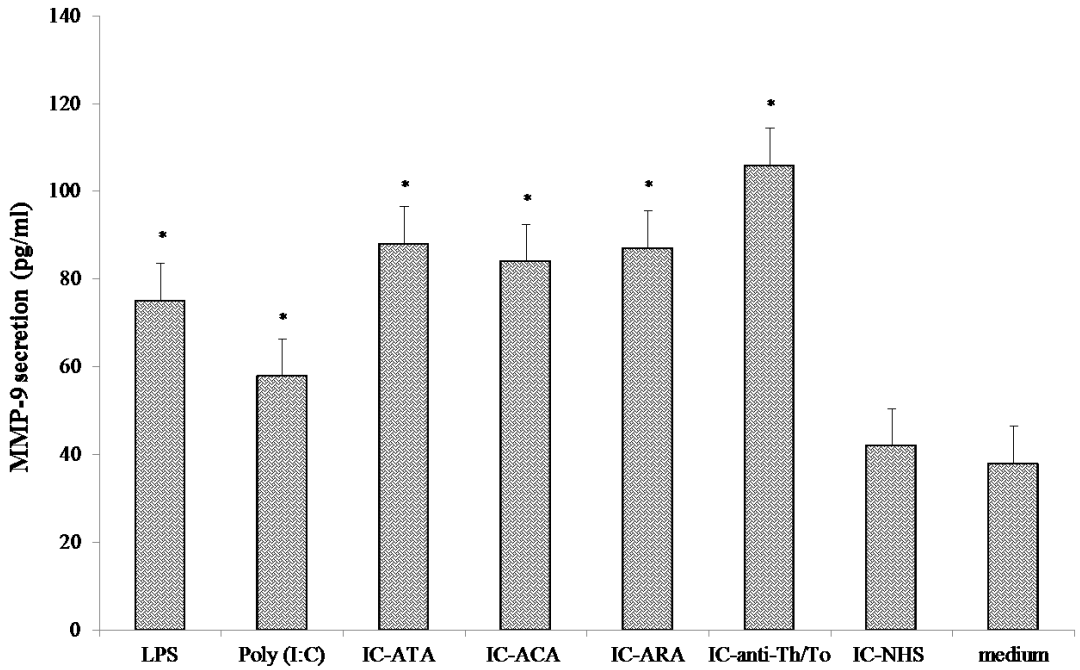


Figure 11. MMP-9 protein levels (pg/ml) in the supernatants of skin fibroblasts from healthy subjects incubated for 48 hours with LPS (1 μg/ml), Poly(I:C) (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) as compared to culture medium. Experiments were run in triplicates.

- **IFN-α and IFN-β mRNA expression in fibroblasts stimulated with scleroderma and control immune complexes**

To evaluate the effects of scleroderma ICs on the expression of type I IFNs, skin fibroblasts from healthy subjects were stimulated for 24 hours with ICs purified from SSc and healthy subjects (dilution 1:2) and with Poly(I:C) (1 μg/ml). As evinced in **Figure 12**, incubation with scleroderma-specific ICs triggered a significant increase in both IFN-α and IFN-β mRNA expression levels. In particular, such increase was rather marked for ICs anti-Th/To; a less prominent effect was reported for ACA ICs, ARA ICs and –to even a lesser extent- for ATA ICs. ICs from healthy subjects did not modulate IFN-α and IFN-β expression as compared to the medium, whereas Poly(I:C) up-regulated both IFN-α and IFN-β mRNA levels ($p<0.01$).

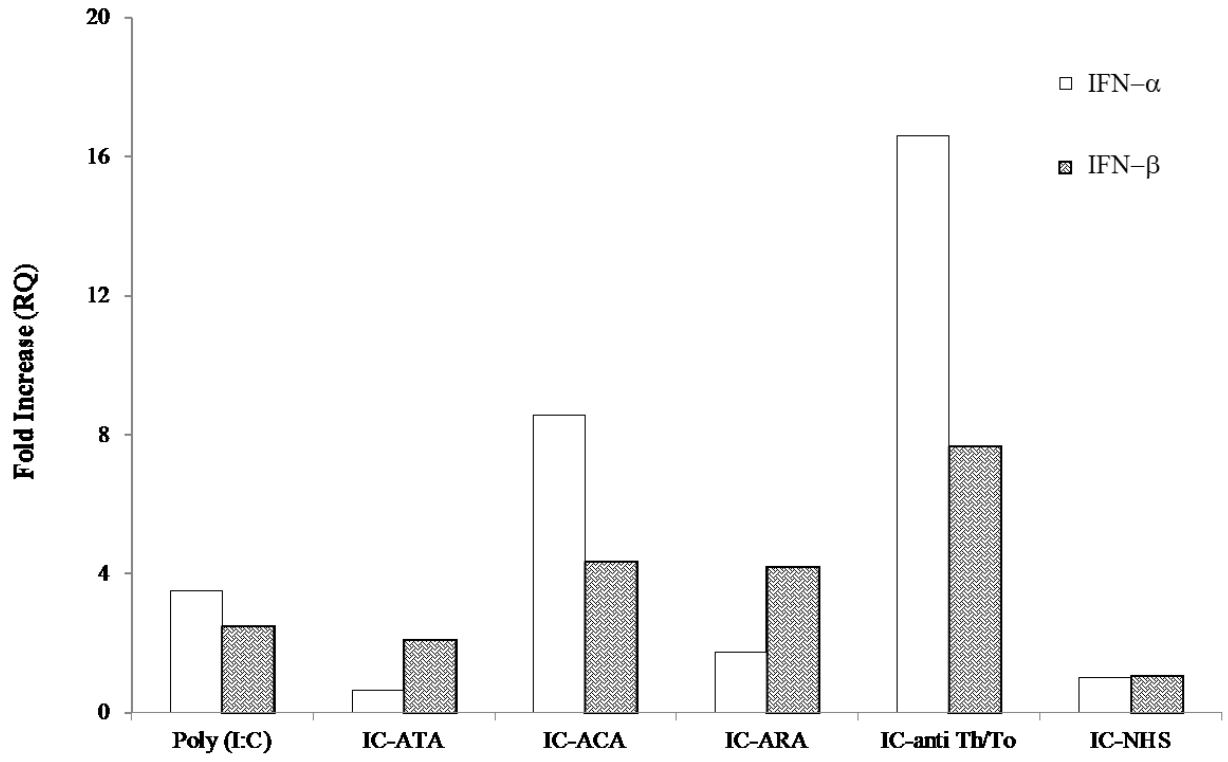


Figure 12. IFN-α e IFN-β mRNA expression levels (expressed as Fold change) in skin fibroblasts from healthy donors incubated for 24 hours with LPS (1 μg/ml), Poly(I:C) (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) and culture medium. Experiments were run in triplicates.

- **TLR3 expression on fibroblast cell surface**

Cell surface expression of TLR3 by skin fibroblasts was confirmed by flow cytometry, with 47.4% of positive events.

