KEY PLAYERS IN SYSTEMIC SCLEROSIS: THE IMMUNE SYSTEM AND BEYOND

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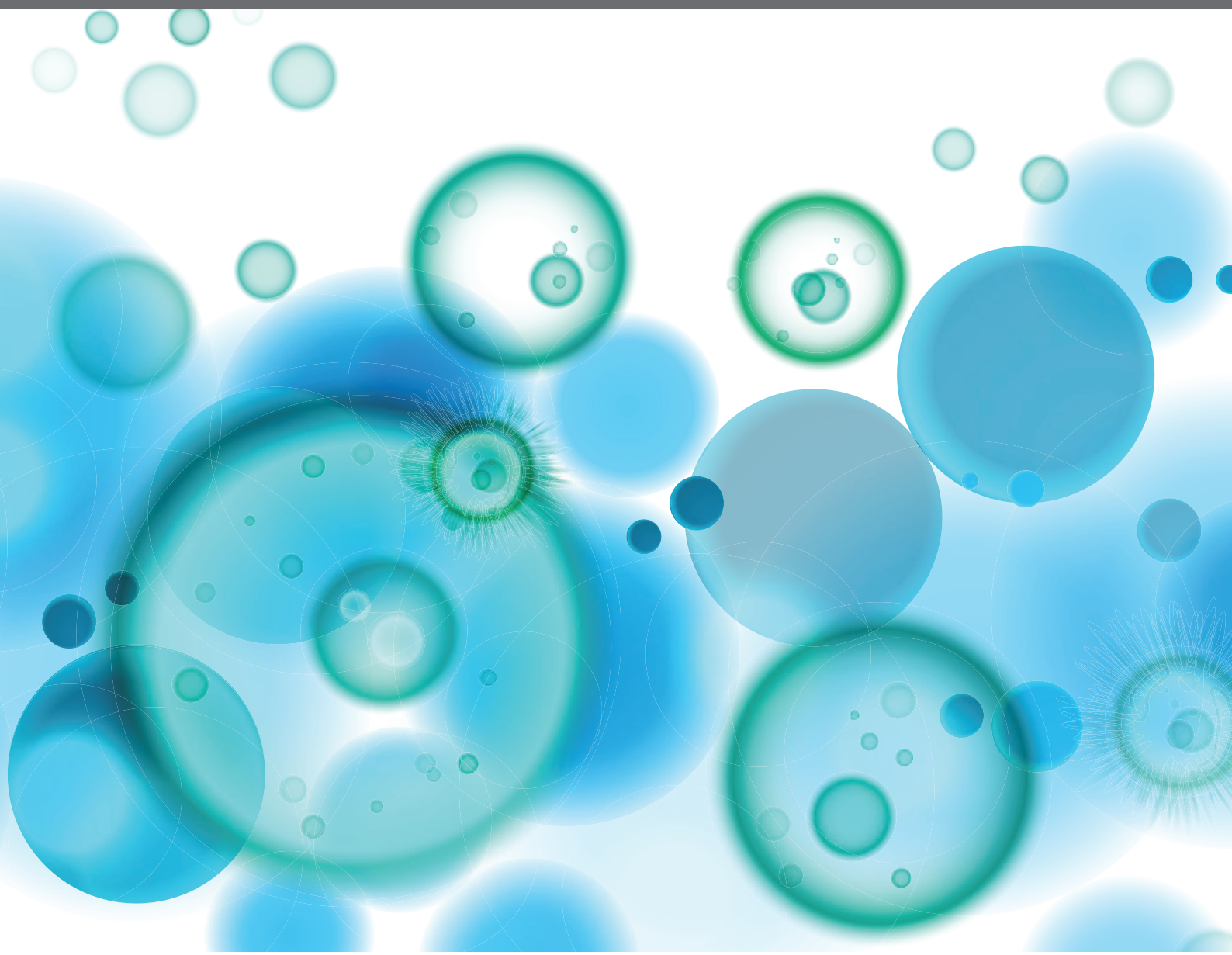

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KEY PLAYERS IN SYSTEMIC SCLEROSIS: THE IMMUNE SYSTEM AND BEYOND

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EDITORIAL
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Editorial: Key Players in Systemic Sclerosis: The Immune System and Beyond

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Editorial on the Research Topic

Key Players in Systemic Sclerosis: The Immune System and Beyond

In these times of Covid-19 pandemic, during which medical research is mobilized in the fight against the coronavirus, patients continue to suffer from other acute and chronic diseases and research on them must continue. Systemic sclerosis (SSc) is one of these diseases, both chronic and complex, whose evolution can be fatal and whose clinical repercussions, variable from one patient to another, can result in a profound alteration of autonomy and quality of life. The current crisis of the coronavirus should not make us forget this reality.

At this point, we must recall the well-known generalities about this disease (1). Systemic Sclerosis is a rare multifaceted disease, whose development is related to the interplay of three major pathophysiological mechanisms, *i.e.* vascular impairment, autoimmunity and uncontrolled fibrogenesis. These mechanisms lead to the development of the main disease-related manifestations including skin, lung, heart, and gastrointestinal tract involvement. Fibrosis primarily affects the skin dermis. However, the lungs, heart, and/or digestive tract may also be involved. Vascular impairment leads to the development of Raynaud's phenomenon, telangiectasia, digital ulcers, pulmonary arterial hypertension and/or vascular renal crisis. These features render SSc a very peculiar autoimmune disorder. Autoimmunity in SSc is mediated *via* the immune cell activation and production of autoantibodies (Abs). The latter are mainly directed against three target autoantigens, namely Topoisomerase 1 (anti-SCL70 Abs), CENP-A/B (anti-centromere Abs) or RNA-polymerase III. Sustained immune and inflammatory stimulation promote pathological fibroblast activation, which results in excessive extracellular matrix protein production and accumulation in tissues.

The clinical presentation of the disease is heterogenous. SSc may present in three classical forms, including limited cutaneous, diffuse cutaneous, and 'sine scleroderma' subsets, and may associate with other connective tissue diseases. SSc has a poor prognosis, globally with a severe vital prognosis and potential long-term disability. Its treatment is mainly palliative and based on symptomatic therapies, vasodilators, immunosuppressants, targeted therapies and antifibrotic drugs. Therefore,

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advances in our understanding of the crucial actors and mechanisms that underlie this disease are essential for improving therapy (hopefully, through the development of innovative therapeutic approaches) and patient outcomes.

In fact, a myriad of questions regarding SSc pathogenesis still remain unresolved. By exploring the most recent advances and discoveries in this field, this Research Topic aimed to transition our understanding of SSc pathogenesis from its old conception (based on the three main mechanisms, *i.e.* autoimmunity, fibrogenesis and vascular involvement) to a modern view.

When we proposed this topic to Frontiers in Immunology (https://www.frontiersin.org/research-topics/7231/key-players-in-systemic-sclerosis-the-immune-system-and-beyond#overview), the most recent advances in SSc had included the following key points (2): (i) fibroblast activation and endothelial impairment are likely dependent on environmental, genetic and epigenetic factors, with a specific contribution of endogenous and/or exogenous oxidative stress; (ii) a link was recently established between cancer and some specific forms of SSc [with particular autoantibodies (i.e. anti-RNA polymerase III and related Abs)], illustrating that the immune response could be directed against malignant targets; (iii) metabolic pathways (such as those implicating PPAR-γ) were also likely important for SSc development and could represent innovative therapeutic targets; (iv) while autologous hematopoietic stem cell transplantation following intensive immunosuppressant therapy had convinced more and more SSc specialists of its interest, progenitor cell-based therapies [such those using mesenchymal stromal cells (MSC)] were under development with great promises; (v) additionally, several pharmacological approaches were also under development to counteract fibrosis and vascular impairment in SSc.

The present Research Topic is entitled "Key players in Systemic Sclerosis - The immune system and beyond…", since key players in SSc are not limited to the immune system. This is one of the most original specificities of the disease. So, in the present Research Topic, we proposed 20 articles (including 10 review articles and 10 original articles) in the field of SSc, focusing on various cellular, molecular and environmental actors involved in SSc, in relationship with the immune system, and on innovative therapies for SSc. So, you can read in the present book, two original articles on Abs [anti-Ro (Gkoutzourelas et al.), and antiphospholipid antibodies (Sobanski et al.)], illustrating the variety of Ab repertoire in SSc, and discussing the capacity of SSc for overlapping. Herein, you can also find articles on the role of immune system and its interaction with other key players (such as fibroblasts and endothelial cells): the role of the innate immune system (Bhattacharyya et al.; Laurent et al.), the costimulatory pathways (Boleto et al.), the Regulatory T Cells (Frantz et al.), the microchimerism (Di Cristofaro et al.), the relationships between inflammatory cytokines and fibroblasts (Dufour et al.), the induction of endothelial microparticles release by natural killer cells (Benyamine et al.). Otherwise, other key players are presented: the central role of myofibroblasts is extensively reviewed (van Caam et al.), while a novel resistance mechanism to apoptosis is described in fibroblasts and vascular smooth muscle cells (Takata et al.); the deleterious effects of oxidative stress is discussed considering endothelial-to-mesenchymal transition (Thuan et al.), whereas the beneficial role of Nrf2-antioxidant response is illustrated in the murine model of HOCl-induced scleroderma (Kavian et al.; Kavian et al.). The intriguing relationships between SSc and cancer are also presented in a review article (Maria et al.), underlining the similarities between fibrosis and cancer development as well as the most recent data on paraneoplastic forms of SSc. The phenomenon of vascular leaking is also presented as a key player in the early phase of SSc (Bruni et al.), and the stages of fibrosis progression are now documented in the murine HOCl-induced model (Maria et al.). Finally, therapeutic approaches based on autologous hematopoietic stem cell transplantation (Del Papa et al.) or MSC (Maria et al.; Peltzer et al.; Maria et al.; Rozier et al.) are also presented in this book. We thank all the authors for their contribution.

Three years and one pandemic later, the content of this book remains fully relevant and continues to shed light on new ways of understanding and treating SSc. We would now like to highlight some of the data and concepts described in this book, and discuss them in light of other recent studies.

We encourage the reading of the review article on the innate immune system (Laurent et al.). While macrophages and type I interferon have been considered for many years as key players in SSc, several other components of the innate immune system (such as pathogen-recognition receptors, platelet-derived danger-associated molecular patterns, innate lymphoid cells, and plasmacytoid dendritic cells) exhibit now an emerging role. Research on the subject is ongoing and promising. A recent study (3) demonstrates the role of innate lymphoid cells-2 (ILC2) both in human and murine SSc, and more precisely the interplay between these cells and cytokines (namely, TGFβ and IL10) in the development of fibrosis. Interestingly, TGFβ could induce the switch of ILC2 from an 'inflammatory' phenotype ($KLRG1^{high}$) to a 'natural' phenotype ($KLRG1^{low}$), which exhibits a more potent capacity for fibrogenesis. Even more interesting, pharmacological TGFβ blockade with the anti-fibrotic agent pirfenidone did not significantly reduce the fibrotic process, while a combined treatment with pirfenidone and IL10 did. This therapeutic response was associated with a reduced number of skin infiltrating ILC2 and an enhanced expression of KLRG1. These findings illustrate the complexity of the disease as well as the variety of interactions between key players, both at the molecular and cellular levels, and suggest that combined therapies may be the future.

T-cell costimulation pathways (such as CD28/CTLA-4, ICOS-B7RP1, CD70-CD27, CD40-CD154, or OX40-OX40L) may be key players in the pathogenesis of SSc, as suggested by several experimental findings (Boleto et al.). Notably, the blockade of T-cell costimulation with abatacept (CTLA-4-Ig) improved the various manifestations of SSc in pre-clinical animal models (4, 5) and these promising results were corroborated by some preliminary clinical reports (6, 7). Interestingly, abatacept prevented the development of inflammation-driven fibrosis, but demonstrated no efficacy in the treatment of late and non-inflammatory dermal fibrosis (4). Unfortunately, a recent trial (ASSET trial, NCT02161406) did not confirm the promises

ofabatacept for patients with early diffuse SSc, considering the modified Rodnan skin thickness score (mRSS) at 12 months as primary endpoint (8). Nevertheless, according to this trial, abatacept remains promising when considering some secondary endpoints, *i.e.* the "Health Assessment Questionnaire disability index" (HAQ) and the composite score called "American College of Rheumatology Combined Response Index in diffuse cutaneous Systemic Sclerosis (ACR CRISS)". These two scores reflect respectively the disability induced by SSc and the probability of improvement in response to treatment in patients with early diffuse cutaneous SSc. Undoubtedly, all these findings illustrate also the complexity and heterogeneity of the disease, as well as the difficulty to monitor SSc and to determine the different components of the disease for a given patient (inflammatory versus dry fibrosis, for example). It also reminds us that encouraging results obtained in animals do not presume a success in humans. So, these -almost- negative results with abatacept suggest that CD28 pathway inhibition alone is insufficient to significantly impact skin disease in patients with early diffuse SSc, and suggest the co-targeting of CD28 with another costimulatory molecule to gain efficacy. For instance, the development of a dual CD28/ICOS antagonist (ALPN-101) may be of interest, since the blockade of both CD28 and ICOS in an acute graft versus host mouse model led to improved survival in ALPN-101-treated mice compared to mice receiving a CD28-CD80/CD86 pathway antagonist (belatacept; CTLA-4-Ig) only (9).

This book also includes a short collection of articles on cell-based therapies in SSc (Maria et al.; Peltzer et al.; Maria et al.; Rozier et al.), and we would like to shortly discuss the progress of knowledge in this field, more precisely on MSC-based therapies. The questions concerning the source and origin of MSC are still under debate, in particular the use of an autologous versus allogeneic approach (23). Concerning MSC and SSc, 12 studies are registered today on the site "ClinicalTrials.gov", with several distinct methodological approaches. So, we can expect to get answers to many questions in the months and years to come. Another perspective in the field of MSC is represented by their extracellular vesicles (EVs), that function as mediators of intercellular communication and natural transporters of bioactive molecules (proteins, RNA, DNA) to their microenvironment or systemically. Actually, MSC-derived EVs exhibit the main functions of MSC, and thus could constitute an attractive alternative to MSC, *i.e.* not suffering from the potential issues associated with expanded cells. In addition, EVs possess the advantage to be safer (anuclear) and could be stored at room temperature as lyophilized product (10). Recently, our group demonstrated the beneficial effects of EVs obtained from adipose tissue-derived MSC (ASC), both *in vivo* in the HOCl-induced SSc model (11) and *in vitro* in a TGFβ1-induced model of human myofibroblasts, mimicking the characteristics of fibroblasts isolated from SSc patients (12). We also demonstrated that miR-29a-3p (contained in the EVs) plays a pivotal role for the therapeutic effects of ASC, probably by targeting fibrotic, remodeling, apoptotic, and epigenetic processes (11). These findings make even more sense when considering the different key players and mechanisms described in this book. Finally, in parallel with the development of pharmacological molecules, research on cell therapies is also progressing, with interesting therapeutic prospects using the cells or their derived vesicles.

SSc is definitely a complex disease with many key players, which for a long time escaped our understanding and, which we know better, day by day. The challenge of SSc remains still very important, both pathophysiologically and therapeutically. We hope you enjoy reading this book. We hope that it will open up new perspectives for all SSc specialists, and also for all others in their own fields of research.

AUTHOR CONTRIBUTIONS

All three authors contributed to the editing of the Research Topic, writing and reviewing of the present Editorial article. All authors contributed to the article and approved the submitted version.