- **TLR3 and TLR9 mRNA expression in fibroblasts stimulated with scleroderma and control immune complexes**

The modulation of TLR3 and TLR9 mRNA expression in skin fibroblast cultures was evaluated by Real-Time PCR after 6 hour stimulation with ICs from scleroderma patients, control ICs and TLR3 agonist, Poly(I:C) (1 μg/ml). As shown in **Figure 13**, SSc-ICs significantly up-regulated the mRNA levels of both TLR3 and TLR9. The most marked increase was registered in TLR9 expression, in particular after incubation with ARA ICs. Poly(I:C) stimulation drove an up-regulation of its receptor TLR3 and, to a lesser extent, of TLR9.

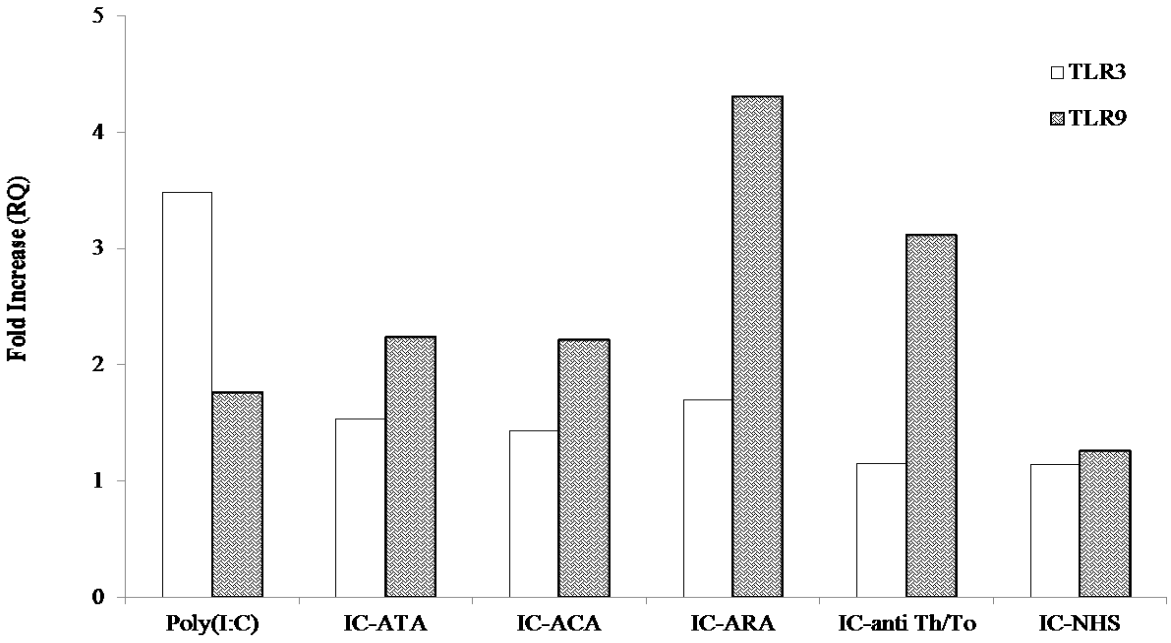


Figure 13. TLR3 and TLR9 mRNA expression levels (expressed as Fold change) in skin fibroblasts from healthy donors incubated for 6 hours with Poly(I:C) (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) and culture medium. Experiments were run in triplicates.

- **Activation of intra-cellular signaling pathways in fibroblasts stimulated with scleroderma and control immune complexes**

Fibroblast monolayers (96 well plate) were incubated with ICs purified from sera of SSc patients with different autoantibody specificities and of healthy subjects (dilution 1:2) and TLR4 synthetic agonist (LPS, 1 μg/ml). All stimuli were prolonged for 30 minutes, total proteins were then extracted.

As shown in **Figure 14**, stimulation with ARA and anti-Th/To ICs, and also LPS, resulted in a significant activation of NFκB p65 as compared to the medium ($p<0.01$). Conversely, fibroblast incubation with ATA and ACA ICs, as well as ICs from healthy donors, did not elicit a significant NFκB phosphorylation.

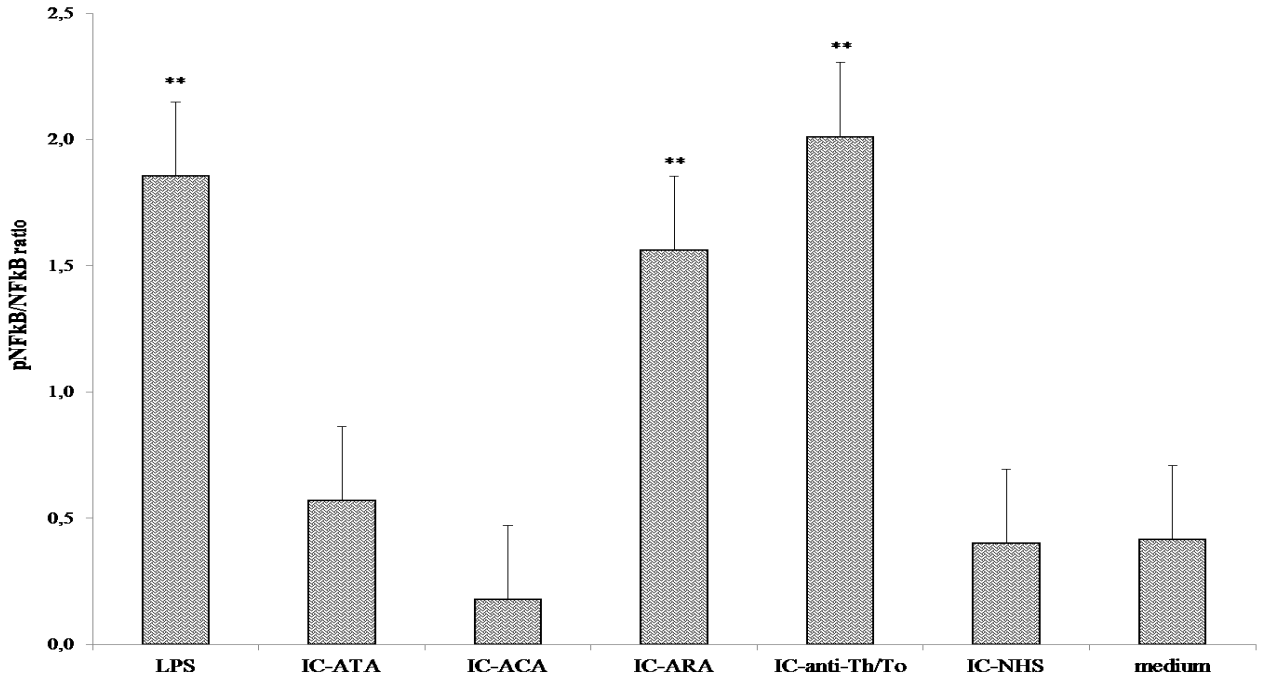


Figure 14. Activation of NFκB (expressed as pNFκB/NFκB ratio) in skin fibroblasts from healthy subjects incubated for 30 minutes with LPS (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) and culture medium. Experiments were run in triplicates.

As shown in **Figure 15**, incubation with ARA and anti-Th/To ICs, as well as with LPS, induced a significant activation rate of p38MAPK as compared to the medium ($p<0.01$). A less striking, but still significant, activation of p38MAPK was observed when fibroblasts were stimulated with ATA and ACA ICs ($p<0.05$). Conversely, fibroblast incubation with control ICs did not elicit a significant p38MAPK phosphorylation.

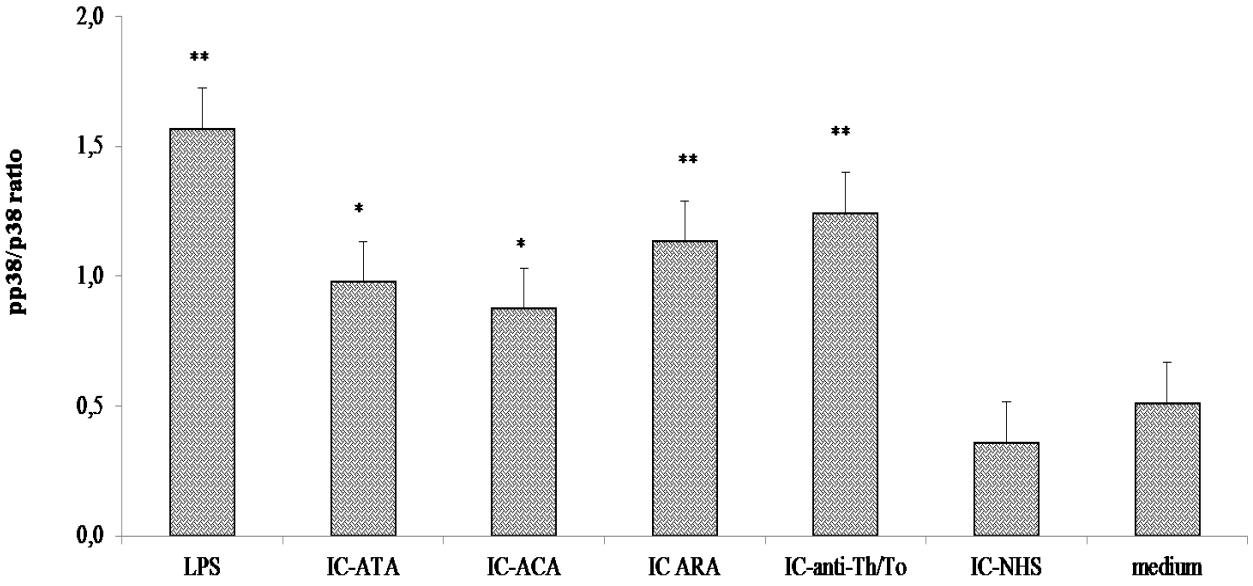
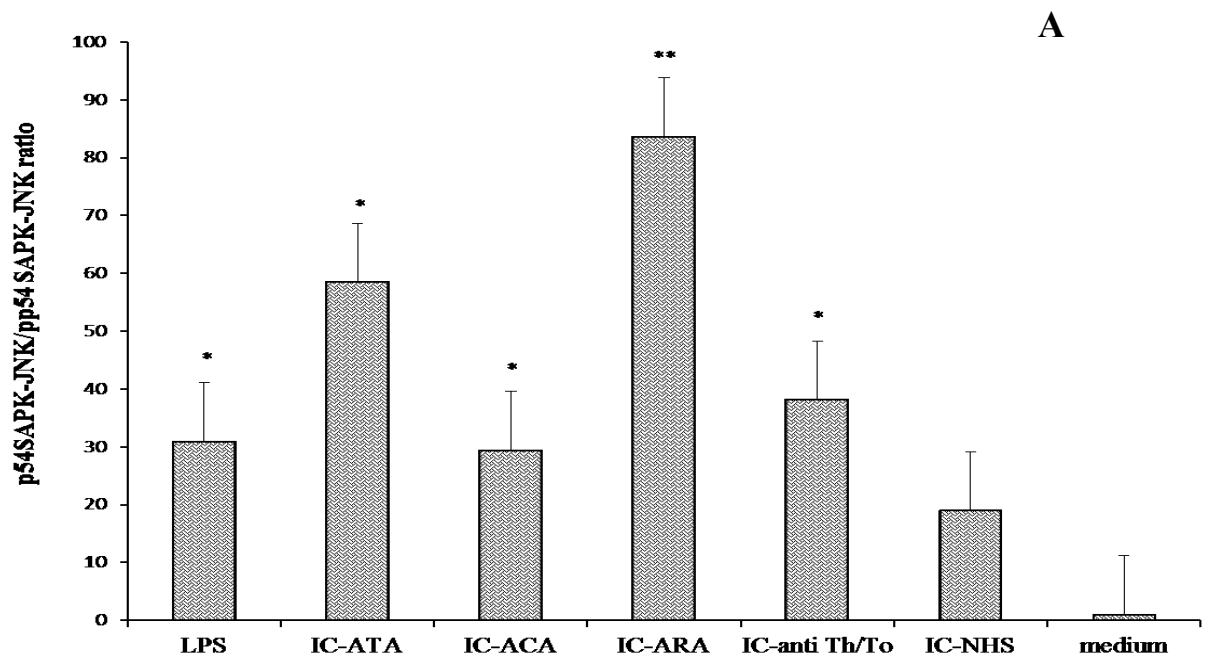


Figure 15. Activation of p38 (expressed as pp38/p38 ratio) in skin fibroblasts from healthy donors incubated for 30 minutes with LPS (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) and culture medium. Experiments were run in triplicates.

Stimulation with all scleroderma immune complexes and LPS ($p<0.05$) induced a significant increase in the phosphorylation rate of both p54 and p46, the two phosphorylation sites of SAPK-JNK. A differential activation was registered in the two phosphorylation sites: ARA ICs emerged as the autoantibody specificity responsible of the highest increase in the activation rate of p54 ($p<0.01$), whereas anti-Th/To ICs were the scleroderma immune complexes to elicit the most significant increase in p46 phosphorylation rate ($p<0.01$). A not significant modulation of the phosphorylation of both subunits of SAPK-JNK was reported in response to control immune complexes and medium alone.