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Natural Killer Cells Exhibit a Peculiar Phenotypic Profile in Systemic Sclerosis and Are Potent Inducers of Endothelial Microparticles Release

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The pathophysiology of systemic sclerosis (SSc) involves early endothelial and immune activation, both preceding the onset of fibrosis. We previously identified soluble fractalkine and circulating endothelial microparticles (EMPs) as biomarkers of endothelial inflammatory activation in SSc. Fractalkine plays a dual role as a membrane-bound adhesion molecule expressed in inflamed endothelial cells (ECs) and as a chemokine involved in the recruitment, transmigration, and cytotoxic activation of immune cells that express CX3CR1, the receptor of fractalkine, namely CD8 and γδ T cells and natural killer (NK) cells. We aimed to quantify circulating cytotoxic immune cells and their expression of CX3CR1. We further investigated the expression profile of NK cells chemokine receptors and activation markers and the potential of NK cells to induce EC activation in SSc. We performed a monocentric study (NCT 02636127) enrolling 15 SSc patients [15 females, median age of 55 years (39–63), 11 limited cutaneous form and 4 diffuse] and 15 healthy controls. Serum fractalkine levels were significantly increased in SSc patients. Circulating CD8 T cells numbers were decreased in SSc patients with no difference in their CX3CR1 expression. Circulating γδ T cells and NK cells numbers were preserved. CX3CR1 expression in CD8 and γδ T cells did not differ between SSc patients and controls. The percentage and level of CX3CR1 expression in NK cells were significantly lowered in SSc patients. Percentages of CXCR4, NKG2D, CD69-expressing NK cells, and their expression levels were decreased in NK cells. Conversely, CD16 level expression and percentages of $CD16^+$ NK cells were preserved. The exposure of human microvascular dermic EC line (HMVEC-d) to peripheral blood mononuclear cells resulted in similar NK cells degranulation activity in SSc patients and controls. We further showed that NK cells purified from the blood of SSc patients induced enhanced release of EMPs than NK cells from controls. This study evidenced a peculiar NK cells phenotype in SSc characterized by decreased chemokine and activation receptors expression, that might reflect NK cells involvement

in the pathogenic process. It also highlighted the role of NK cells as a potent mechanism inducing endothelial activation through enhanced EMPs release.

Keywords: natural killer cells, endothelial microparticles, fractalkine, CX3CR1, systemic sclerosis

INTRODUCTION

Systemic sclerosis (SSc) is a complex systemic autoimmune disease characterized by small vessel vasculopathy and multi-organ fibrosis with a lung involvement, highly related to morbidity and mortality (1). The damage of endothelial cells (ECs) is believed to be an early event in the natural history of SSc and may involve environmental, genetic factors, and excessive innate immune responses (2). The disruption of vascular integrity and the subsequent acquisition of an activated endothelial phenotype favor the local recruitment of activated leukocytes and sustain the development of the vasculopathy and tissue fibrosis. Activated immune cells also release soluble inflammatory cytokines and pro-fibrotic growth factors that further activate SSc fibroblasts. The cross talk between activated ECs, fibroblasts, and immune effectors is a major mechanism underlying the pathogenesis and clinical progression of the disease (3–6). Therefore, a better understanding of the quantitative and functional alterations of immune cells is needed in order to shed light on the still unclear pathogenic processes.

Natural killer (NK) cells and γδ T lymphocytes are at the boundary between innate and adaptive immunity (7–9). They are key cytotoxic effectors of the anti-infectious and anti-tumor defenses. Both NK cells and γδ T cells comprise various subsets. Among NK cells, $CD56^{dim}$ cells are the main cytotoxic circulating subset whereas $CD56^{bright}$ NK cells are rather proinflammatory and tissue-trafficking cells (10, 11). Regarding γδ T cells, Vδ2 T cells is the main circulating subset whereas Vδ1 subset is mainly intra-epithelial. NK cells and γδ T cells share expression of non-rearranged activating receptors such as DNAM-1 and NKG2D that sense ligands overexpressed by stressed cells. The antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism can be triggered in NK cells and γδ T cells, after engagement of the CD16 receptor (FcγRIIIa) by the Fc fragment of immunoglobulins. These cells also share non-MHC restricted cytotoxic activity that involve the perforin/granzyme pathway, Fas/Fas ligands interactions, ADCC, and the release of inflammatory cytokines.

Natural killer cells subsets are further defined by their expression of chemokine receptors which influences NK cell migration toward organs. Chemokine receptors are modulated during NK cell activation and contribute to NK cell homeostasis (12). Among them, NK cells express CXCR3 which can bind various proinflammatory chemokines including CXCL4, a biomarker of SSc (13). They also express CXCR4 that specifically binds to stromal-derived-factor 1 SDF-1 (CXCL12) (14, 15). NK cells and to a lesser extent γδ T cells and CD8 T cells express the receptor for its sole ligand fractalkine (CX3CL1) (16). Fractalkine is both a soluble chemokine released after metalloproteases cleavage and an endothelial membrane-bound adhesion molecule. Hence, CX3CR1 is both a receptor that enables the migration of NK cells in response to fractalkine chemokine and an adhesion receptor that can mediate the activation and transmigration of NK cells when binding endothelial membrane-bound fractalkine (16). Interestingly, the endothelial expression and secretion of CX3CL1/fractalkine have been identified as major triggers favoring the recruitment of mononuclear cells expressing CX3CR1 in the affected skin and lung tissue of patients with SSc (17). Variations in the gene encoding the CX3CR1 have been identified as individual susceptibility factors associated with SSc and SSc-pulmonary arterial hypertension (PAH) (18). Furthermore, the disruption of the interaction between fractalkine and CX3CR1 has been shown to dampen the fibrosis process in a murine model of cytokine-induced SSc (19). The identification of the fractalkine/CX3CR1 pathway in SSc pathogenesis thus offers new perspectives for targeted therapy that could limit the inflammatory fibrosis and immune-mediated vascular injury (20).

The release of soluble factors (21–26) and endothelial microparticles (EMPs) (21, 27, 28) have been shown to reflect endothelial injury results (29). We recently identified increased circulating levels of fractalkine and EMPs as a signature of endothelial inflammatory activation and disrupted homeostasis in SSc patients with potential value as a biomarker of organ involvement and disease severity (21). EMPs are also considered as "miniature cells" loaded with bioactive molecules (RNA, proteins, cytokines, and lipids) with a key role in the regulation of intercellular cross talks that sustain inflammation, hemostasis, angiogenesis, and fibrosis (30–32). These EMPs have been shown to be associated with skin and lung fibrosis (27) and to be deleterious *via* the induction of an oxidative burst in a murine model of SSc (33). However, the mechanisms that drive this EMPs release remain poorly understood. The recruitment and activation of NK cells toward target vessel wall and NK cells-mediated microvascular injury were suggested in the pathogenesis in autoimmune vasculitis (34). Interestingly, we recently identified that NK cells are major providers of inflammatory cytokines and endotheliotoxic effects associated with antibody-mediated vasculopathy (35) and impairment of endothelial progenitor cell regenerative functions (36).

Our study thus aimed to investigate the features of NK cells and their potential role as cytotoxic effectors of EC activation and damage in SSc.

MATERIALS AND METHODS

Patients

We performed a monocentric study (NCT 02636127). Fifteen patients with SSc were recruited in the Department of Internal Medicine of Marseilles. All the enrolled patients had a score ≥9 according to the 2013 EULAR/ACR 2013 criteria for SSc (37). The patients were not treated with immunosuppressive drugs except for low-dose steroids under 10 mg/day. Among patients,

there were 15 women with a median age of 55 years (39–63 years) (**Table 1**). Age-matched healthy volunteers ($n = 15$) were recruited as controls. The control group consisted of 15 women with a median age of 54 years (40–61). This study was carried out in accordance with the recommendations of French directives regarding Biomedical Research and the local ethics committee review board of Marseilles, "Comité de Protection des Personnes Sud Méditerranée." The protocol was approved by the "Comité de Protection des Personnes Sud Méditerranée." All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Clinical and Standard Biological Assessment

All patients had a physical examination and underwent a blood sample. Clinical characteristics of the study population are summarized in **Table 1**.

Systemic sclerosis patients were classified as having limited cutaneous SSc or diffuse cutaneous SSc according to the criteria established by LeRoy et al. (38). The disease duration, the presence of Raynaud's phenomenon, pitting scars, digital ulcers, digital gangrene, or telangiectasias were recorded. The modified Rodnan Skin thickness score (mRSS) was graded on a scale of 0–3 with a maximum total score of 51. Disease severity was measured on a scale of 0–4 according to Medsger's severity scale (39).

Pulmonary involvement was determined by pulmonary function tests including forced vital capacity and diffusing lung capacity for carbon monoxide (DLCO) and DLCO divided by alveolar volume (DLCO/VA). Fibrosis was diagnosed on Chest Computed Tomography imaging with qualitative criteria consisting in the presence of honey combing, ground glass opacities, reticular abnormalities, traction bronchiectasis, and septa thickening. Systolic pulmonary arterial pressure was measured by transthoracic echocardiography and pulmonary hypertension was confirmed by right heart catheterization.

Antinuclear antibodies were assessed by Indirect Immunofluorescence analysis on HEp-2 cells. Anti-centromere and anti-topoisomerase I antibodies were measured by EliA.

TABLE 1 | Characteristics of the patients.

	SSc patients ($n = 15$)
Male/female	0/15
Age (years), median ± IQR	55 (39–63)
Cutaneous form: limited/diffuse	11/4
Anti-centromere/anti-topoisomerase I auto-antibodies	7/6
mRSS	8.5 (3–27)
Disease duration ≤3 years/>3 years	5/10
Medsger Severity Scale	
Grade 0–1–2 versus Grade 3–4	0–4–5 versus 2–4
Pitting scars	10
Digital ulcers	5
Telangiectasias	10
Pulmonary function tests	
– TLCO (%)	57 (45–71)
– TLCO/VA (%)	67.8 (56.1–76.5)
– FVC (%)	100.1 (68.38–111.8)
Lung fibrosis on HRCT scan	4
Pulmonary arterial fibrosis	3

Quantitative variables are described using median and IQR (first quartile–third quartile). mRSS, modified Rodnan sclerosis score; DLCO, diffusing capacity of the lung for carbon monoxide; FVC, forced vital capacity; VA, alveolar volume; HRCT, high resolution computed tomography; IQR, interquartile range; SSc, systemic sclerosis.

Cell Culture

Peripheral blood mononuclear cells (PBMCs) from SSc patients and healthy controls were isolated by density gradient centrifugation (Lymphocyte Separation Medium, Abcys, Eurobio).

Freshly isolated PBMCs were stained with monoclonal antibodies for flow cytometry analysis and the remaining PBMCs were frozen until they were used for functional tests.

Natural killer cells were isolated using StemSep Column-Based Cell Isolation kit (StemCell Technologies). The purity of NK cells assessed was determined by flow cytometry and average purity was 93.4%.

Human adult dermal microvascular endothelial cells (HMVEC-d) were obtained from Lonza (Passage 3). They were cultured with EC Growth Medium-MicroVascular (EGM2-MV) (Lonza) and used at Passage 5 for functional tests.

Flow Cytometry Analysis

2×10^5 PBMCs were washed in Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, Thermofisher) and incubated at 4°C for 20 min with specified mAb. Following incubation and washing, samples were analyzed on NAVIOS-3 lasers instrument (Beckman Coulter, Miami, FL, USA), using Kaluza software.

Cytotoxic cell subsets were gated among human PBMCs by flow cytometry using CD45 Krome Orange (Clone J33, Beckman Coulter), CD3 ECD (clone UCHT1, Beckman Coulter), CD56 PC7 (clone NKH-1, Beckman Coulter), CD8 Pacific Blue (clone B9.11, Beckman Coulter), Anti-TCR Pan γ/δ PC5 (clone IMMU510, Beckman Coulter), Anti-TCR Pan α/β PC5 (clone BMA031), Anti-TCR Vδ2-Pacific Blue (clone IMMU389, Beckman Coulter), and Anti-TCR Vδ1 Fitc (clone TS8.2, Thermo Scientific).

The percentage of positive cells and their levels of expression for the following markers were studied with the following labeled antibodies: CX3CR1-PE (clone 2A9, Medical and Biological Laboratories), CD184 (CXCR4)-PE (clone 12G5, Beckman Coulter), CD183 (CXCR3)-Alexa Fluor 488 (clone #49801, R and D systems), CD314 (NKG2D)-PE (clone ON72, Beckman Coulter), CD226 (DNAM1)-Fitc (clone DX11, Becton Dickinson), CD69 PE (clone TP1.55.3, Beckman Coulter), CD16-APC-AlexaFluor750 (clone 3G8, Beckman Coulter), CD107a (LAMP-1)-Fitc (clone H4A3, Becton Dickinson), CD107b (LAMP-2)-Fitc (clone H4B4, Becton Dickinson), and their matched-control istotypes IgG2a PE, IgG1 PE, IgG1 Fitc, IgG1 APC AlexaFluor750.

CD107 Degranulation Assay

HMVEC-d were cultured overnight in 24 well-plates (2×10^5 cells/well) with Endothelial Basal Medium (EBM2, Lonza) supplemented with either 25% fetal bovine serum (FBS) (Gibco, Thermofisher) or 25% FBS plus IFNγ (Tebu-bio, 50 ng/ml) and TNFα (Euromedex, 20 ng/ml) or 25% healthy control serum or

25% SSc patient serum. All sera were heat inactivated prior to experiments.

Residual rabbit anti-thymocyte globulins (ATG) were obtained from the residual samples that could not be used in clinic. ATG (50 µg/ml) was added to HMVEC-d during 15 min at 37°C, then removed. Cells were washed with DPBS before the addition of PBMCs.

For analysis of CD107 expression, thawed PBMCs (5×10^5 cells/well) were added to HMVEC-d target cells after the overnight culture and incubated at 37°C in the presence of anti-CD107a/b antibodies. After 4 h, PBMCs were collected, washed in PBS and the level of CD107a/b expression was measured by flow cytometry among viable NK cells, defined as $CD45^+DAPI^-CD3^-CD56^+$ cells.

Enumeration of EMPs Release by Flow Cytometry

For EMPs release tests, HMVEC-d were cultured overnight in 96 well-plates (50,000 cells/well) with Endothelial Basal Medium (EBM2, Lonza) supplemented with either 25% FBS (Gibco, Thermofisher) or 25% FBS plus IFNγ (Tebu-bio, 50 ng/ml) and TNFα (Euromedex, 20 ng/ml) or 25% healthy control serum or 25% SSc patient serum. All sera were heat inactivated prior to experiments.

1×10^5 PBMCs or 2×10^5 purified NK cells of SSc patients or age-matched healthy controls were added to the overnight culture in the conditions containing their autologous serum or healthy control serum and conversely.

Supernatants were collected after the overnight culture and were subjected to two successive centrifugations (2,500 *g* for 15 min at room temperature) to remove dead cells and debris-like apoptotic bodies. Annexin V-Fitc and fluorescent antibody reagents were procured from Beckman Coulter (Villepinte, France): CD54 (ICAM-1) PE (clone 84H10), CD45 PC7 (clone J.33).

Endothelial microparticles were enumerated by high sensitivity flow cytometry following standardization as previously described (40, 41). 30 µl of supernatant was incubated with the appropriate amount of specific antibody plus 10 µl of Annexin V-Fitc. Each stained sample was analyzed on CytoFLEX cytometer (Beckman Coulter). Briefly, a standardized side scatter (SSC) microparticle gate was defined using megamix + forward scater (FSC) and SSC beads. Lower limit was defined in SSC just above 0.16 µm bead and upper limit integrated all 0.5 µm bead, still in SSC. This gate is equivalent to a 0.3–1 µm FSC gate, allowing a standardized analysis of small vesicles below 1 µm. Fluorescence gains of CytoFLEX were optimized using sphero 8 peaks beads. EMPs were defined as Annexin V^+/$ICAM1^+CD45^-$ events. The absolute EMP counts (events per µl) were determined using volume measure provided by the instrument (use of a peristaltic pump). The percentage of increase of EMPs was expressed in reference to the medium condition (EBM2 + 25% FBS).

Analysis of Soluble Fractalkine and IL-6 Levels

Circulating levels of sfractalkine (CX3CL1) were measured in serum using commercially available ELISA kits from R&D System Inc. (Minneapolis, MN, USA). IL-6 levels were measured in culture supernatants using Human Cytokine/Chemokine Magnetic Bead Panel (Milliplex, Millipore, MO, USA). Assays were performed according to the manufacturer's instructions.

Statistical Analysis

Results were expressed as median ± interquartile range (25th–75th percentile, IQR). Statistical analyses were performed using Spearman correlation, Wilcoxon test, and Mann–Whitney *U* test. p Values <0.05 were considered significant. Analyses were performed using GraphPad Prism program version 5.

RESULTS

Decreased CD8 T Cells but Conserved Numbers of NK Cells and γδ T Cells Circulating Cytotoxic Immune Cells in SSc Patients

We first aimed to determine whether absolute numbers of circulating cytotoxic immune cells, namely CD8 T cells, NK cells, or γδ T cells, were affected in SSc patients. We found that the median number and percentage of circulating CD8 T cells were significantly decreased in SSc patients [279/mm^3 (218–356) and 19% of leukocytes (13–21)] compared with healthy controls [473.5 (278–763) and 26% (18–33); $p = 0.0246$ and $p = 0.0142$] (**Figure 1A**). The numbers of circulating NK cells (**Figure 1B**), NK cells $CD56^{dim}$ (**Figure 1C**), γδ T cells (**Figure 1D**), and γδ T cells subpopulations, Vδ1 (**Figure 1E**) and Vδ2 T cells (**Figure 1F**), as well as their percentages (*data not shown*) were similar between SSc patients and healthy controls.

Increased Fractalkine Soluble Levels and Lower Expression of Fractalkine Receptor CX3CR1 Expression in NK Cells from SSc Patients

As we and others previously demonstrated soluble fractalkine to be a marker of endothelial inflammation and severity of SSc, we aimed to assess fractalkine levels in the studied population. Here, we found that patients with SSc had significantly higher median levels of fractalkine [855.2 pg/ml (683.1–1,077)] than healthy controls [539.3 pg/ml (457.6–731); $p = 0.0006$] (**Figure 2**).

Next, we assessed the surface expression of CX3CR1 namely fractalkine receptor, within circulating cytotoxic immune cell subsets of SSc patients and controls. We first analyzed the percentages of $CX3CR1^+$ cytotoxic immune T cell subsets. The proportion of $CX3CR1^+$ CD8 T cells (**Figure 3A**) and γδ T cell subsets (**Figure 3B**) including Vδ1 (**Figure 3C**) and Vδ2 T cells (**Figure 3D**) were comparable in both groups. The percentage of $CX3CR1^+$ NK cells was significantly lower in SSc patients [87.63% (74.54–98.28)] than in healthy controls [99.03% (96.95–99.21), $p = 0.0023$] (**Figure 4A**).

The level of CX3CR1 surface expression of circulating CD8 T cells assessed by flow cytometry with the median fluorescence intensity (MFI) did not differ between SSc patients and healthy

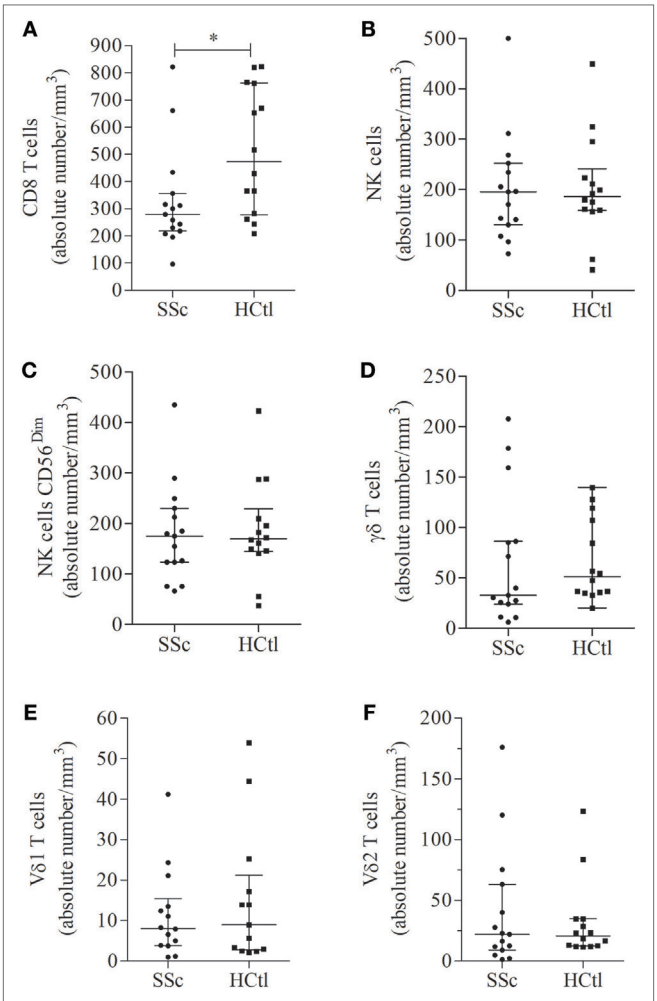

FIGURE 1 | Number of circulating cytotoxic immune cells in systemic sclerosis (SSc) patients. Percentages of circulating immune cells were assessed in the peripheral blood of SSc patients ($n = 15$) with flow cytometry and compared with healthy controls (HCtl) ($n = 14$). Absolute numbers of circulating cells including CD8 T cells **(A)**; natural killer (NK) cells **(B)**; NK cells $CD56^{dim}$ **(C)**; γδ T cells **(D)**; Vδ1 **(E)**; and Vδ2 T cells **(F)** were obtained based on the white blood lymphocyte count and expressed as number of cells per mm^3. Results were expressed as median ± interquartile range. Statistical difference was established using Mann–Whitney *U* test. $^*p < 0.05$.

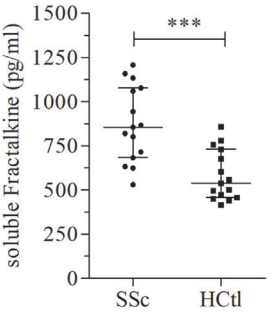

FIGURE 2 | Soluble fractalkine levels in systemic sclerosis (SSc) patients. Soluble fractalkine levels were quantified with ELISA, in the serum of SSc patients ($n = 15$) and healthy controls (HCtl) ($n = 15$). Results were expressed as median ± interquartile range and statistical significance was established using the non-parametric Mann–Whitney *U* test. $^{***}p < 0.001$.

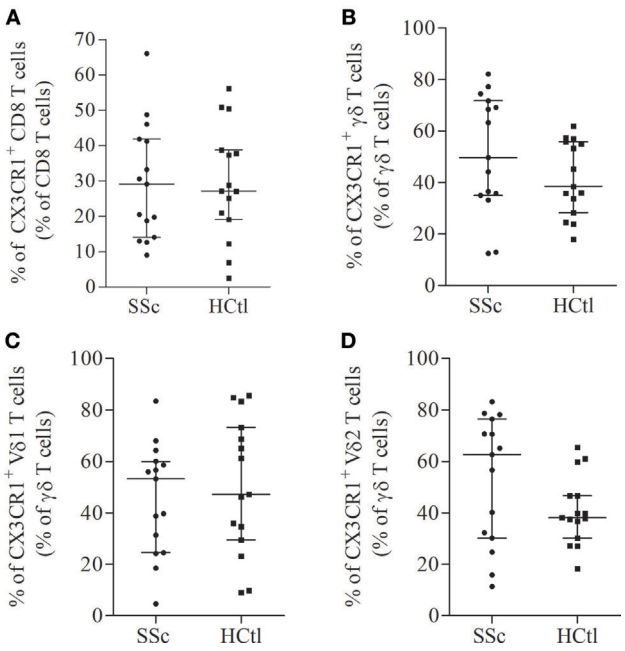

FIGURE 3 | CX3CR1 expression in circulating cytotoxic immune cells from systemic sclerosis (SSc) patients. Percentages of CX3CR1 expressing immune cells were evaluated with flow cytometry analysis on the surface of CD8 T cells **(A)**; γδ T cells **(B)**; Vδ1 **(C)**; and Vδ2 T cells **(D)** from SSc patients ($n = 15$) in comparison with healthy controls (HCtl) ($n = 15$). Results were expressed as median percentages and median ± interquartile range.

controls. CX3CR1 expression was significantly lower in SSc NK cells: [MFI of 4.01 (3.03–7.4)] compared to values observed in healthy controls, [MFI of 5 (4.46–6.13); $p = 0.0225$; **Figure 4B**]. No statistical difference was noted regarding CX3CR1 expression in γδ T cells, the Vδ1 and Vδ2 T cell subsets (*data not shown*).

While no correlation was noted between circulating fractalkine levels and percentages of CX3CR1 expressing NK cells, CD8 T cells, γδ T cells, and Vδ2 T cells, fractalkine levels were inversely correlated with the percentage of $CX3CR1^+$ Vδ1 T cells in SSc patients ($r_S = -0.55$; $p = 0.0337$).

Collectively, our data show in SSc patients a lower proportion of $CX3CR1^+$ NK cells subsets and a downregulation of CX3CR1 on NK cells.

Decreased Expression of CXCR4 Chemokine Receptor in NK Cells from SSc Patients

We sought to assess the expression of CXCR3 and CXCR4 expression in NK cells from SSc patients and healthy controls.

No statistical difference was noted regarding CXCR3 level of expression and percentages of $CXCR3^+$ NK cells between patients and controls (*data not shown*).

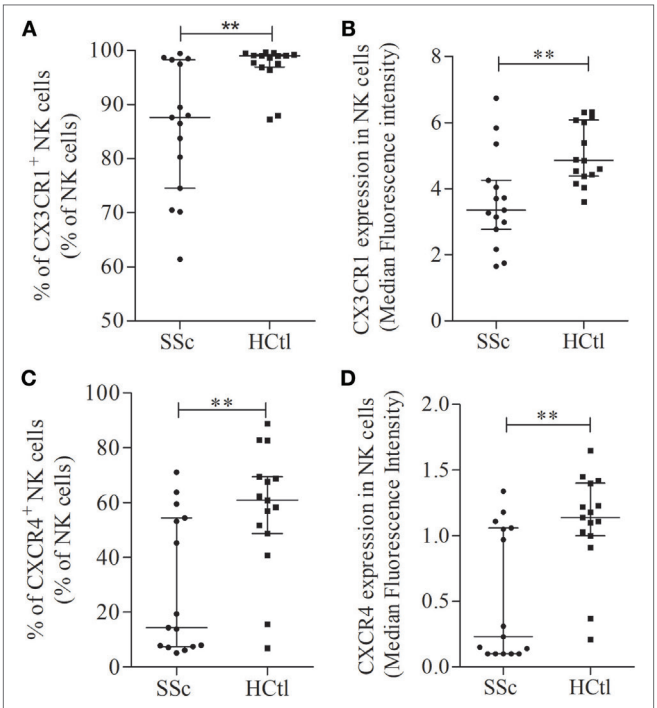

FIGURE 4 | Chemokine receptors expression in natural killer (NK) cells in systemic sclerosis (SSc) patients. CX3CR1 **(A,B)** and CXCR4 expression levels and percentages **(C,D)** were assessed with flow cytometry analysis in whole NK cell from SSc patients ($n = 15$) in comparison with healthy controls (HCtl) ($n = 15$). Results were expressed as percentages of positive cells among NK cells **(A,C)** and median fluorescence intensity **(B,D)**. Results were depicted as median ± interquartile range. Statistical difference was established using Mann–Whitney U test. $^{**}p < 0.005$.

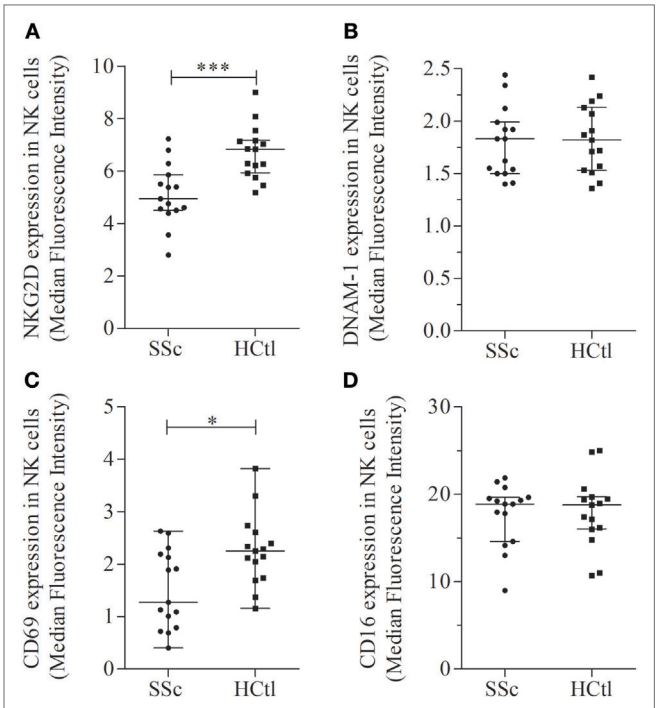

FIGURE 5 | Activation receptors and markers in natural killer (NK) cells of systemic sclerosis (SSc) patients. NKG2D **(A)**, DNAM-1 **(B)**, CD69 **(C)**, and CD16 **(D)** expression levels were evaluated with flow cytometry analysis of NK cells among peripheral blood mononuclear cells from SSc patients and healthy controls (HCtl). Results were expressed as median fluorescence intensity (MFI) and median ± interquartile range. Statistical difference was established using Mann–Whitney U test. $^{*}p < 0.05$; $^{***}p < 0.001$.

The percentage of CXCR4$^+$ NK cells [14.36% (7.49–54.45) in SSc versus 60.9% (48.79–69.44) in SSc; $p = 0.009$; **Figure 4C**], and CXCR4 expression level at the surface of NK cells [0.23 (0.1–1.1) in SSc versus 1.14 (1–1.4); $p = 0.0032$; **Figure 4D**] were significantly lower in patients than in controls.

Decreased NKG2D and CD69 Expression and Conserved DNAM1 Expression in NK Cells of SSc Patients

We next further characterized the phenotype of NK cells from SSc patients according to their expression of the NKG2D and DNAM1 activating receptors and of CD69 and CD16 stimulatory/activation receptors.

We observed that percentages of NKG2D$^+$, DNAM-1$^+$, CD16$^+$, CD69$^+$ NK cells were similar between patients and healthy controls (*data not shown*).

The level of NKG2D expression was significantly lower in NK cells from SSc patients in comparison with healthy controls [MFI of 5.15 (4.68–6.03) in SSc patients versus 6.92 (5.95–7.27) in healthy controls; $p = 0.0009$] (**Figure 5A**). The level of DNAM1 expression (**Figure 5B**) was not statistically different between patients and controls. However, DNAM1 expression was inversely correlated with TLCO in SSc patients ($r_S = 0.66$; $p = 0.0368$).

The level of CD69 expression was significantly decreased in SSc patients [1.27 (0.79–2.19)] compared with healthy controls [2.25 (1.74–2.61); $p = 0.0161$] (**Figure 5C**). The level of CD16 expression was similar between SSc patients and controls (**Figure 5D**).

Collectively, our data demonstrate a peculiar NK cell phenotype in SSc patients characterized by (i) a decreased expression of CX3CR1 and CXCR4 in NK cells (percentages and intensity of expression) and (ii) a lower expression level of the NKG2D activating receptor and of the CD69 activation marker.

Clinical Association Between Expression Percentages of CX3CR1$^+$ and CXCR4$^+$ NK Cells and Clinical Characteristics of the Patients

A high heterogeneity was observed among SSc patients regarding CX3CR1 (**Figures 4A,B**) and CXCR4 expression on NK cells (**Figures 4C,D**). Hence, we set up to assess whether clinical characteristics of SSc patients were different between patients with high or low CX3CR1 and CXCR4 expression. When patients were segregated in two groups defined by the median percentage of CX3CR1 expressing NK cells (87.63%), a higher frequency of pulmonary fibrosis identified by Chest High Resolution Computed Tomography ($p = 0.0289$) and anti-topoisomerase 1 antibodies

($p = 0.0483$) was observed in patients with the higher percentages of CX3CR1$^+$ NK cells.

Conversely, when patients were segregated using median percentage of expression of CXCR4 on NK cells (9.65%), the clinical characteristics of SSc could not be associated with percentages of CXCR4$^+$ NK cells.

Conserved Ability of NK Cells to Degranulate Toward Microvascular Endothelial Target

Natural killer cells from SSc patients were previously found to have either normal of decreased natural cytotoxic activity toward the erythroleukemic cell line K-562. However, few studies have investigated their ability to degranulate toward EC targets.

Here, we used a human dermal microvascular EC line (HMVEC-d) as a target to investigate the NK cell natural cytotoxicity. For this, we assessed NK cell ability to degranulate, after exposure of PBMCs from SSc patients and healthy controls to HMVEC-d targets, in the presence of sera from SSc patients or healthy controls. The levels of exocytosis of CD107a$^+$ and CD107b$^+$ cytotoxic granules in NK cells were analyzed through multiparametric flow cytometry analysis, within the CD3$^-$CD56$^+$ NK cell subset gated among CD45$^+$ PBMCs.

In addition, we evaluated the ADCC potential of NK cells from SSc patients and controls in response to thymoglobulin (ATG)-coated HMVEC-d targets.