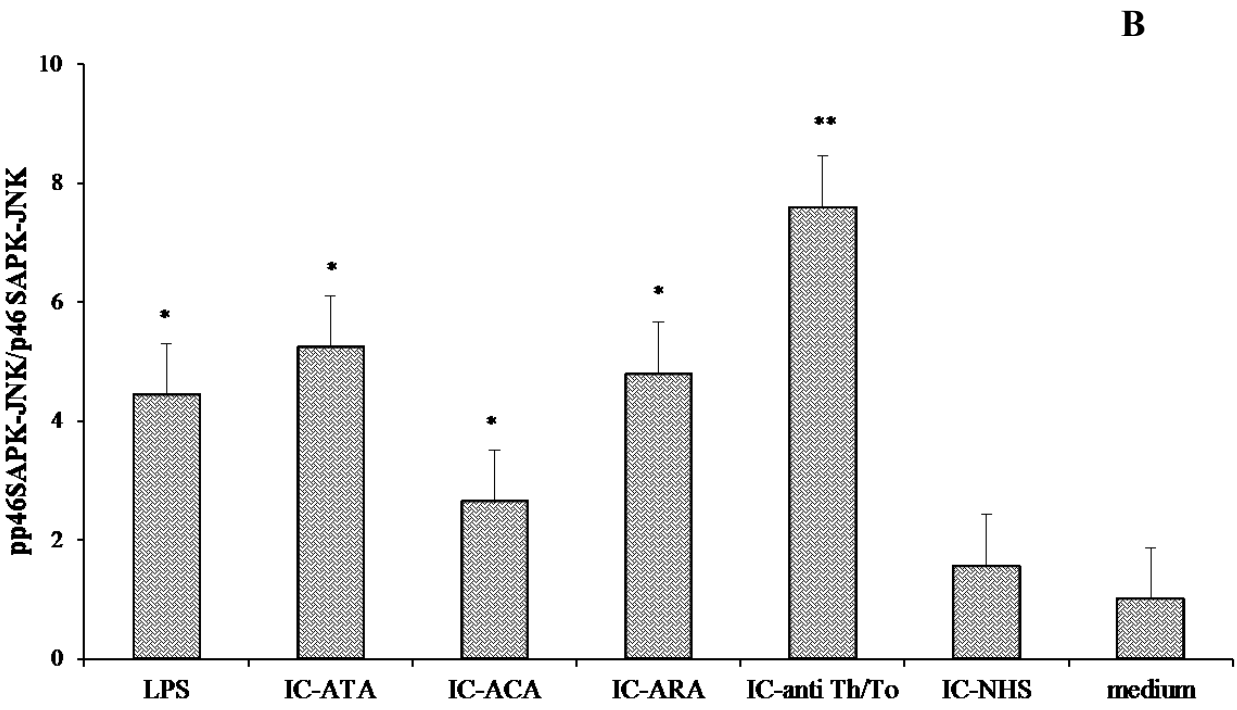


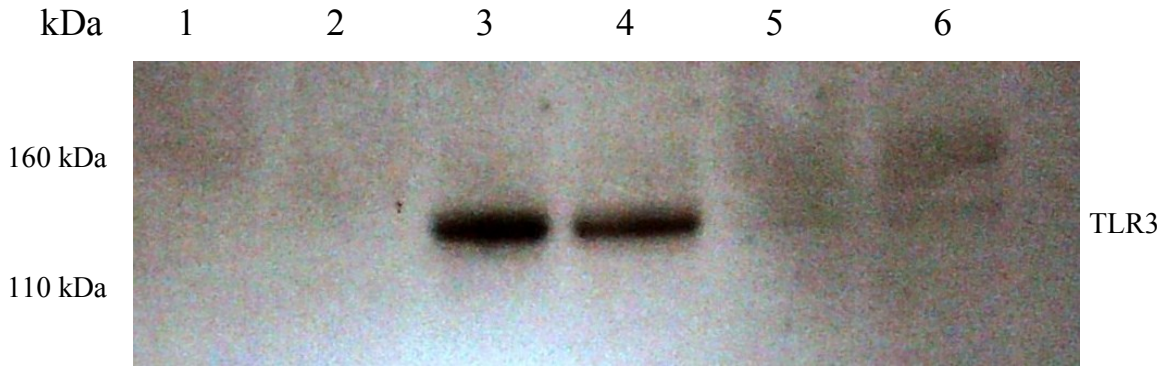
Figure 16. Activation of p54SAPK-JNK (expressed as pp54SAPK-JNK/p54SAPK-JNK, panel A) and p46SAPK-JNK (expressed as pp46SAPK-JNK/p46SAPK-JNK, panel B) in skin fibroblasts from healthy donors incubated for 30 minutes with LPS (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) and culture medium. Experiments were run in triplicates.

To address the potential role of TLR3 in mediating IC-induced effects on fibroblasts, experiments were conducted also using *tlr3*-silenced fibroblasts.

- **Evaluation and confirmation of *tlr3* silencing efficiency in fibroblasts**

Skin fibroblasts from healthy subjects were transiently transfected with a *tlr3* specific siRNA and a control siRNA for 72 hours. In preliminary experiments, HSS110816 was selected as the most efficient siRNA to silence *tlr3*. The effect of *tlr3* siRNA transfection on mRNA expression was quantitatively evaluated by Real-Time PCR. *tlr3* siRNA strongly down-regulated TLR3 expression, inducing a 95% reduction in its mRNA levels ($p<0.01$ as compared to Lipofectamine 2000-treated cells). Negative control siRNA and Lipofectamine 2000 alone did not affect TLR3 expression levels. The modulation of TLR3 expression was evaluated also in *tlr3*-silenced fibroblasts incubated with Poly(I:C) (1 μg/ml) for 24 hours.

The efficiency of *tlr3* silencing was demonstrated also at a protein level by Western blot analysis. As shown in **Figure 17**, the protein signal was strongly attenuated in *tlr3* silenced cells (lane 1), *tlr3* silencing also prevented TLR3 up-regulation induced by Poly(I:C) (1 μg/ml) stimulation (lane 2). The protein signal was reported when cells were incubated with Poly(I:C) (1 μg/ml) and Poly(I:C) plus Lipofectamine 2000 (lane 3 and 4, respectively). No TLR3 expression could be detected when cells were treated with Lipofectamine 2000 and control medium (lane 5 and 6, respectively).



1. *tlr3* siRNA
2. *tlr3* siRNA + Poly(I:C)
3. Poly(I:C)
4. Poly(I:C) + Lipofectamine 2000
5. Lipofectamine 2000
6. Culture medium

Figure 17. Protein expression of TLR3 in *tlr3*-silenced untreated or Poly(I:C) (1 μg/ml)-treated fibroblasts and naïve fibroblasts incubated with Poly(I:C) (1 μg/ml), Poly(I:C) (1 μg/ml) plus Lipofectamine 2000, Lipofectamine 2000 alone and culture medium. Experiments were run in triplicates.

- **Modulation of ICAM-1 expression in *tlr3*-silenced fibroblasts stimulated with scleroderma and control immune complexes**

tlr3-silenced skin fibroblasts cultured in a 96-well plate were incubated for 48 hours with scleroderma and control ICs, Poly(I:C) and LPS. As presented in **Figure 18**, *tlr*3 silencing significantly attenuated the up-regulation of ICAM-1 induced by ICs from SSc patients and by Poly(I:C) ($p<0.01$) but not LPS. ICAM-1 levels were similar when cells were treated with control ICs and culture medium. No difference in ICAM-1 expression level was observed when fibroblasts were treated with Lipofectamine 2000.

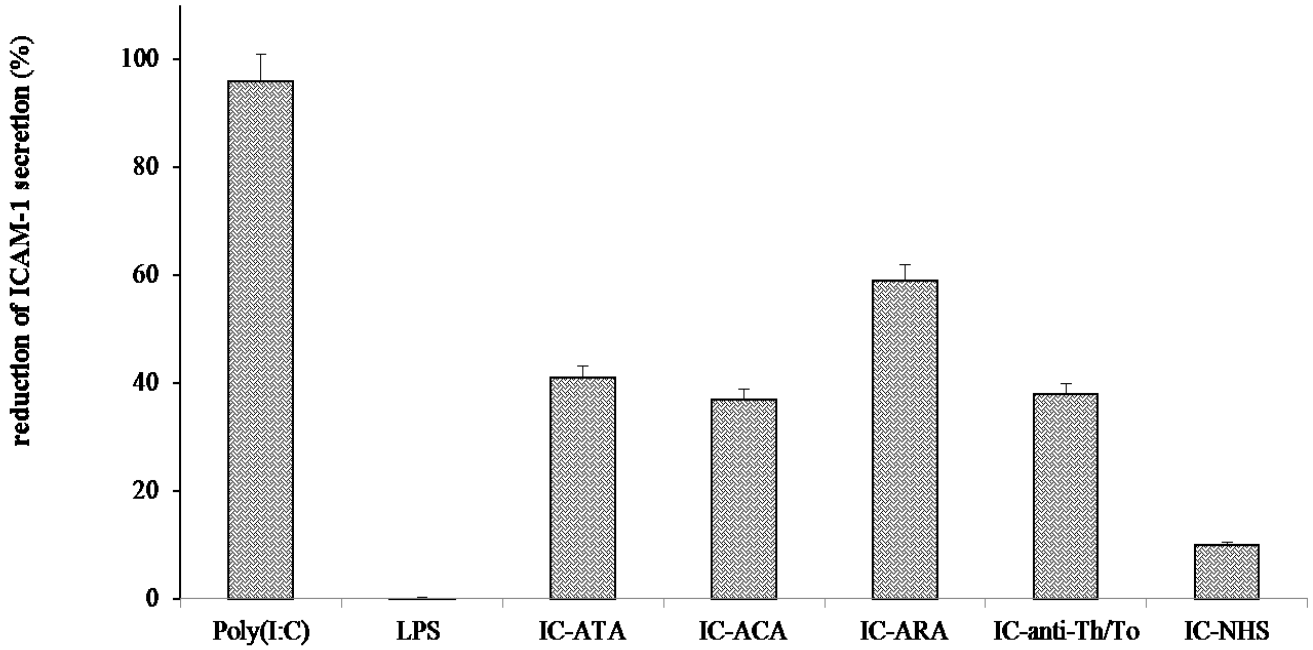


Figure 18. Reduction (%) of ICAM-1 expression levels in *tlr*3-silenced skin fibroblasts from healthy subjects incubated for 48 hours with Poly(I:C) (1 μg/ml), LPS (1 μg/ml), scleroderma ICs (1:2) and control ICs (1:2). Experiments were run in triplicates.

- **Modulation of IL-6 levels in *tlr3*-silenced fibroblasts stimulated with scleroderma and control immune complexes**

tlr3-silenced skin fibroblasts cultured in a 96-well plate were incubated for 48 hours with scleroderma and control ICs, Poly(I:C) and LPS. *tlr*3 silencing significantly affected the up-regulation of IL-6 modulated by ICs from SSc patients ($p<0.01$). In particular, the reduction of IL-6 secretion was rather prominent when cells were incubated with ATA and ARA ICs and less marked for ACA-stimulated fibroblasts ($p<0.01$). Conversely, *tlr3* silencing did not attenuate IL-6 levels in supernatants from cells treated with control ICs and culture medium. As expected, *tlr3* silencing significantly inhibited Poly(I:C)- ($p<0.01$) but not LPS-induced IL-6 secretion. No difference in IL-6 was registered when fibroblasts were treated with the transfection vector, Lipofectamine 2000. The rates of the reduction of IL-6 secretion observed in the different experimental conditions after *tlr3* silencing are presented in **Figure 19**.

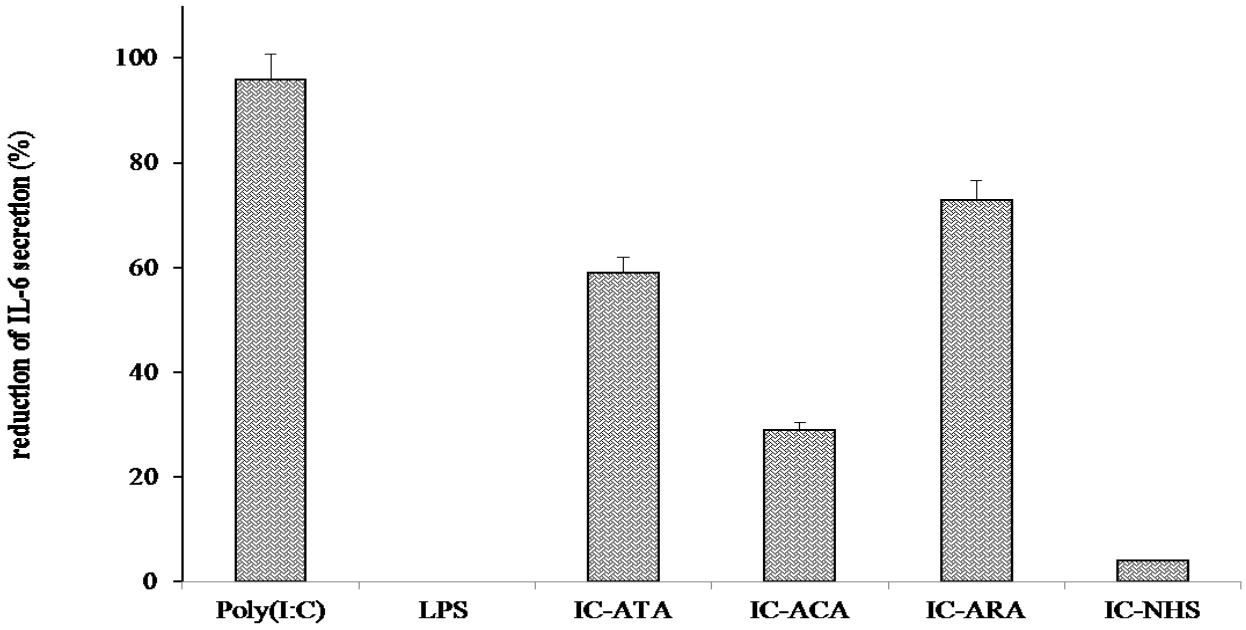


Figure 19. Reduction (%) of IL-6 levels in the supernatants of *tlr*3-silenced skin fibroblasts from healthy subjects incubated for 48 hours with Poly(I:C) (1 μg/ml), LPS (1 μg/ml), scleroderma ICs (1:2) and control ICs (1:2). Experiments were run in triplicates.

- **Modulation of IL-8 levels in *tlr3*-silenced fibroblasts stimulated with scleroderma and control immune complexes**

IL-8 levels were evaluated by commercial ELISA kit in the supernatants of *tlr3*-silenced fibroblasts cultured in a 96-well plate and incubated for 48 hours with scleroderma and control ICs (dilution 1:2), Poly(I:C) (1 μg/ml) and LPS (1 μg/ml). *tlr3* silencing did not affect IL-8 secretion by fibroblasts incubated with ICs isolated from scleroderma serum samples (data not shown). In *tlr3*-silenced cells, Poly(I:C) stimulation but not LPS significantly affected IL-8 secretion ($p<0.01$).