The degranulation potential of NK cells from both patients and controls was significantly enhanced in response to ATG [% of CD107$^+$ SSc NK cells of 22 (18.56–23.82) versus 4.46 (3.3–5.78); $p = 0.0313$; % of CD107$^+$ healthy controls NK cells of 28.59 (22.23–33.81) versus 5.4 (4.8–6.59); $p = 0.0313$], whereas NK cells ADCC potentials were similar when using NK cells from patients or healthy controls ($p = 0.1563$) (**Figure 6A**).

Peripheral blood mononuclear cells from SSc patients were then cocultured with either autologous serum or with serum of healthy controls and conversely. We observed a trend for a lower ability of degranulation of NK cells from SSc compared with healthy donors in the presence of serum of SSc patients ($p = 0.0938$) (**Figure 6B**).

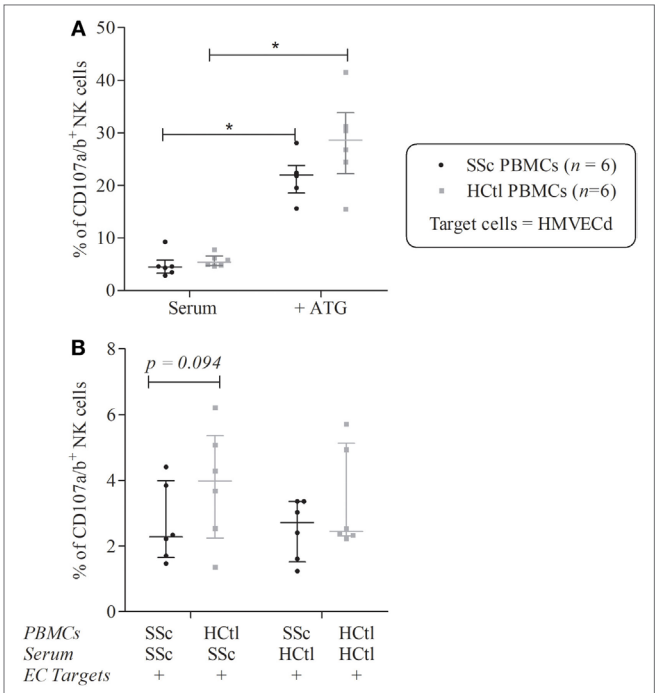

FIGURE 6 | Natural killer (NK) cells degranulation of systemic sclerosis (SSc) patients toward microvascular endothelial cell (EC) target. Cytolytic degranulation of NK cells was assessed by flow cytometry analysis of CD107a/b expression of NK cells among peripheral blood mononuclear cells (PBMCs) after a 4-h coculture with human microvascular dermal ECs (HMVEC-d) with an effector/target ratio of 2/1. **(A)** PBMCs from patients with SSc ($n = 6$) and healthy controls (HCtl) ($n = 6$) were cultured with HMVEC-d with or without thymoglobulin (ATG). **(B)** PBMCs from SSc patients ($n = 6$) and HCtl ($n = 6$) were cultured with HMVEC-d and either SSc serum or HCtl serum and conversely. Cumulative data from five independent experiments. Results were expressed as median percentages ± interquartile range. Statistical significance was established using the non-parametric paired Wilcoxon *U*-test. *$p < 0.05$.

Increased EMPs Release Induced by NK Cells from SSc Patients

As NK cells from SSc patients exhibited a specific phenotype, we aimed to assess the functional consequence of such phenotype upon microvascular ECs activation. In order to evaluate endothelial activation and injury, we monitored the EMPs release from HMVEC-d. EMPs were gated as described above and the AnnexinV$^+$, CD45$^-$, ICAM1$^+$ EMPs were quantified.

As expected, the overnight culture of HMVEC-d with IFNγ and TNFα significantly boosted their release of EMPs [671.7 (436.2–839.9) versus 141.6 (90.5–199); $p = 0.002$] (**Figure 7A**).

Exposure of HMVEC-d to serum of SSc patients resulted in higher endothelial EMPs release than when the same cells were exposed with FBS [196.1 (130.4–233) versus 141.6 (90.5–199); $p = 0.0273$] but no significant difference was noted when comparing the effect of serum from healthy controls or SSc patients (**Figure 7A**).

The addition of PBMCs either obtained from healthy controls or SSc patients increased HMVEC-d-derived EMPs release when compared to the sole addition of autologous sera [281.1 (188–418.7) versus 152.1 (95.94–250.2); $p = 0.0195$ for healthy controls; 208.4 (175.5–526.7) versus 196.1 (130.4–233); $p = 0.0371$ for SSc patients] (**Figure 7A**). However, no statistical difference was noted between the induction of EMPs by PBMCs from SSc patients and healthy donors (**Figure 7A**).

We further assessed the specific effect of purified peripheral NK cells isolated from SSc patients compared with healthy controls. The coculture of HMVEC-d with purified NK cells from SSc patients in the presence of autologous SSc serum was associated with more EMPs release than NK cells from healthy controls with their autologous serum [371.9 (203.8–567.3) versus 239.7 (176.2–390.2); $p = 0.0488$] (**Figure 7B**).

The coculture of HMVEC-d with NK cells from SSc and control serum trended to result in more EMP release than NK cells from healthy controls with autologous serum [337.7 (249.7–518.6) versus 239.7 (176.2–390.2); $p = 0.0742$]. However, the addition of SSc serum to NK cells from healthy controls did not modify

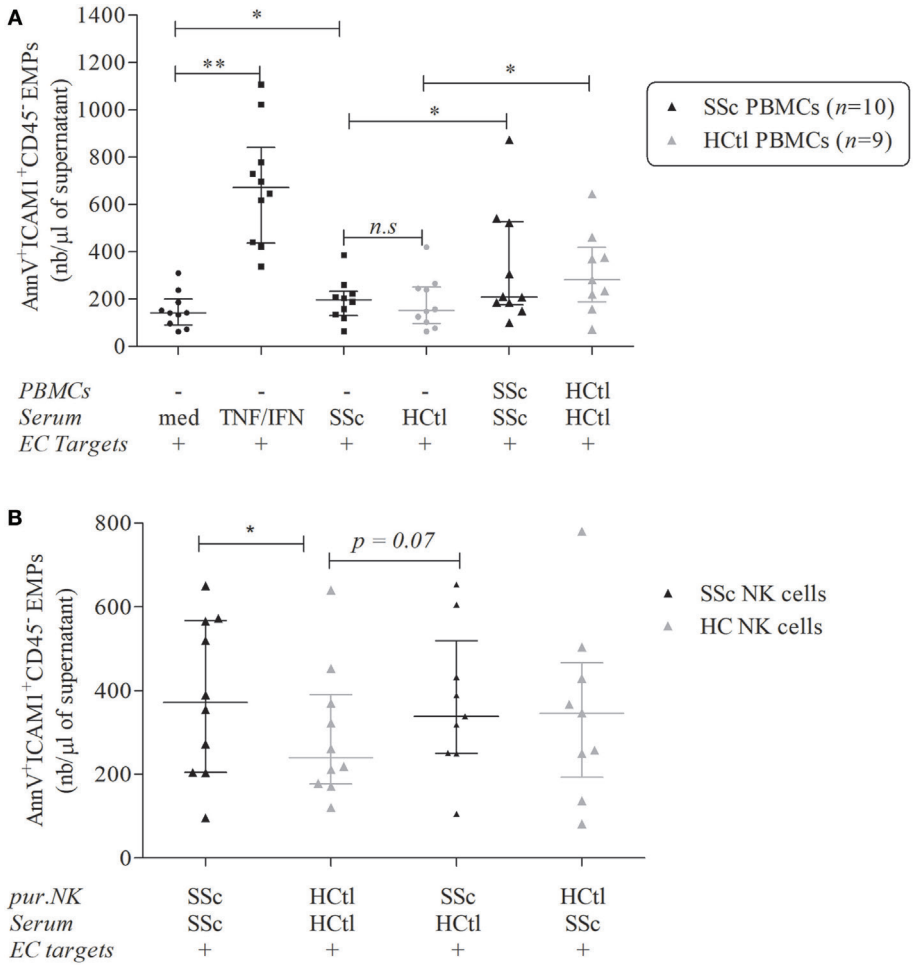

FIGURE 7 | Endothelial microparticles (EMPs) release from microvascular endothelial cells (ECs) induced by natural killer (NK) cells from systemic sclerosis (SSc) patients. EMPs were obtained from the supernatant of overnight-cultured human microvascular dermal EC (HMVEC-d) line. **(A)** HMVEC-d were used as ECs targets and cultured with medium (med), i.e., EBM2 + 25% FCS ± IFNγ (50 ng/ml)/TNFα (20 ng/ml) or serum of SSc patients or healthy controls (HCtl) ± their autologous peripheral blood mononuclear cells (PBMCs) (n = 10 and n = 9, respectively) with a PBMCs/HMVEC-d target ratio of 1/1. **(B)** HMVEC-d were cultured with EBM2 medium with serum from SSc patients or healthy controls and purified NK cells (pur. NK) from SSc patients (n = 10) or healthy controls (n = 9–10) with a NK cells/HMVEC-d ratio of 2/1. Results were expressed as median number of EMPs per microliter ± interquartile range. Cumulative data from 10 independent experiments. Statistical significance was established using the non-parametric paired Wilcoxon U-test. *$p < 0.05$, **$p < 0.005$.

the HMVEC-d EMPs release. These findings suggested that the observed enhancement of EMPs release might be mediated by NK cells from SSc patients (**Figure 7B**). In this line, we observed that IL-6 levels in the supernatants of coculture NK cells from SSc patients and healthy controls were positively correlated with EMPs release ($r_S = 0.56$; $p = 0.0218$).

In addition, we observed a trend to a negative correlation between the percentage of expression of CX3CR1 on NK cells and the enhancement of EMPs releasing triggered by NK cells from SSc patients and their autologous serum ($r_S = -0.6$; $p = 0.0968$).

DISCUSSION

Our study evidenced quantitative and phenotypic features that characterize cytotoxic immune cells in SSc with a special focus on NK cells. Circulating CD8 T cells were decreased while circulating γδ T cells and NK cells numbers were preserved in SSc patients. Circulating NK cells from SSc patients exhibited decreased expression of CX3CR1 (fractalkine receptor), and CXCR4 (SDF-1 receptor) chemokine receptors, as well as of the NKG2D activating receptor and CD69 activation marker. DNAM-1 expression was similar in SSc patients and controls but inversely correlated with lung involvement assessed by DLCO in patients. CD16 expression was maintained in SSc patients analyzed in reference with controls. NK cells from SSc patients displayed preserved antibody-dependent degranulation ability compared with healthy controls and a trend to a decreased potential to exert natural cytotoxicity in the presence of their autologous serum. Interestingly, NK cells purified from SSc patients induced higher endothelial activation through microvascular microparticles release than healthy controls.

Our observation of decreased circulating CD8 T cells in SSc patients was consistent with previous reports (42–44). Other studies have also shown an increase of CD8 T cells in the bronchoalveolar fluid of SSc patients with interstitial lung disease (ILD) (45) which may suggest their recruitment to the lung where they would exert their cytotoxicity.

In the present study, SSc patients exhibited preserved numbers and percentages of NK cells and γδ T cells in their peripheral blood. Few studies have assessed NK cells and γδ T cells circulating compartments in SSc patients and reported discrepant results. Some studies found normal (43, 46), or decreased NK cells (47) and normal (48, 49) or decreased γδ T cells counts (50–52). These apparently conflicting results might partly result from differences in the gating strategy to define cell populations and/or characteristics of both patients and controls.

As we and others have previously identified soluble fractalkine to belong to the endothelial inflammation signature of SSc (17, 21), we investigated whether the expression of the CX3CR1 fractalkine receptor in cytotoxic immune cells could be affected in the context of enhanced release of this chemokine. Our analysis did not conclude to significant alterations in CX3CR1 expression in CD8 T cells and γδ T cells. Hasegawa et al. have previously described an increased expression of CX3CR1 in circulating CD8 T cells of SSc patients with diffuse cutaneous form compared with controls, whereas no difference was found between SSc patients with limited and diffuse cutaneous involvement (17). However, the low number of patients with the diffuse cutaneous form in our study ($n = 4$) may explain the lack of difference evidenced.

Strikingly, we observed a marked decrease in the percentages and expression level of CX3CR1 in NK cells of SSc patients. Hasegawa et al. have shown a conserved expression of CX3CR1 in $CD16^+$ cells, defined as NK cells, but did not assess the other markers defining the NK cells subset, such as the lack of CD3 expression and the expression of CD56 (17). This difference of cytometry gating strategy of NK cells could explain the discrepancy between our data.

We observed a decreased expression of CX3CR1 in NK cells that might suggest its engagement with membrane-bound endothelial fractalkine and the recruitment of NK cells, chemoattracted by soluble fractalkine toward organ, through inflamed endothelium. Consistent with this hypothesis, Hasegawa et al. found increased CX3CR1 expression in inflammatory cells, colocalizing with CX3CL1 fractalkine-expressing endothelium in target organs such as lung and skin in SSc patients (17). Hence, we cannot rule out that soluble fractalkine levels are linked to CX3CR1 expression within SSc targeted tissues.

As demonstrated for NKG2D activating receptor, the decreased CX3CR1 expression on NK cells could rely on TGFβ, given that these two receptors have been demonstrated to be downregulated following TGFβ exposure (53, 54). Such event could be expected, as increased TGFβ signaling has been involved in the pathogenesis of SSc (55).

We noticed a rather high heterogeneity of CX3CR1 expression on NK cells in SSc patients. We thus further analyzed the variations in CX3CR1 expression levels within NK cells according to the clinical characteristics of patients and found a positive association with the pulmonary fibrosis and anti-topoisomerase 1 antibodies. Due to the low number of subjects in this study, no definitive conclusion can be drawn. Nevertheless, such observations suggest that the specific activation of the fractalkine/CX3CR1 pathway may favor NK cells-based mechanisms associated with the development of organ-specific involvement. Further investigations specifically addressing this point remain required in a larger cohort of patients.

We found no correlation between fractalkine levels and the level of expression of CX3CR1 in CD8 T cells, NK cells, and γδ T cells. Qualitative factors such as polymorphic variants of CX3CR1 could affect the binding affinity of CX3CR1 to fractalkine ligand. This may account for the lack of correlation between fractalkine and CX3CR1 observed here (18). However, the percentage of Vδ1 $CX3CR1^+$ cells was inversely correlated with the level of circulating fractalkine. This may also suggest the recruitment of this tissular subset of γδ T cells toward target organs in SSc, such as evidenced in skin biopsies (48) and in the bronchoalveolar fluid of SSc patients (56).

The finding of decreased CX3CR1 expression NK cells in SSc patients led us to further characterize the expression of chemokine receptors and activation markers/receptors in this specific subset. We found decreased expression of CXCR4 but conserved expression of CXCR3, in comparison with circulating NK cells from healthy controls. CXCR4 expression and percentages of CXCR4 expressing cells were recently shown to be decreased in circulating monocytes, CD8 T cells, and B cells in SSc (57). In SSc, increased tissular expression of CXCR4 and its SDF-1 ligand have been identified in dermal ECs of SSc patients (58). Hence, this decreased CXCR4 expression might reflect chronic stimulation of this receptor by SDF-1 or SDF-1 driven migration of $CXCR4^+$ NK cells toward skin tissue.

As chemokine receptors CX3CR1 and CXCR4 were decreased in SSc patients, we wondered whether the activating receptors CD16, DNAM-1, and NKG2D that participate in NK cells cytotoxicity were also modified. We observed that CD16 and DNAM-1 expression were not affected in SSc patients. However, DNAM-1 expression level was inversely correlated with the TLCO, reflecting ILD and/or PAH. This was consistent with a previous study that evidenced decreased DNAM-1 expression in circulating NK cells of SSc patients with ILD and PAH (47). Interestingly, we also found that NKG2D expression and percentage of NKG2D-expressing cells were decreased in SSc patients. Chronic exposure to membrane-bound or soluble NKG2D ligands can induce NKG2D down-modulation in NK cells. However, to date in literature, no information on NKG2D ligands expression in SSc is available to our knowledge. Here, our preliminary data found an increased relative expression of the ULBP3 transcript, NKG2D ligand, following HMVEC-d exposure to SSc sera (*data not shown*). This might suggest an increased release of ULBP3 by ECs in SSc patients and a subsequent negative feedback that would lead to the NKG2D internalization.