Preliminary experiments were also conducted using HUVEC as *in vitro* model, in order to investigate the potential effects elicited by scleroderma ICs on the endothelium, a key player in the pathogenesis of scleroderma.

- **ICAM-1 expression in HUVEC stimulated with scleroderma and control immune complexes**

HUVEC monolayers (96 well plate) were incubated with ICs purified from sera of SSc patients with different autoantibody specificities and of healthy controls (dilution 1:2), TLR3 (Poly(I:C), 1 μg/ml) and TLR4 synthetic agonists (LPS, 1 μg/ml). All stimuli were prolonged for 48 hours. As shown in **Figure 20**, scleroderma ICs up-regulated ICAM-1 as compared to the medium, all to a similar extent regardless of the antigenic specificity ($p<0.05$). No increase in ICAM-1 expression was observed after stimulation with ICs from healthy controls. Both TLR ligands induced a significant increase in the protein levels of ICAM-1 as compared to the medium ($p<0.05$).

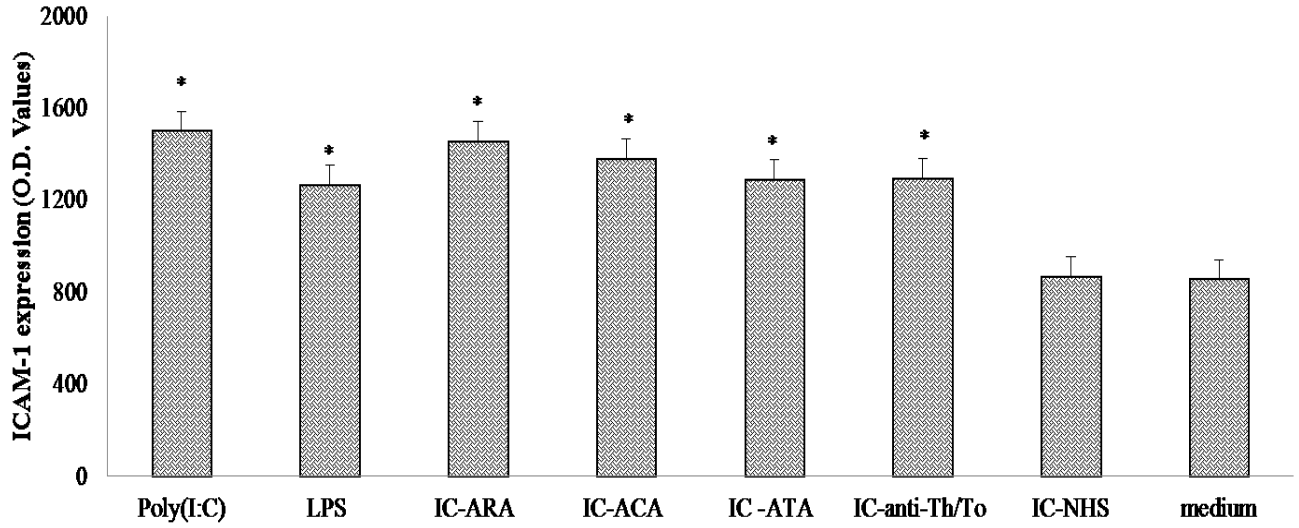


Figure 20. ICAM-1 expression levels (Optical density [OD] values) in HUVEC incubated for 48 hours with Poly(I:C) (1 μg/ml), LPS (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) as compared to culture medium. Experiments were run in triplicates.

- **TLR3 expression on HUVEC cell surface**

Cell surface expression of TLR3 by HUVEC was confirmed by flow cytometry, with 65.36% of positive events.

- **Activation of p38MAPK in HUVEC stimulated with scleroderma and control immune complexes**

HUVEC monolayers (96 well plate) were incubated with ICs purified from sera of SSc patients with different autoantibody specificities and of healthy donors (dilution 1:2) and TLR4 synthetic agonist (LPS, 1 μg/ml). All stimuli were prolonged for 30 minutes, total proteins were then extracted.

Incubation with ARA and LPS induced a significant activation rate of p38MAPK as compared to the medium ($p<0.01$, **Figure 21**). Conversely, HUVEC incubation with ATA, ACA and anti-Th/To ICs did not result in significant p38MAPK activation. Similarly, control ICs did not elicit a significant p38MAPK phosphorylation.

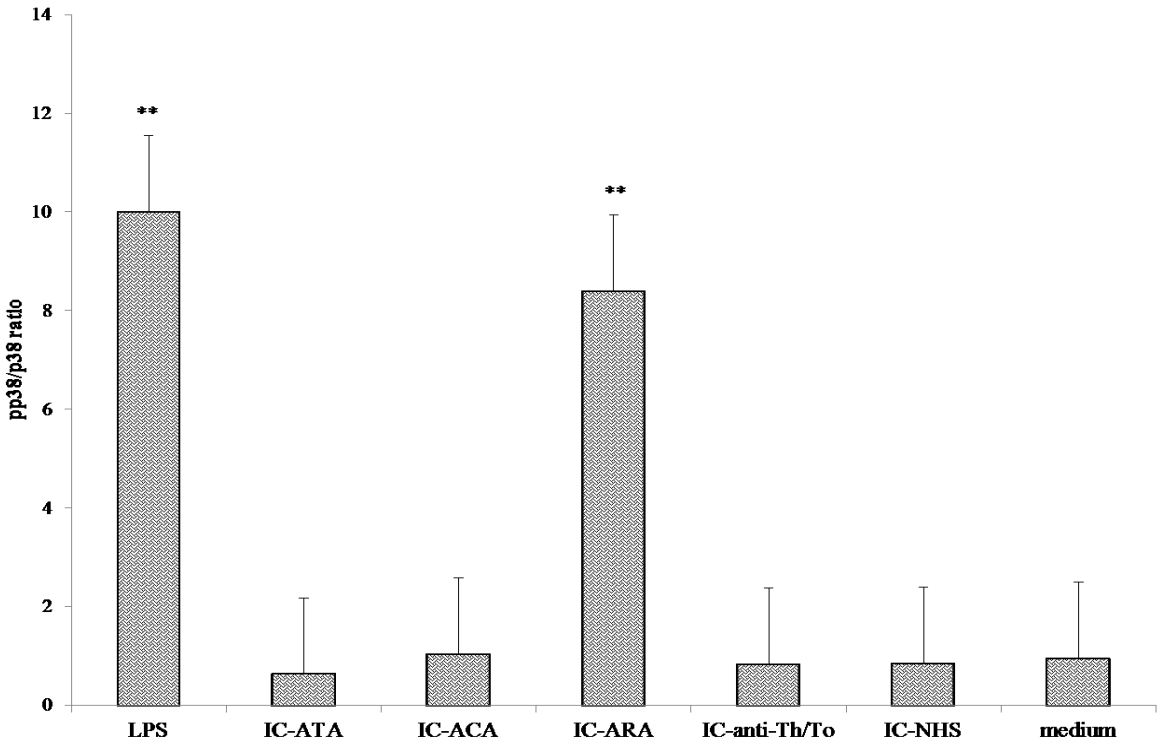


Figure 21. Activation of p38 (expressed as pp38/p38 ratio) in HUVEC incubated for 30 minutes with LPS (1 μg/ml), scleroderma ICs (1:2), control ICs (1:2) and culture medium. Experiments were run in triplicates.

Discussion

The evidence raised in this study strongly suggests that ICs containing scleroderma-specific autoantibodies might exert a pathogenic role in the induction phase of SSc. Indeed, experiments envisaged in this work show that scleroderma ICs can disrupt the functionality of fibroblasts and endothelial cells, two key players in the promotion of tissue fibrosis. In particular, data suggest that incubation with ICs isolated from SSc patients but not from healthy subjects is sufficient to perturb fibroblast functionality *in vitro*, with the induction of a pro-inflammatory and pro-fibrotic phenotype. Indeed, upon treatment with scleroderma ICs, skin fibroblasts from healthy donors over-express ICAM-1, IL-6, IL-8, MMP-9 and type I IFNs, all mediators involved in scleroderma pathogenesis. Similar results, unfortunately still incomplete, have been reported in HUVEC, whose response to stimulation with SSc-IgG comprises the up-regulation of the adhesion molecule ICAM-1. These data implicate that SSc ICs might participate in disease pathogenesis early on disease course, providing an additional player in the complex interplay between endothelial damage, immune deregulation and fibroblast activation that ultimately leads to scleroderma.

These findings could thus contribute to the collapse of a long-standing dogma in rheumatology, namely the non-pathogenicity of scleroderma-specific ANAs. The potential pathogenicity of SSc-ICs fits well with the strong diagnostic and prognostic predictive value that scleroderma autoantibodies exert in clinical settings, with their appearance predating disease onset by few months or several years. Noteworthy, positivity for scleroderma-specific autoantibodies has emerged as the strongest risk factor influencing the evolution into definite SSc[150]. Nevertheless, a very elegant study has recently suggested a causal link between the expression of a mutant ARA antigen, the production of specific autoantibodies and SSc onset[77]. Indeed, a striking temporal clustering between solid tumours, particularly breast cancers, and the onset of ARA-positive SSc has recently emerged[49,50]. Most interestingly, the POLR3A locus, coding for the antigenic target of ARA, was altered in cancer tissue specimens from scleroderma patients carrying ARA. This mutation implied the synthesis of an immunogen POLR3A gene product, leading to a T-cell driven production of specific ARA ultimately resulting in scleroderma onset[77]. Interestingly, patients with

SSc exhibit increased DNA damage in peripheral blood cells as evaluated by the comet assay. In particular, polymorphic sites in DNA repair genes might affect efficiency in repairing DNA damage, resulting in the production of autoantibodies[152].

The nuclear localization of target antigens, preventing their accessibility, has been advocated as the strongest objections against the pathogenicity of scleroderma antibodies. Dendritic and B cells could engage SSc antibodies via FcγR; this is not the case of skin fibroblasts and endothelial cells, which do not express FcγR. Novel perspectives about the potential pathogenic role of SSc peculiar autoantibodies have been addressed in previous works, which demonstrated that the nuclear antigens targeted by ATA and ACA, respectively topoisomerase I and CENP-B, can adhere to the cell surface of cells involved in SSc pathogenesis, resulting in cellular activation upon autoantibody binding[70,71,73,75,76].

This study addresses an alternative hypothesis to overcome this issue, postulating that the interaction of ICs from scleroderma patients with target cells might be mediated by innate immunity sensors as TLRs. At this regard, it should be mentioned that scleroderma-specific antibodies bind – either directly or indirectly via bridge proteins- to nucleic acids. Indeed, ATA target topoisomerase I, which is a DNA-nicking enzyme that can also associate with several proteins bound to RNA fragments[151]; ACA react against proteins assembled with centromeric DNA; ARA recognize the enzyme RNA polymerase, involved in RNA replication while anti-Th/To antibodies bind to ribonuclear proteins associated with small RNA fragments[35]. This bulk of observations suggests that nucleic acid-containing scleroderma ICs might be engaged by target cells as fibroblasts and endothelial cells via TLRs, receptors deputed to sensing nucleic acids, among several PAMPs and DAMPs. In this work, research attention was first focused on TLR3, whose role in scleroderma pathogenesis has been progressively recognized[136,137]. It should be mentioned that both skin fibroblasts and HUVEC express TLR3 not only in intra-cellular compartments but also on the cell membrane, as demonstrated by flow cytometry analysis. To address the potential role of TLR3 as receptor for scleroderma ICs, dermal fibroblasts obtained from healthy subjects were transiently

silenced for *tlr3*. *tlr3*-silencing results in a marked –but not complete- reduction of the expression of ICAM-1 and IL-6 induced by SSc-ICs, whereas IL-8 synthesis in response to IC incubation is not significantly attenuated. As expected, treatment with siRNA does not induce TLR3 activation: this synthetic sequence is 25 ribonucleotide long, whereas TLR3 activation requires a minimum length of 45 ribonucleotides[135].

These data suggest that TLR3 is involved in mediating IC effects on fibroblasts, but additional receptors might recognize scleroderma autoantibody/antigen complexes. The observation that pathologic ICs induce higher levels of IFN-α compared to IFN-β might imply the preferential involvement of TLRs recruiting MyD88 as adaptor protein. Research attention has been thus catalyzed by TLR7, TLR8 and TLR9 as candidate receptors. *tlr9*-silencing has first been planned, and experimental conditions for *tlr9*-silencing in fibroblasts have already been set.

When recruitment of intra-cellular mediators is analyzed in response to scleroderma ICs, a differential rate of activation is observed for each of the considered antibody specificities. Incubation with ARA and anti-Th/To ICs initiates a significant activation of NFκB, p38MAPK and SAPK-JNK, whereas ATA and ACA stimulation elicits a less significant effect. This is a rather interesting finding, since the differential pathogenic effects of scleroderma ICs might account for the characteristic clinical phenotypes associated with each autoantibody specificity.

The nucleic acids incorporated in scleroderma ICs might contain both DNA and RNA: pre-treating IC preparation with DNase (Worthington) and RNase (Worthington), an experiment that will be shortly performed, will allow to better characterize the nucleic acid composition of different ICs. Nucleic acids incorporated in SSc-ICs might be of endogenous as well exogenous nature. DNA and RNA are released from damaged and necrotic self-cells, and a defective clearance of apoptotic bodies has been reported in many systemic autoimmune conditions. Consistently, the gene coding for *DNASE1L3*, an enzyme involved in DNA fragmentation during apoptosis, has been identified as one of the strongest susceptibility loci for SSc[52]. Nucleic acids from pathogens might also be incorporated in ICs; interestingly, EBV has been shown to infect the majority of fibroblasts and

endothelial cells in the skin of SSc patients, with the expression of EBV noncoding small RNAs and the increased expression of immediate-early lytic and latency mRNAs and proteins[145].