As NK cells from SSc patients exhibited decreased expression of cytotoxic receptor NKG2D, we aimed to precise their activation state assessing the activation marker CD69 and showed a decrease of CD69. CD69 was identified as an early activation marker but was more recently associated with tissue residency of $CD56^{bright}$ NK cells (10). Thus, the decrease of CD69 expression

in NK cells might reflect circulating NK cells exhaustion but does not preclude NK cells intra-tissular activation in SSc target organs.

Altogether, lowered expressions of CX3CR1, CXCR4, NKG2D, and CD69 point out a phenotypic signature of NK cells associated with SSc. We assume that this phenotype might impact the trafficking of NK cells subset and recruitment into the targeted organs. The decrease of chemokine receptors and CD69 might reflect the loss of tissue retention signals and might be associated with the dysregulation of NK cells localization and activation.

We further investigated whether such NK cell phenotype would impact NK cell natural and antibody-dependent cell-mediated cytotoxic function in the context of this autoimmune disease. NK cells from SSc patients displayed preserved ADCC compared with donors consistent with a similar CD16 expression profile among NK cells. The evaluation of cytolytic $CD107^+$ granules release by NK cells revealed a slight decreased ability to exert natural cytotoxicity through perforin/granzyme pathway. This was consistent with the observation of Horikawa et al. who evidenced decreased NK cells cytotoxicity toward the erythroleukemic cell line K-562 (46). Using a microvascular dermal EC line, Sgonc et al. demonstrated that NK cells from SSc patients displayed anti-EC antibody-dependent cytotoxicity mediated by Fas/Fas ligand interaction but not by the perforin–granzyme pathway (59). Conversely, in our study, the lack of CD16 engagement in NK cells of SSc patients in the presence of SSc sera and HMVEC-d cell line (*data not shown*) did not argue for a major contribution of such cytotoxic mechanism. Our results rather indicate a potential role of the NK cells-endothelium cross talk in the tissue recruitment of NK cells, endowed with the ability to release paracrine factors driving endothelial activation.

In this line, we show that circulating purified NK cells from SSc patients induced higher endothelial activation through microvascular EMPs release than healthy controls. This was observed both with SSc serum and healthy control serum rather suggesting an intrinsic capacity of SSc NK cells to promote this effect. Interestingly, we observed that percentages of CX3CR1 expressing NK cells tended to negatively correlate with the enhancement of EMPs release. This observation suggests a potential implication of CX3CR1 in NK cells-mediated endothelial activation that should be implemented in a larger number of patients.

This greater NK cells-induced EMPs release might also involve IL-6 secretion given that IL-6 levels in coculture supernatants were correlated with EMPs release. IL-6 released by NK cells has been shown to be an early event in SSc associated with SSc onset (60) and IL-6 secretion was demonstrated to be correlated with EMPs release and vascular inflammation associated with coronary disease (61).

The EMPs can promote or aggravate pre-existing vascular dysfunction in cardiovascular diseases (30) and drive endothelial senescence in response to environmental stress (62). Our results suggest that EMPs generated in response to endothelial interactions with NK cells may contribute to the amplification of endothelial dysfunction and of stress-induced senescence processes favoring the progression of vasculopathy during SSc.

In conclusion, this study identifies a peculiar profile of the chemokine and activating receptors of NK cells in SSc, which may reflect NK cells involvement in the SSc pathogenic process. It also highlights the role of NK cells as a potent immune inducer of endothelial activation through vesiculation, with a potential implication of CX3CR1 in this mechanism.

We acknowledge the limits of this study, partly inherent to the low number of patients. However, it is the first one to demonstrate that purified NK cells from SSc patients have a higher potential to activate EMPs release in the presence of autologous SSc serum, thus providing a mechanistic hypothesis linking NK cells to endothelial activation in this disease. Further studies are needed to define the actors and mechanisms involved in the cross talk between NK cells and ECs and the consequence of such EMPs release.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of French directives regarding Biomedical Research and "Comité de Protection des Personnes Sud Méditerranée." The protocol was approved by the "Comité de Protection des Personnes Sud Méditerranée." All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AB, JM, FS, FDG, BG, and PP designed the study and wrote the paper. AB, BG, GK, KM, PR, and MR-G included patients. SM included healthy controls. AB, LL, CD, and SR performed experiments. AB collected and analyzed data. All of the authors have reviewed the manuscript for critical content and approved the final version of the manuscript.

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Innate Immunity in Systemic Sclerosis Fibrosis: Recent Advances

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Systemic sclerosis (SSc) is a heterogeneous autoimmune disease characterized by three interconnected hallmarks (i) vasculopathy, (ii) aberrant immune activation, and (iii) fibroblast dysfunction leading to extracellular matrix deposition and fibrosis. Blocking or reversing the fibrotic process associated with this devastating disease is still an unmet clinical need. Although various components of innate immunity, including macrophages and type I interferon, have long been implicated in SSc, the precise mechanisms that regulate the global innate immune contribution to SSc pathogenesis remain poorly understood. Recent studies have identified new innate immune players, such as pathogen-recognition receptors, platelet-derived danger-associated molecular patterns, innate lymphoid cells, and plasmacytoid dendritic cells in the pathophysiology of SSc, including vasculopathy and fibrosis. In this review, we describe the evidence demonstrating the importance of innate immune processes during SSc development with particular emphasis on their role in the initiation of pathology. We also discuss potential therapeutic options to modulate innate immune cells or signaling in SSc that are emerging from these recent advances.

Keywords: innate immunity, systemic sclerosis, fibrosis, sterile inflammation, future therapeutic

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INTRODUCTION

Systemic sclerosis (SSc) is a complex autoimmune disease interconnecting vasculopathy, autoimmunity, and fibrosis features. A large body of evidence has indicated that the adaptive immune system with autoreactive T cells and autoantibodies produced by B cells plays a central role in SSc pathogenesis (1). In addition, inflammatory cytokines produced by the innate immune cells have been detected in the affected tissues of both the early and late stage of SSc, suggesting a role of innate immunity both at the onset and progression of the disease (2–6). This notion was recently reinforced by genomic and genetic approaches that have been undertaken to decipher key and conserved pathophysiological pathways within organs across disease forms (7–9). Apart from genomic approaches, the study of mechanisms governing normal tissue repair has revealed physiological pathways that may be disrupted during SSc as well. The concept of unresolved tissue repair leading to sustained fibrosis has emerged based on a persistent sterile inflammation that converts a self-limited repair response to a non-resolving pathological fibrosis (10, 11). However, the initial events leading to such sterile inflammation remain unclear. Recent data showing that an imbalance in danger-associated molecular pattern (DAMP) release and/or pathogen-recognition receptor (PRR) signaling leads to sustained inflammatory cytokine production by fibroblasts or macrophages may provide the missing link in early events of SSc pathophysiology (11). In addition, plasmacytoid dendritic cell (pDC)

activation (12, 13) and type I interferon (IFNα/β, IFN-I) production has also been recently shown to contribute to SSc.

In this review, we focus on recent evidence highlighting the contribution of innate immunity during the course of SSc pathogenesis, primarily at the early stages of disease. We also discuss potential therapeutic options that may modulate innate immune cells or signaling in SSc patients.

WHAT CAN BE LEARNED FROM GENETIC STUDIES ON INNATE IMMUNE FUNCTION DURING SSc?

Major technological and analytical advances in the past 10 years have allowed the extraction of critical information from transcriptomic data such as lineage-specific gene expression, networks of interactions, and functional information (14–17). This yielded a novel field of study in the integrated comprehension of SSc pathogenesis, identifying a major contribution of innate immunity.

By analyzing three independent gene expression data sets from SSc skin biopsies, the group of Whitfield proposed interconnected functional modules involved in SSc pathogenesis, two of which involve innate immunity and are dominated by IFN, IFN-inducible genes, and type 2 macrophage (M2) signatures. The three other subnetworks were linked to adaptive immunity, fibrotic processes [response to transforming growth factor beta (TGF-β) and extracellular matrix (ECM) disassembly/wound healing], cell cycle, proliferation, and apoptosis (9). The same group recently identified a common pathogenic signature related to an "innate immune-fibrotic axis" that includes IFN-I and alternatively activated macrophages commonly referred as M2 macrophages and describes new specific pathways and hubs active in the skin and lung (8). Among shared networks, the authors found that the "innate immunity-fibrotic network" is conserved between skin and lung while the internal composition and interactions of gene expression in those tissues vary.

Such large-scale genomic studies paved the way for multiple experimental approaches to determine the molecular processes involved in patients and to establish novel therapeutic options targeting specific organs or shared pathophysiological processes.

EMERGING CONCEPT: SSc AS AN OVER-REPAIR PATHOLOGY

The ability of an organism to efficiently recover from injury whether traumatic, infectious, chemical, or internal is pivotal to maintain its integrity (18). During tissue repair, innate immune cell plasticity actively contributes to the development of an abnormal microenvironment, leading to a shift in the balance between the pro-inflammatory and pro-reparative sides of tissue repair, as recently reviewed (10).

Early SSc is characterized by a perivascular leukocyte infiltrate mainly composed of macrophages and T lymphocytes, reminiscent of the process induced during normal wound healing (19, 20). Whereas normal wound healing is accompanied by a remodeling or resolving stage, abnormal wound healing with chronic activation of immune cells such as macrophages or stromal cells like myofibroblasts fails to resolve fibrosis during SSc. Hence, SSc, specifically diffuse cutaneous forms of the disease, could be considered as a general form of over-repair. The initial trigger of the injury is still unknown, but several lines of recent evidence have brought new hypotheses on its nature.

Role of Sterile Inflammation in Unresolving Tissue Fibrosis During Scleroderma: Importance of DAMP/PRR Imbalance

Recognition of pathogen-associated molecular patterns (PAMPs) or endogenous DAMPs by innate immune cells as well as non-immune cells is the first line of response to pathogen or sterile tissue injury. DAMPs, mainly produced by epithelial cells, are heterogeneous in form encompassing early produced and highly diffusible Ca^{2+}, H_2O_2, reactive oxygen species (ROS), adenosine tri-phosphate, self-nucleic acids, but also proteins like high-mobility group protein 1, heat shock protein, S100 proteins, and fragments of the ECM. The recognition of PAMPs and DAMPs relies on cell surface, endosomal, and cytosolic PRRs that include toll-like receptors (TLRs), Nod-like receptor, Rig-I-like receptors (RLRs), cyclic GMP-AMP synthase, and receptor for advanced glycation end products. Innate immune signaling triggered by DAMPs during sterile inflammation or the persistence of pathogens such as endogenous viruses might represent an important pathway responsible for converting self-limited regenerative repair into an unresolved fibrotic process during SSc. Hence, innate immune signaling *via* TLRs was recently proposed as a key driver of persistent fibrotic response in SSc and other fibrotic-related diseases (11).

Overexpression of TLR4 and its two co-receptors CD14 and myeloid differentiation factor 2 (MD-2) has been described in SSc-affected skin and lung. TLR4 expression was mainly associated with macrophages, fibroblasts, and myofibroblasts (21). In the skin, TLR4 expression correlated to fibrosis severity measured by modified Rodnan skin score. *In vivo*, chronic TLR4 activation leads to sustained nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling, resulting in macrophage activation and a profibrotic profile (22). Work from the Varga lab recently demonstrated that endogenous DAMP activation of TLR4 can contribute to converting self-limited tissue repair responses into uncontrolled ECM deposition during SSc [for recent review, see Ref. (11)]. They proposed that fibronectin, containing alternatively spliced exons encoding type III repeat extra domain (EDA), and tenascin-C are constitutively produced by SSc fibroblasts leading to their accumulation in the skin but also in the blood. Together, fibronectin-EDA and tenascin-C act as strong profibrotic factors during SSc by binding to fibroblasts TLR4, leading to enhanced production of collagen and alpha-smooth muscle actin (α-SMA) expression (23, 24). Deletion of EDA or tenascin-C or disruption of TLR4 signaling resulted in reduced fibrotic response in a murine model of SSc. Furthermore, tensional forces generated within a rigid fibrotic microenvironment were reported to favor exposure of the EDA domain of fibronectin (25), suggesting that increased stiffness of the matrix in fibrotic tissue could favor the bioavailability and profibrotic activity of fibronectin-EDA.

Altered expression of multiple DAMPs/TLRs beyond TLR4 has been described during SSc. Indeed, increased expression of TLR9 was found in human SSc skin biopsies in both early and late stages of the disease and was mainly associated with α-SMA-positive myofibroblasts (26), and a TLR9 signature was detected in SSc skin. *In vitro* treatment of normal cutaneous fibroblasts with the TLR9 ligand unmethylated-CpG-oligodeoxynucleotides (CpG ODN) induced a profibrotic profile involving autocrine TGF-β production. Collectively, these results support the role of TLR9 signaling in SSc. Furthermore, expression of TLR2 (27) and TLR3 (28) is also increased in SSc skin fibroblasts. TLR2 was shown to respond to the endogenous ligand amyloid A, resulting in NFκB activation and increased interleukin (IL)-6 secretion causing inflammation (27). However, the role of TLR3 in SSc pathogenesis remains controversial. TLR3 activation by polyinosinic:polycytidylic acid (poly I:C) stimulates IFN-I production by fibroblasts, which in turn reduces their ability to produce ECM components (28). Conversely, such stimulation was shown to promote the expression of TGF-β by fibroblasts thus contributing to the overall fibrosis (29).

In addition to TLRs, other PRRs have been described to play a role in SSc pathogenesis. The IFN-I stimulatory property of poly I:C on SSc patient fibroblasts was shown not only to rely on TLR3 but also on intracellular RLRs (28). The inflammasome, specifically the NLRP3-inflammasome, was shown to contribute SSc pathogenesis *in vivo* (30) through the induction of the microRNA miR-155, which in turn favors excessive ECM production by fibroblasts, exacerbating SSc (31).

Studies on the contribution of TLR signaling to fibrosis in SSc as well as other fibrotic diseases have generated conflicting results (22, 26, 32–35), suggesting that whether TLR activation leads to pro- or anti-fibrotic effects depends on many factors. The nature of the stimulation (chronic vs acute), of the responding cells (immune or non-immune cells), as well as disease stage (inflammatory vs remodeling) might modulate the effects of TLRs in the fibrotic process. Profibrotic effects of TLR activation seem related to fibroblast and macrophage activation in the context of chronic stimulation, whereas epithelial and other immune cell activation in the context of acute stimulation might lead to anti-fibrotic effects. Although additional PRRs have recently been implicated in SSc, further studies are required to identify their endogenous ligands and mechanisms leading to disease. Nevertheless, PRRs and their signaling pathways may represent multiple novel therapeutic targets in SSc.

Old Players, New Pathways: Type-2 Macrophages, Platelets, and Mastocytes

Macrophages and platelets have emerged as key players not only during tissue homeostasis and repair but also fibrosis, recently reviewed in Ref. (36, 37).

We and others have defined the profibrotic role of platelets in SSc. The Distler group has shown that serotonin [5-hydroxytryptamine (5-HT)] stored in platelets strongly induces ECM synthesis in interstitial fibroblasts *via* activation of 5-HT_{2B} receptors (5-HT_{2B}) in a TGF-β-dependent manner (38). Our group discovered a pathophysiological loop active in SSc that links vasculopathy and fibrosis. Indeed, we showed that platelet activation induced the production of thymic stromal lymphopoietin (TSLP) by dermal microvascular endothelial cells in an IL-1β-dependent manner. TSLP was found to be strongly expressed in SSc skin endothelial cells and correlated to the severity of skin fibrosis. *In vitro*, TSLP was able to induce a profibrotic profile in both normal and SSc fibroblasts (39, 40).