Many aspects still remain to be clarified, and forthcoming experiments will be aimed at assessing the effects elicited by scleroderma ICs on additional pathogenic mediators in both healthy fibroblasts and HUVEC. ET-1 (Hs00174961_m1, Life Technologies), mTOR (Hs00234508_m1, Life Technologies), PPAR-γ (Hs01115513_m1, Life Technologies) and IRF3 (Hs01547283_m1, Life Technologies) mRNA levels will be tested by Real-Time PCR, CTGF (mouse monoclonal anti-human CTGF antibody, Abcam) and α-SMA (mouse monoclonal anti-human α-SMA antibody, Abcam) will be evaluated by Western Blotting, MCP-1 and MMP (MMP-2 and MMP-9) secretion in supernatants will be detected by a commercial ELISA kits (R&D Systems) (**Figure 20**).

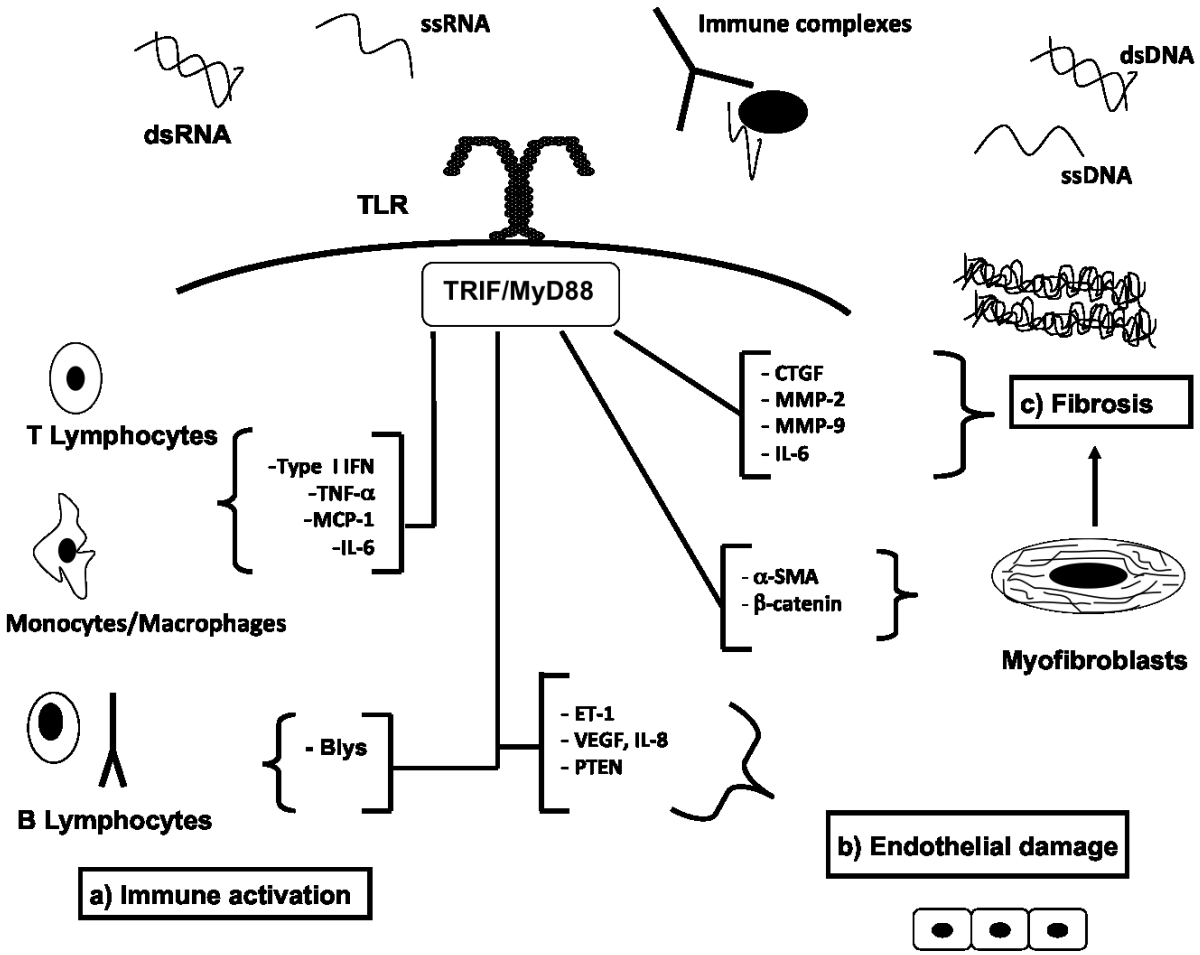


Figure 20. Mediators involved in scleroderma pathogenesis whose levels in response to SSc-ICs will be evaluated in future experiments (Original figure).

TLR: Toll-like Receptor; ss: single stranded; ds: double stranded; CTGF: connective tissue growth factor; MMP: matrix metalloproteinase; IL: interleukin; α-SMA: α-smooth muscle actin; ET-1: endothelin-1; VEGF: vascular endothelial growth factor; PTEN: tumour suppressor, phosphatase and tensin homolog; IFN: interferon; TNF-α: tumour necrosis factor –α; MCP-1: monocyte chemotactic protein 1; Blys: B lymphocyte stimulator.

Inhibitors of several intra-cellular downstream mediators will be used to further characterize the signaling cascade engaged by scleroderma ICs: LY294002 (PI3K inhibitor, Cell Signaling Technology), MG132 (NFκB inhibitor, Life Technologies), SB202190 (p38MAPK inhibitor, Cell Signaling Technology) and *mTOR* siRNA (s602, s 603 and s604, Life Technologies). To better

characterize the cellular localization of TLRs engaged by scleroderma ICs, treatment with bafilomycin A1, an endosome acidificator (Millipore, Temecula, CA, USA), will be employed. In addition, it would be interesting to investigate the effects elicited by SSc-specific autoantibodies in other effector cells involved in the pathogenesis of the disease, such as endothelial cells, T and B cells.

The burden of data presented in this study allows to better understand the role of scleroderma ICs. Surely SSc autoantibodies should not be advocated as the only player in scleroderma etiopathogenesis; however, they could provide an additional factor contributing to the interplay between immunity, vascular damage and excessive fibroblast activation culminating in the tissue fibrosis characteristic of scleroderma. The production of autoantibodies might be favoured by environmental factors together with a predisposing genetic milieu, as documented by the strong association with specific HLA assets and polymorphisms in genes coding for TLRs and down-stream mediators. The emerging pathogenic role of scleroderma-specific autoantibodies could affect every day clinical practice, strongly highlighting the pivotal importance of an early diagnosis. Indeed, it might be envisaged that instituting treatment very early on disease course might potentially prevent disease evolution. In addition, a further characterization of the pathogenicity of scleroderma-specific autoantibodies and down-stream mediators might allow identifying potential pharmacological targets: the development of novel agents would be much needed, given the current lack of effective treatment.

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