Infiltration of macrophages in the early skin lesions of SSc patients, particularly in perivascular areas, was first detected over 20 years ago and could lead to secondary activation of adaptative system (19, 20). Since then, numerous studies have established the involvement of macrophages in SSc pathogenesis, notably their alternatively activated counterpart called M2 macrophages as reviewed in Ref. (41). Soluble CD163, a putative marker of M2 macrophages, was shown to be elevated in SSc patients' blood and associated with their poor clinical outcome (42). These observations have been reinforced by the recent genetic studies showing a prominent M2 macrophage signature in SSc-affected skin and lung (8). However, the activation of lung macrophages in SSc patients with pulmonary fibrosis is distinct from that observed in SSc skin. Activated lung-resident macrophages display a specific increase in the expression of genes related to lipid and cholesterol trafficking, suggesting a switch in their metabolism. Thus, while M2 macrophages are central to the fibrotic process both in skin and lung during SSc, distinct stimuli derived from the organ-specific microenvironment might differentially shape the plasticity of macrophages. In the recent FASSCINATE trial, molecular profiling of skin biopsies revealed that IL-6 receptor blockade by tocilizumab resulted in a reduced M2 macrophage signature observed in SSc skin (43). Accordingly, the blockade of cAMP-specific phosphodiesterase-4, which inhibits differentiation of M2 macrophages as well as IL-6 production, led to an amelioration of fibrosis in a murine model of SSc induced by bleomycin treatment (44). The same group demonstrated that nintedanib, a tyrosine kinase inhibitor targeting vascular endothelial-, fibroblast-, and platelet-derived growth factor receptors, effectively blocked myofibroblast differentiation and reduced pulmonary, dermal, and myocardial fibrosis in transgenic Fra2 mice. This effect was primarily mediated by preventing M2 macrophage accumulation in the affected tissues (45). However, the mechanisms leading to aberrant M2 macrophage polarization and the precise pathways through which M2 macrophages contribute to tissue fibrosis remain unclear. One elegant study by Eming et al. provided novel mechanistic insight to the role of M2 macrophages in fibrosis. Using a murine model of wound healing, IL-4Ra activation by IL-4 and IL-13 was demonstrated to induce the production of resting like molecule alpha by M2 macrophages, which in turn stimulates the production of enzyme lysyl-hydroxylase-2 (LH-2) ultimately contributing to persistent profibrotic collagen cross-linking in fibroblasts (46). This process was shown to be critical for transformation of the tissue into a persistent scar. In humans, Relm-β induces LH-2 in fibroblasts, and expression of both factors was reported to be increased in lipodermatosclerosis, a condition associated with excessive skin fibrosis. Whether this process contributes to SSc is still unknown. The fine mapping of specific macrophage subsets across tissues and during the course of disease, as well as elucidating of the molecular mechanisms underlying

macrophages-induced abnormal resolution, will pave the road to the development of new drugs that prevent/limit fibrosis.

Studies in patients and animal models of SSc have demonstrated that mast cells infiltrate the fibrotic skin (47, 48). This infiltration was associated with more severe disease phenotypes (48), but the function and net contribution of mast cells to fibrosis is only beginning to be understood. Mast cells have been suggested to be an important source of TGF-β and thus contribute to the overall fibrosis (49). Furthermore, a recent report using transgenic mice that develop spontaneous skin fibrosis showed a major role for mast cells specifically in inducing inflammation of the skin and the production of ECM and α-SMA by fibroblasts (50). Together with recent observations showing that mast cell deletion ameliorates experimental SSc *in vivo* (47, 51), these results indicate that mast cell targeting in SSc patients may represent an effective therapeutic approach.

Finally, other innate immune players such as natural killer (NK) cells (52, 53) and neutrophils (54) were shown to display altered properties and phenotypes in the blood of SSc patients. However, further studies are required to evaluate the role of NK cells and neutrophils in the SSc pathogenesis, especially in the settings of murine experimental models.

NEW KIDS ON THE BLOCK: pDC AND INNATE LYMPHOID CELL (ILC)

Plasmacytoid dendritic cells are innate immune cells specialized in the production of copious amounts of IFN-I (55), and thus play a key role in the initiation of antiviral immune responses (56, 57). IFN-I production by pDCs requires recognition of viral nucleic acids by TLR7 and TLR9, respectively (56, 57). pDCs were also shown to produce IFN-I in response to self-nucleic acids and consequently contribute to the development of multiple inflammatory and autoimmune disorders (58–62). An IFN-I signature, reflected by increased expression of numerous IFN-I-stimulated genes has been reported in patients with SSc (12). Furthermore, genome-wide association studies in SSc have identified polymorphisms in genes involved in the regulation of IFN-I expression in pDCs, particularly IFN-regulatory factor (IRF)-5, IRF-7, and IRF-8 (12). Approximately half of SSc patients (~50%) display an IFN-I signature within their peripheral blood mononuclear cells (63–65) and in fibrotic skin (66). The association between IFN-I signature and SSc disease activity remains controversial as no major impact of the IFN-I signature on pathological features of SSc, including extent of skin fibrosis, autoantibody specificities, and interstitial lung disease, has been reported (63, 65). However, when the profile of IFN-induced chemokines was specifically analyzed in a large cohort of SSc patients, an association was then identified with more severe SSc (67). As pDCs are an important source of IFN-I, numerous groups have investigated their role in SSc. pDCs were indeed detected in the affected skin of SSc patients (65, 68) as well as in the fibrotic skin of mice after bleomycin treatment (13). Furthermore, mice lacking fibrillin-1 (*Fbn1*), which spontaneously develop a stiff skin syndrome that recapitulates the skin fibrosis observed in SSc patients, show a high infiltration of pDCs in the affected skin (69). The frequency of pDCs is reduced in the circulation of SSc patients, likely due to their preferential recruitment into the fibrotic skin (13). Anti-topoisomerase I and anti-nuclear autoantibodies in SSc patients were shown to form immune complexes with apoptotic cell-derived constituents *in vitro* and consequently stimulate IFN-I production by pDCs (70, 71) upon uptake *via* FcγRII and the stimulation of TLR7/9 (70, 71). While such "interferongenic" properties of immune complexes may contribute to the aberrant IFN-I production, an IFN-I signature was not associated with the production of specific autoantibodies detected in the sera of SSc patients (70), suggesting that additional factors may contribute to pDC activation *in vivo*. Furthermore, pDCs in the peripheral blood or fibrotic skin of SSc patients spontaneously secrete CXC motif ligand (CXCL)-4 and IFNα (13, 68). High levels of CXCL4 in the circulation of SSc patients were associated with disease severity including skin fibrosis and pulmonary arterial hypertension (68). CXCL4 was described to potentiate pDC ability to produce IFN-I *in vitro* largely in response to TLR9 stimulation. In addition, CXCL4 was shown to induce both the expression of TLR8 and the ability to produce IFN-I in response to its specific ligands in pDCs (13). Recently, the pathogenic role of TLR8 was confirmed *in vivo* using transgenic mice that express human TLR8 and develop exacerbated skin fibrosis after bleomycin treatment compared with control animals (13). However, whether such exacerbation of disease in TLR8 transgenic animals is dependent on pDCs remains unknown, and the association between CXCL4 levels and the IFN-I signature in SSc patients has not yet been characterized. Ah Kioon et al. showed that bleomycin-induced skin fibrosis is strongly attenuated after selective pDC depletion (13). Furthermore, this model of fibrosis was associated with an IFN-I signature and increased expression of CXCL4 in the affected skin, and pDC depletion significantly reduced the occurrence of these parameters. From a therapeutic standpoint, pDC depletion ameliorated established bleomycin-induced skin fibrosis, indicating that pDCs are critical even in the maintenance of skin fibrosis. This constitutes the first study showing the deleterious impact of pDCs on SSc development *in vivo* (13). Overall, pDCs play a critical role in SSc pathogenesis; however, the molecular mechanisms through which they contribute to the disease require further investigation. This recent progress nevertheless positions SSc as another autoimmune pathology that may benefit from therapeutic targeting of pDCs using depleting or inhibitory antibodies (72).

Innate lymphoid cells were recently described as novel components of the immune system that may be considered as innate counterparts of polarized T helper cells (73). Nevertheless, knowledge on the role of ILCs in SSc remains limited. Wohlfahrt and colleagues have shown elevated numbers of ILC2 in both the peripheral blood and the affected skin of patients with SSc compared with healthy individuals, and their number correlated with the extent of cutaneous fibrosis (74). However, the increased frequency of ILC2 in SSc peripheral blood was not observed in a different study, which instead reported an elevated frequency of CD4 + ILC1 and NKp44 + ILC3 (75).

Nevertheless, in animal models of lung fibrosis induced by bleomycin, IL-33, an alarmin that has been reported to be elevated in SSc patients (76), induced the expansion of ILC2s producing the profibrotic cytokine IL-13 (77). Hence, further investigations

TABLE 1 | Potential therapeutics and therapeutics in latest clinical trials specific to innate immunity and fibrosis in SSc.

Innate immunity targeted physiopathological pathways	Target	Molecules	Drug name/ trade name	Clinical trial in SSc	Primary end-point	Result
(A) Chronic sterile inflammation						
	TLR4/MD-2 inhibition	Selective TLR4 inhibitor, lipid A mimetic	E5564/Eritoran	None for SSc, tested in sepsis (lack of efficiency)		
		Anti-TLR4	NI-0101			
		Selective TLR4 inhibitor, small molecule	T5342126	None		
	TLR4/MD-2 inhibition of DAMP	Tenascin-C A1 domain specific blocking antibody	F16	None		
		Fibronectin-EDA specific blocking antibody	F8	None		
	TLR4 downstream signaling	Small molecule binding the Cys747 of the intracellular domain of TLR4	TAK-242	None for SSc, tested in sepsis (lack of efficiency)		
	TLR7/8/9	Small molecule or oligonucleotides	CpG-52364, DV-1179, IMO 3100, IMO-8400	None		
	NFκB	PDE4 inhibitor	Crisaborole/ Eucrisa	None for SSc but Pilot Study Evaluating the Efficacy of a Topical PDE4 Inhibitor for Morphea NCT03351114	Change in dermal thickness of sentinel plaque from Baseline to 12 weeks	
	pDC	Anti-BICD2 antibody	BIIB059	None		
	Type 1 IFN	Type 1 interferon receptor sub-unit 1 blocking antibody	MEDI-546	Phase I open-label study in diffuse cutaneous SSc NCT00930683	Safety and tolerability of single or multiple intravenous doses	Decreased type I IFN gene expression in whole blood and skin for subjects with positive scores at baseline
(B) Abnormal resolution						
	Fibroblasts	Selective CB2 agonist	JBT-101/ Lenabasum	Phase II + open-labeled extension	Safety and reduction of the mRSS score	Reduction of 8.4 points in the mRSS score in the open-label extension
		Selective CB2 agonist	JBT-101/ Lenabasum	Phase III RESOLVE-1 trial NCT03398837	Change from baseline in mRSS	Expected results in 2020
	Type-2 macrophages	Anti-IL-6 receptor alpha blocking antibody	Tocilizumab/ Roactemra	Phase II FASSCINATE trial NCT01532869	Safety and difference in mean change from baseline in mRSS at week 24	Primary end-point not reached but diminished type-2 signature in the treated arm
		Tyrosine kinase inhibitor	Nintedanib	Phase III SENSCIS trial NCT02597933	Efficacy and safety in SSc patients with interstitial lung disease at week 52	
		PDE4 inhibitor	Crisaborole/ Eucrisa	No clinical trial in SSc, but pilot study evaluating the efficacy of a topical PDE4 inhibitor for morphea NCT03351114	Change in dermal thickness of sentinel plaque from Baseline to 12 weeks	
	TGF-β	TGF-β isoforms 1, 2, and 3 blocking antibody	Fresolimumab	Phase I open-label trial NCT01284322	Safety and efficacy (molecular assessment of TGF-β responsive genes and improvement in the mRSS)	Inhibition of TGF-β-regulated gene expression and improvement in the mRSS in the fresolimumab treated group

(*Continued*)

TABLE 1 | Continued

Innate immunity targeted physiopathological pathways	Target	Molecules	Drug name/ trade name	Clinical trial in SSc	Primary end-point	Result
		Soluble guanylate cyclase activator blocking TGF-β-induced release of ECM components from fibroblasts	BAY63-2521/ Riociguat	Phase II RISE-SSc trial NCT02283762	Safety and efficacy (change in mRSS at week 52) in patients with diffuse cutaneous SSc	

BDCA-2, blood dendritic cell antigen 2; DAMP, danger-associated molecular pattern; IFN, interferon; mRSS, modified Rodnan skin score; SSc, systemic sclerosis; TGF-β, transforming growth factor beta; TLR, toll-like receptor; PDE4, phosphodiesterase-4; ECM, extracellular matrix; pDC, plasmacytoid dendritic cell; MD-2, myeloid differentiation factor 2; CB2, cannabinoid receptor type 2.

are warranted to determine the role of ILC2 in the development of SSc fibrosis.

FUTURE DIRECTIONS AND THERAPEUTIC AVENUES

Significant progress has recently been made in understanding the contribution of innate immunity to SSc fibrosis. Although the precise molecular mechanisms of their action must be further defined, promising new therapeutic targets for SSc have already emerged. Such strategies include blockade of TLR4/MD-2, TLR9, or downstream signaling molecules to limit chronic sterile inflammation, modulation of macrophage polarization to promote resolution and matrix remodeling, and targeting pDCs/IFN-α. This therapeutic challenge is ongoing with many attractive new therapeutic candidates, some of which are currently being tested in Phase III clinical trials (**Tables 1A,B**). Both the evaluation of potential side effects and identification of biomarkers of patients who would benefit from such therapies are warranted in order to maximize the efficacy of treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Interleukin-6 and Type-I Collagen Production by Systemic Sclerosis Fibroblasts Are Differentially Regulated by Interleukin-17A in the Presence of Transforming Growth Factor-Beta 1

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Functional cytokine networks have been poorly characterized in systemic sclerosis (SSc). While interleukin-17A (IL-17A) is increased in SSc skin and other organs, its role is still debated, particularly considering fibrogenesis. We uncover here a dual function of IL-17A in the presence of transforming growth factor-β 1 (TGF-β), the master pro-fibrotic cytokine. In the one hand, we report an unexpected synergic activity resulting in enhanced production of IL-6 by dermal fibroblasts; in the other hand, a substantial inhibition of type I collagen (col-I) production. IL-17A or TGF-β enhanced the production of IL-6 by 8- to 16-folds when compared to control in healthy donors (HD) and SSc cultures. However, the joint presence of IL-17A and TGF-β resulted in robustly exuberant responses with levels of IL-6 up to 100-folds higher than those observed in untreated cells. Inhibition of NFκB signaling pathway preferentially inhibited the production of IL-6 driven by IL-17A in HD fibroblasts, while inhibition of PI3K preferentially inhibited the production of IL-6 driven by TGF-β. Interestingly, when p38 MAPK was inhibited, substantial reduction of IL-6 production was observed for both IL-17A and TGF-β. Consistently with the inhibition experiments, the combined stimulation of fibroblasts by IL-17A and TGF-β resulted in 1.8-fold increase in p38 MAPK phosphorylation ($P = 0.025$), when compared to levels of phosphorylated p38 MAPK induced by IL-17A alone. Furthermore, the enhanced phosphorylation of p38 MAPK in the joint presence of IL-17A and TGF-β was unique among the signaling molecules we examined. As expected, TGF-β induced SMAD2 phosphorylation and col-I production. However, in fibroblasts cultured in the joint presence of TGF-β and IL-17A, SMAD2 phosphorylation was decreased by 0.6-folds ($P = 0.022$) when compared to that induced by TGF-β alone. Remarkably, in this condition, the production of col-I and fibronectin was significantly decreased in both HD and SSc. Thus, IL-17A and TGF-β reciprocally

influence each other effector functions in fibroblasts. Intracellular molecular switches may favor synergic or antagonistic activities, which are revealed by specific readouts. The implications of these data in the context of SSc are far reaching, particularly in terms of therapeutic approaches since IL-6, IL-17A, and TGF-β are all putative targets of treatment.

Keywords: systemic sclerosis, interleukin-17A, transforming growth factor-beta, interleukin-6, type-I collagen, monocyte chemotactic protein-1, fibrosis

INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disorder characterized by fibrosis of the skin and internal organs, vasculopathy, and dysregulated immuno-inflammatory responses. Fibrosis is a characteristic aspect of the disease, bears a high token of morbidity and mortality (1), and is caused by an excess of extracellular matrix (ECM) deposition over degradation (2, 3). The driving factors leading to pathological fibrosis are object of controversies (4, 5), but it is likely that inflammatory mediators including, but not exclusively, interleukin-17A (IL-17A) (6), transforming growth factor-β 1 (TGF-β) (7) and IL-6 (8, 9) play a major role (10).

IL-17A is a pro-inflammatory cytokine mainly but not exclusively produced by Th17 cells involved in protection against extracellular bacteria and fungi as well as in autoimmunity (11). IL-17A levels and/or Th17 cells have been reported to be increased in SSc peripheral blood, bronchoalveolar lavage fluid, and skin (12–18), although some reports point to decreased serum levels (19, 20).

The role of IL-17A in the development of fibrosis is controversial (21). Concordant data generated in various animal models of fibrosis point to a pro-fibrotic activity (22–24). In contrast, studies in humans using *in vitro* fibroblast cultures suggest that IL-17A rather controls fibrosis (24–26) by inhibiting collagen synthesis and the transdifferentiation of fibroblasts to myofibroblasts induced by TGF-β (18, 24, 26). Furthermore, the number of IL-17A+ cells appears to be inversely correlated to the extent of skin fibrosis (18) and to increase with disease duration (27), thus pointing to an antifibrotic activity *in vivo*. Nonetheless, IL-17A and Th17 cells have potent pro-inflammatory properties including the induction of several mediators dysregulated in SSc, including IL-8, IL-6, monocyte chemotactic protein-1 (MCP-1), matrix metalloproteinases (MMP) by dermal fibroblasts, in addition to enhancing their proliferation capacity (12, 26, 27).

TGF-β is considered a master pro-fibrotic cytokine with important immunomodulatory properties regulating inflammation, adipogenesis, chondrogenesis, osteogenesis, epithelial cell differentiation and proliferation, hemopoiesis, and wound healing. It binds to TGF-β receptor type-2 (TGFβR2), thus recruiting and phosphorylating signal transducer TGF-β receptor type-1 (TGFβR1). TGF-β is secreted as a latent protein, which needs to be activated mostly by protease-mediated cleavage favored by integrin-mediated release (28). It was reported that the levels of TGF-β are increased in the skin of SSc patients (7, 29–32) and that TGF-β-induced gene signature is strongly increased in SSc skin and positively correlates with the severity of the disease (33). A recent pilot trial targeting TGF-β with fresolimumab has shown some efficacy in reducing skin fibrosis (34).

IL-6 is a multifunction cytokine that plays a key role in acute phase responses, regulates cell proliferation, activation, and differentiation (35), and IL-6 serum levels are increased in SSc (8, 9, 36). Furthermore, IL-6 induces collagen production by dermal fibroblasts in the presence of trans-signaling by soluble IL-6 receptor (37) and participates to imbalanced degradation of ECM that is controlled by MMP and their inhibitors (38). IL-6 blockade by both passive or active immunization strategies or IL-6 genetic deletion reduces fibrotic responses in animal models of fibrosis (39–41). Furthermore, an IL-6 targeted therapy in SSc appears to be promising (42) and is currently assessed in a phase 3 clinical trial (NCT02453256).

The interplay between IL-17A and TGF-β has been only partially assessed (18, 24) and here we address the question to which extent their coordinate action affects fibroblast responses. We report for the first time that they may simultaneously have synergic or antagonistic activities depending on the readout used to evaluate fibroblast responses.

TABLE 1 | Clinical characteristic of the fibroblast donors.

	SSc	HD
Age, mean (range), years	61.2 (46–78)	42.1 (26–60)
Sex (M/F)	3/6	1/8
Disease duration, mean (range), months	108 (12–324)	N/A
Form (limited/diffuse)	4/5	N/A
MRSS (mean, range)	12.4 (4–28)	N/A
ANA positivity (yes/no) (n = 8)	7/1	N/A
ANA specificity (ACA/ATA) (n = 6)	4/2	N/A
DLCO, mean (range), % of reference (n = 8)	70.8 (33–112)	N/A
Synovitis	3 of 8	N/A
CK elevation	1 of 9	N/A
DU	3 of 9	N/A
ILD	2 of 8	N/A
GERD	6 of 8	N/A
Prednisone use	4 of 9	none
Prednisone dose >10 mg/day	0 of 9	none
Previous use of immunosuppressive agents	4 of 9	none

DU and synovitis were clinically defined, disease duration refers to the time from the onset of the first non-Raynaud's disease manifestation, ILD was assessed by high resolution CT scan, GERD was determined by gastroscopy. ACA, anticentromere antibody; ANA, antinuclear antibodies; ATA, antitopoisomerase antibody; CK, creatine kinase; DLCO, diffusing capacity of the lungs for carbon monoxide; DU, digital ulcer; GERD, gastroesophageal reflux disease; ILD, interstitial lung disease; IS, immunosuppressant agents; MRSS, modified Rodnan skin score; N/A, not available; HD, healthy donors; SSc, systemic sclerosis.

MATERIALS AND METHODS

Patients

Nine SSc individuals and nine healthy controls were included in this study. All patients met the ACR/EULAR classification criteria for SSc (43) and their clinical presentation classified according to the criteria proposed by LeRoy et al. (44). Clinical characteristics of the patients are shown in **Table 1**. A biopsy was performed in the affected skin of SSc individuals. The control group consisted of age and sex matched individuals who underwent corrective abdomen or breast surgery at the department of plastic surgery of HUG in Geneva (Switzerland). None of the healthy individuals had dermatological disorders and none was under immunosuppressive agents or glucocorticoids. This study was approved by the ethical committee of the institutions involved (06-063, Commission cantonale d'éthique de la recherche, Geneva, Switzerland) and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from each individual.

Reagents

rhIL-17A, rhTGF-β, monoclonal mouse IgG1 TGF-β1, 2, 3 antibody, IL-6, MCP-1, MMP-1, IL-8, pro-collagen Iα1 and fibronectin ELISA DuoSet kits were from R&D Systems (Abingdon, UK); DMEM, PBS, glutamine, penicillin, streptomycin, trypsin, dispase, collagenase type I from Gibco (Paisley, UK); FCS from Biowest (Nuaillé, France); BSA, α-ketoglutaric acid, β-amino propionitrile, L-ascorbic acid, p38 MAPK inhibitor SB203580, and PI3K inhibitor LY294002 from Sigma (St. Louis, MO, USA); MEK1/2 inhibitor U-0126 from Calbiochem (San Diego, CA, USA); TGF-βRI inhibitor SD 208, JNK inhibitor SP 600125 and IKK-2 inhibitor TPCA-1 from Tocris Bioscience (Bristol, UK); LEAF irrelevant control mAbs from Biolegend (San Diego, CA, USA); Complete Protease Inhibitor Cocktail and PhosSTOP phosphatase inhibitor from Roche (Basel, Switzerland); nitrocellulose membranes and chemiluminescence (ECL) blotting analysis system from GE Healthcare (Zurich, Switzerland); phospho-Akt (Ser473), phospho-Smad2 (Ser465/467), phospho-p38 MAPK (Thr180/Tyr182), phospho-NF-κB p65 (Ser536), phospho-IκB-α (Ser32), β-actin and BSA for Western blots from Cell Signaling (Danvers, MA, USA); TMB ELISA substrate from Abcam (Cambridge, UK); EZ4U cell proliferation assay from Biomedica (Vienna, Austria).

Cell Cultures

Human fibroblasts were isolated from skin, as previously described (26). Cells were cultured in DMEM containing 10% FCS, 1% non-essential amino acids, 1% L-glutamine, 1% sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin. Fibroblasts were used at passage 5–8. Twenty thousand cells/well were seeded in 96-well plates for 24 h, then starved for 16 h in the absence of FCS, followed by stimulation with IL-17A (25 ng/ml) and/or TGF-β (2.5 ng/ml). EZ4U cell proliferation assay was used to determine the viability of fibroblasts. When used, inhibitors (SD208, U0126, SB203580, SP600125, Ly294002, TPCA-1) at indicated doses, vehicle (DMSO), 10 μg/ml of TGF-β 1 neutralizing antibody or 10 μg/ml of an irrelevant control antibody were added for 1 h prior to stimulation with IL-17A (25 ng/ml) or TGF-β (2.5 ng/ml), in triplicates. Culture supernatants were harvested after 48 h.

ELISA and Western Blot

IL-6, MCP-1, IL-8, MMP-1, pro-collagen 1αI, and fibronectin were quantified using DuoSet ELISA kits, according to the manufacturer instruction (R&D Systems, Abingdon, UK). For Western blot, cell cultures were treated as previously described (26). Briefly, 20 μg of total protein extract were separated in 10% reducing SDS-PAGE and electroblotted onto nitrocellulose membranes. Blots were incubated with antibodies against phospho-Akt (Ser473), phospho-Smad2 (Ser465/467), phospho-p38 MAPK (Thr180/Tyr182), phospho-NF-κB p65 (Ser536), phospho-IκB-α (Ser32), and β-actin. Horseradish peroxidase-conjugated mouse or rabbit antisera were used to reveal primary binding, followed by detection by an ECL blotting analysis system. Quantification analysis was performed with ImageJ software (http://rsbweb.nih.gov/ij), and values were normalized to β-actin.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 7.02 (Graphpad Software, La Jolla, CA, USA). Shapiro–Wilk normality test was used to evaluate if the residuals follow a Gaussian distribution. Statistical significance was assessed by paired Student's *t*-test. P values <0.05 were considered statistically significant.

RESULTS

IL-6 and MCP-1 Are Synergistically and Specifically Induced in Human Fibroblasts by the Combined Action of IL-17A and TGF-β

Interleukin-17A and TGF-β are both considered of pathogenic importance in SSc. However, relatively little is known on their effects when applied jointly to fibroblasts. To address this issue, we used the fibroblast production of IL-6, MCP-1, and IL-8 as read out and we observed that the joint presence of suboptimal doses of IL-17A and TGF-β induced synergic responses specifically for IL-6 and MCP-1, but not for IL-8 (**Figure 1**). Suboptimal doses of agonistic cytokines were chosen in order to avoid maximal fibroblast responses—as observed with higher levels of IL-17A (data not shown)—thus potentially favoring the quantification of synergistic or antagonistic activities. Of interest, although SSc fibroblasts produced higher basal levels of IL-6, both SSc and HD cells responded equally well to the combination. In particular, it has to be noted that IL-17A and TGF-β were equipotent when used separately to induce the production of IL-6 and their joint action consistently over-enhanced the production of IL-6 by up to 32-folds (**Figure 1A**). To prove that the responses to TGF-β were not due to contaminants, we performed two distinct assays. First, TGF-β neutralization by a specific antiserum abrogated the production of IL-6 induced by TGF-β used alone and reduced

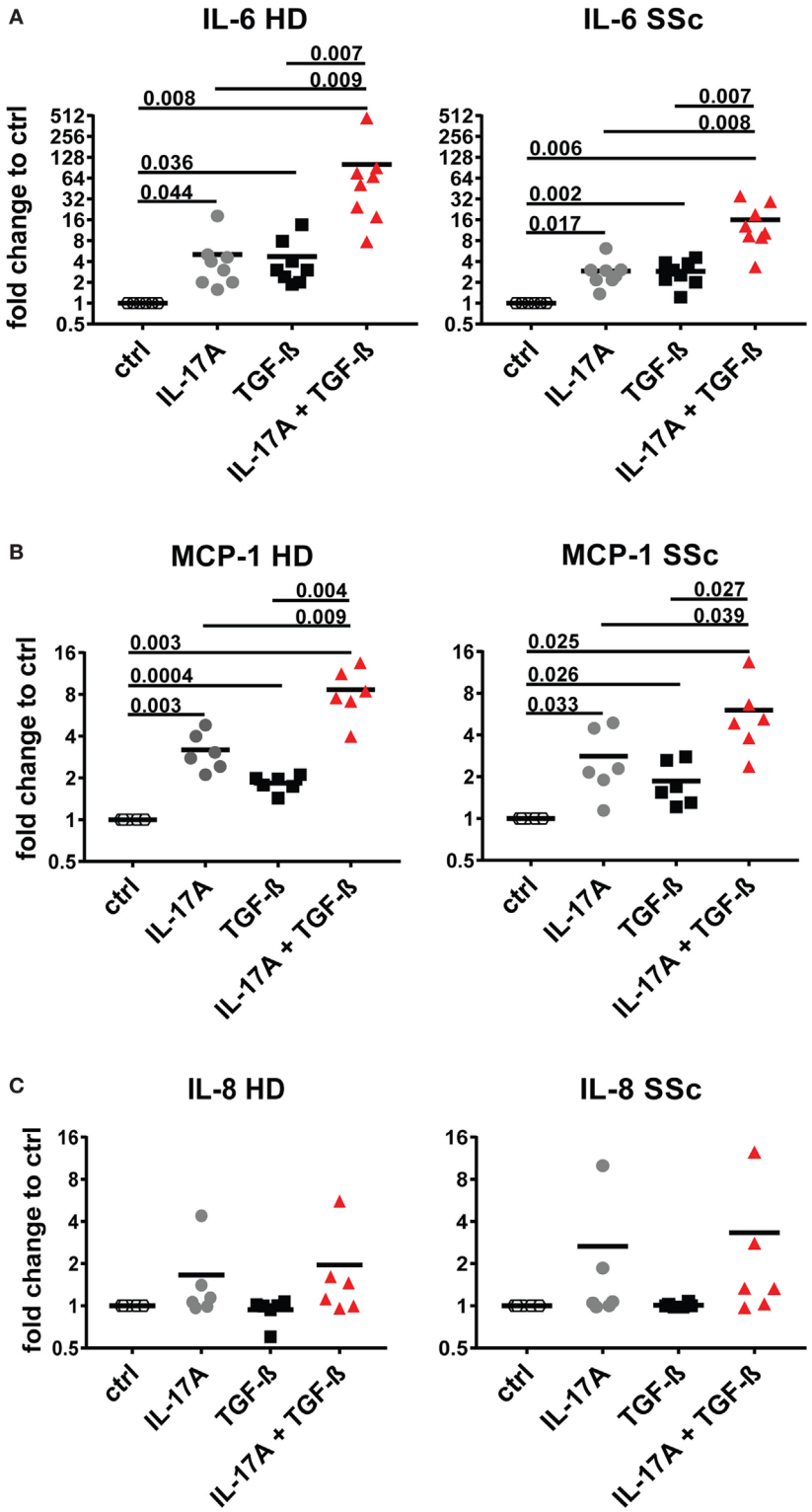

FIGURE 1 | IL-6 and monocyte chemotactic protein-1 (MCP-1) are synergistically and specifically induced in human fibroblasts by the combined action of IL-17A and TGF-β. Primary human dermal fibroblasts from healthy donors (HD) and systemic sclerosis (SSc) patients were cultured in the presence of IL-17A (25 ng/ml), TGF-β (2.5 ng/ml), or their combination for 48 h, in 96-well plates, in triplicates. IL-6 **(A)**, MCP-1 **(B)**, IL-8 **(C)** were assessed by ELISA in culture supernatants. Results are expressed as fold change compared to spontaneous production in control (ctrl) cultures. Basal levels were: 22.7 (±7.3) and 40.7 (±16.7) pg/ml for IL-6; 328.1 (±33.3) and 377.2 (±85.4) pg/ml for MCP-1 and 211.8 (±56.6) and 207.7 (±56.9) pg/ml for IL-8, in HD and SSc, respectively. Significance was assessed by paired *t* test.

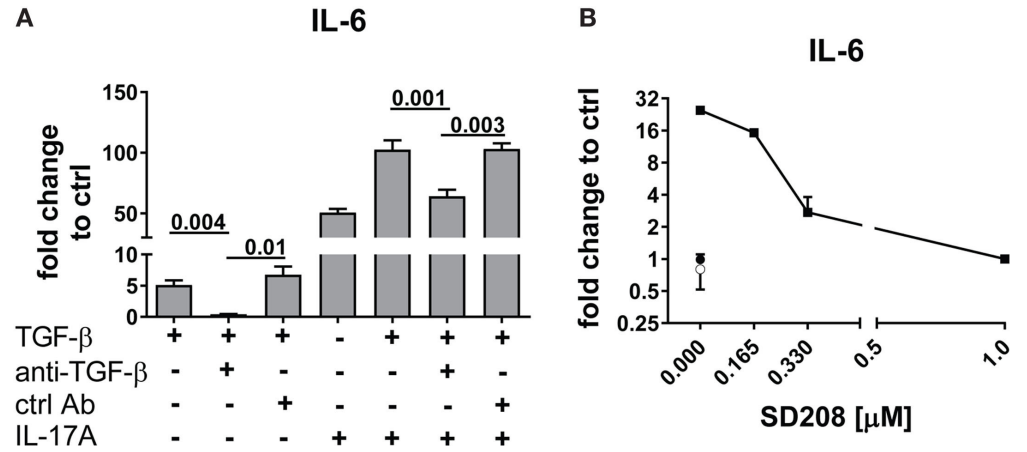

FIGURE 2 | TGF-β inhibition abrogates the synergistic response with IL-17A. HD fibroblasts were treated with **(A)** 10 μg/ml TGF-β 1 neutralizing antibody or 10 μg/ml of an irrelevant ctrl Ab, **(B)** SD208 (TGFβR1 inhibitor) or vehicle for 1 h prior to the addition of IL-17A (25 ng/ml) or TGF-β (2.5 ng/ml) and cultured for 48 h. IL-6 levels in SN were assessed by ELISA. Results are shown as fold change to untreated control cultures (basal level of IL-6 was 3.1 ± 1.1 pg/ml). **(B)** Square: TGF-β (10 ng/ml); empty circle: (SD2018, 1 μM); full circle: vehicle. Significant differences were assessed by paired *t*-test. Bars in **(A)** and symbols in **(B)** represent the mean + SEM of three experiments.

the production of IL-6 to the levels induced by IL-17A when used in combination with IL-17A (**Figure 2A**). Second, inhibition of TGFβR1 signaling by SD208 reduced IL-6 levels in a dose-dependent manner (**Figure 2B**). All together, these results indicate that TGF-β acts *via* its specific receptor and, most notably, that TGF-β synergizes with IL-17A to induce the fibroblast production of IL-6.

Common and Private Signaling Pathways Are Used by IL-17A and TGF-β to Induce IL-6

To unravel the mechanisms explaining the actions of IL-17A and TGF-β, we tested whether signaling pathways known to be involved in IL-6 transcription were used by IL-17A and TGF-β. We focused on IL-6 since it is considered a promising candidate target in SSc treatment (42). We found that, in HD fibroblasts, inhibition of NFκB by TPCA-1 preferentially inhibited the response to IL-17A (**Figure 3A**, left panel), while inhibition of PI3K/Akt by Ly294002 preferentially inhibited the response to TGF-β (**Figure 3A**, right panel). Interestingly, inhibition of p38 MAPK by SB203580, reduced the responses to both IL-17A and TGF-β (**Figure 3A**). By contrast, the inhibition of MEK1/2 with U0126 did not influence the production of IL-6, while inhibition of JNK by SP600125 increased its production in response to IL-17A, but not to TGF-β (**Figure 3A**). Importantly, fibroblast viability was found >90% for all culture conditions (**Figure 3B**).

NFκB and PI3K/Akt Signaling Pathways Are Privately Used by IL-17A and TGF-β and Synergize in the Induction of IL-6

The preferential use of NFκB by IL-17A and PI3K/Akt by TGF-β to stimulate the production of IL-6 (**Figure 3A**) prompted us to test whether these signaling pathways were involved in the synergistic action of IL-17A and TGF-β. The inhibition of NFκB (**Figure 4A**) and PI3K/Akt in HD fibroblasts (**Figure 4B**) decreased in a dose-dependent manner the production of IL-6 driven by the combined action of IL-17A and TGF-β. Notably, in these experiments, PI3K/Akt inhibition by itself did not affect the response to IL-17A, but when combined with NFκB inhibition, it further decreased the production of IL-6 (**Figure 4C**, left panel). Reciprocally, NFκB inhibition by itself did not affect the response to TGF-β, but when combined with PI3K/Akt inhibition, it further decreased the production of IL-6 (**Figure 4C**, middle panel). Moreover, the combined inhibition of NFκB and PI3K/Akt substantially reduced the production of IL-6 triggered by the joint presence of IL-17A and TGF-β (**Figure 4C**, right panel). Of note, suboptimal doses of inhibitors were used to perform the above experiments to favor the assessment of additive outcomes and decrease the likelihood of off-target effects. Furthermore, to ensure the specificity of the inhibitors used, we performed phospho-blot analysis. We observed that Ly294002 specifically inhibited the phosphorylation of Akt, while TPCA-1 specifically inhibited the phosphorylation of IκBα and the downstream p65 transcription factor of the NFκB complex (**Figure 6A**). Thus, NFκB and PI3K/Akt signaling converge in inducing the production of IL-6 by HD fibroblasts in the presence of IL-17A and TGF-β.

p38 MAPK Signaling Pathway Is Common to IL-17A- and TGF-β-Induced IL-6 Production

As reported above, p38 MAPK inhibition reduced the production of IL-6 induced by both IL-17A and TGF-β (**Figure 3A**). Not unexpectedly, substantial inhibition of IL-6 production was observed and this effect was dose-dependent, when HD fibroblasts were stimulated jointly by IL-17A and TGF-β (**Figures 5A,B**). Consistently with the inhibition experiments,

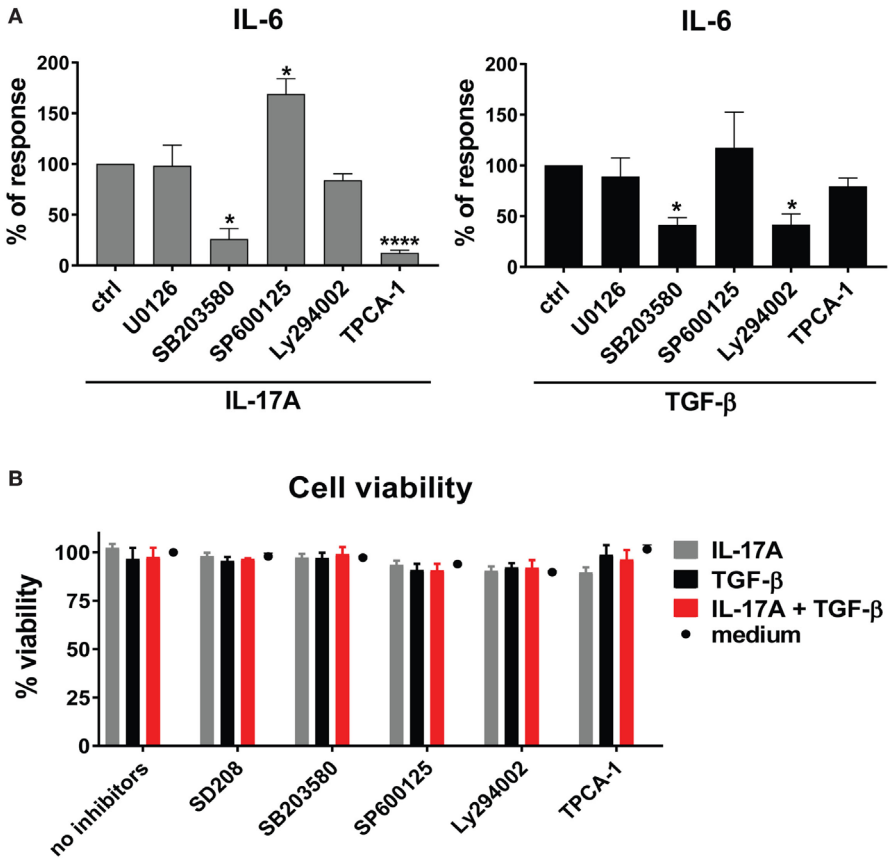

FIGURE 3 | Shared and private signaling pathways are preferentially used by IL-17A and TGF-β to induce IL-6. Healthy donors fibroblasts were treated with optimal doses of inhibitors (20 μM U0126, 20 μM SB203580, 10 μM SP600125, 10 μM Ly294002, or 0.37 μM TPCA-1) or vehicle for 1 h prior to the addition of IL-17A (25 ng/ml) or TGF-β (2.5 ng/ml) and cultured for an additional 48 h, in triplicates. **(A)** IL-6 levels in SN were assessed by ELISA. Results are shown as the percentage of IL-6 production induced by IL-17A or TGF-β in the absence of inhibitors (levels of IL-6 were: 22.8 ± 3.3 pg/ml for IL-17A and 8.8 ± 3.9 pg/ml for TGF-β). Bars represent the mean + SEM of three experiments. Significant differences versus control were assessed by paired *t*-test: $^{*}P < 0.05$, $^{****}P < 0.001$. **(B)**. Fibroblast viability was assessed by EZ4U and found >90% for all culture conditions.

the combined stimulation of HD fibroblasts by IL-17A and TGF-β resulted in 1.8-fold increase ($P = 0.025$) of p38 MAPK phosphorylation, when compared to levels of phosphorylated p38 MAPK induced by IL-17A alone, as assessed by Western blot (**Figure 6**). Furthermore, the enhanced phosphorylation of p38 MAPK in the joint presence of IL-17A and TGF-β was unique among the signaling molecules we examined (**Figure 6**). Thus, p38 MAPK is used by both IL-17A and TGF-β, separately and jointly, to induce the production of IL-6 by HD fibroblasts.

IL-17A Decreases SMAD2 Phosphorylation and Production of Type-I Collagen and Fibronectin Triggered by TGF-β

We and others have reported that IL-17A may decrease the fibroblast response to TGF-β when the ECM response is taken into consideration. We, therefore, explored in the same experimental settings in which IL-17A and TGF-β were displaying synergic activities for the induction of IL-6, whether the same hold true concerning collagen production. The canonical signaling pathway of TGF-β leads to SMAD2 phosphorylation and type-I collagen (col-I) production. We observed that in HD fibroblasts cultured in the joint presence of TGF-β and IL-17A, the phosphorylation of SMAD2 decreased by 0.6-fold ($P = 0.022$) when compared to that induced the TGF-β alone (**Figures 7A,B**). In addition, extending previous reports, IL-17A decreased significantly the production of col-I robustly induced by TGF-β. Most importantly, this was observed in both, HD ($P = 0.007$) and SSc ($P = 0.011$) fibroblasts (**Figure 7C**). Very interestingly, fibronectin was regulated by IL-17A and TGF-β in a similar manner as col-I. IL-17A did not modify fibronectin production, while it decreased the exuberant response induced by TGF-β (**Figure 7D**). Furthermore, when we looked at the ratio of col-I to MMP-1, as a surrogate of ECM turnover, we observed that this ratio was increased in the presence of TGF-β and significantly decreased when IL-17A was added to TGF-β (**Figures 8A,B**). Thus, our data support a dual role in the relationship between IL-17A and TGF-β. On the one hand, they cooperate in inducing IL-6, on the other hand, they exert opposite functions in inducing type-I collagen and fibronectin.

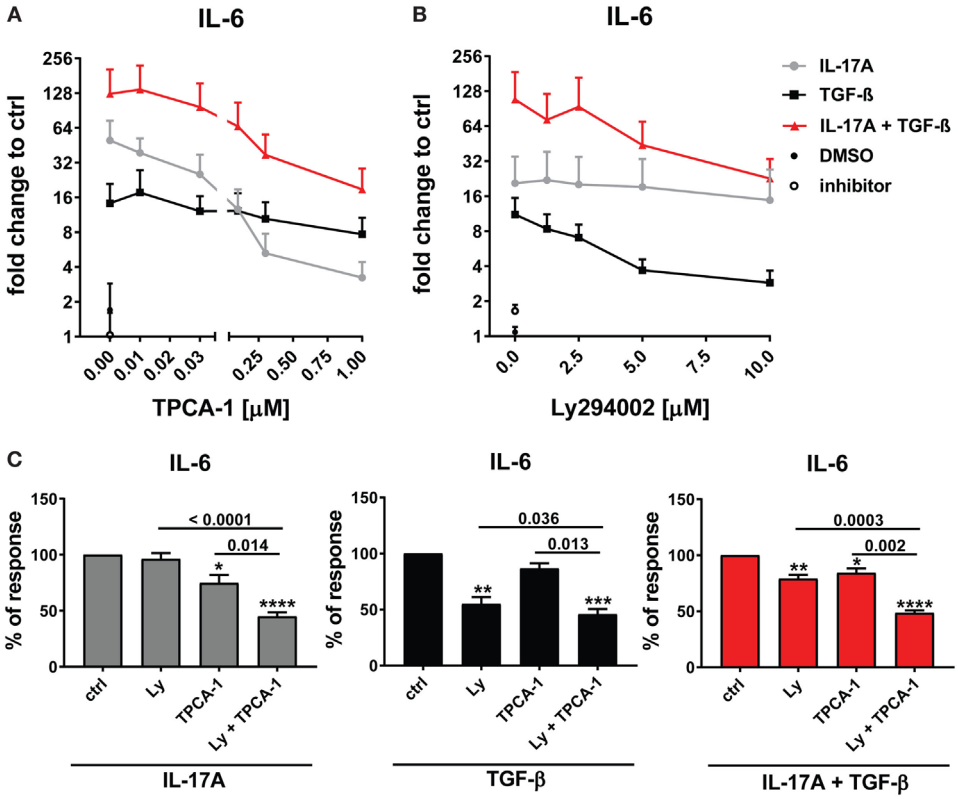

FIGURE 4 | NFκB and PI3K signaling pathways are preferentially used by IL-17A and TGF-β, respectively and together cooperate in inducing IL-6. Healthy donors fibroblasts were treated with the indicated concentrations of **(A)** TPCA-1; **(B)** Ly294002; or **(C)** suboptimal doses of TPCA-1 (0.03 μM) and/or Ly294002 (Ly, 2 μM) for 1 h prior to addition of IL-17A (25 ng/ml) and/or TGF-β (2.5 ng/ml). After 48 h, culture SNs were collected and IL-6 levels were assessed by ELISA. **(A,B)** Results are shown as fold change to untreated cells, mean + SEM is indicated ($N = 4$). Please note the $\log_2$ scale. **(C)** Results are shown as the percentage of IL-6 production induced by IL-17A and/or TGF-β in the absence of inhibitors (levels of IL-6 were: 86.2 ± 11.8 pg/ml for IL-17A, 49.6 ± 10.2 pg/ml for TGF-β, and 257.5 ± 82.5 pg/ml for IL-17A + TGF-β). Bars represent the mean + SEM. Significant differences versus control were assessed by paired *t*-test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.001$.

DISCUSSION

We show here for the first time a synergistic activity of IL-17A and TGF-β for the production of IL-6 (and MCP-1) by SSc and HD dermal fibroblasts, an effect, at least in part, dependent on the convergent signaling mediated by p38 MAPK, NFκB, and PI3K/Akt, as examined in HD. Additionally, our data show an inhibitory role of IL-17A in fibrosis, particularly for its negative effect on TGF-β-mediated col-I production dependent on SMAD signaling (**Figure 9**).

IL-17A is well known to stimulate IL-6 production by synovial-like fibroblasts (46) as well as HD and SSc dermal fibroblasts (27), in addition to other cell types (11). However, relatively few studies essentially performed with lung fibroblasts have documented the capacity of TGF-β to induce IL-6 production (47, 48). Our data are in agreement with such studies and prove that IL-6 production induced by TGF-β is dependent on TGFβR1 and mediated, at least in part, by PI3K/Akt and p38 MAPK-signaling pathways. Interestingly, in one previous study, TGF-β exerted a dual role, favoring IL-6 production when used alone, but reducing the production of IL-6 in fibroblast vigorously stimulated by IL-1 (47). We have not extensively explored the capacity of TGF-β to modulate the production of IL-6 induced by optimally activated

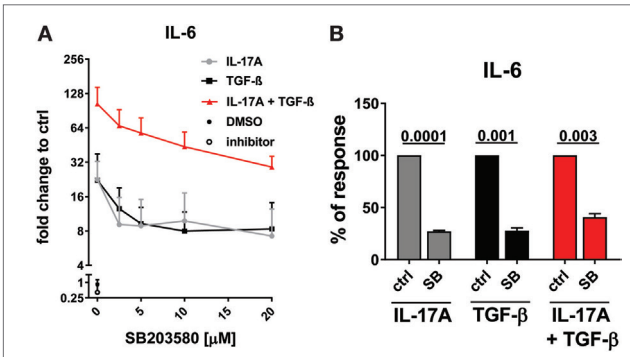

FIGURE 5 | p38 MAPK signaling pathway is common to IL-17A- and TGF-β-induced IL-6 production. Healthy donors fibroblasts were treated with the indicated concentrations of SB203580 **(A)** or 20 μM SB203580 **(B)** for 1 h prior to the addition of IL-17A (25 ng/ml) and/or TGF-β (2.5 ng/ml) in triplicates. After 48 h, culture SNs were collected and IL-6 levels were assessed by ELISA. **(A)** Results are shown as fold change to untreated cells, mean + SEM is indicated, $N = 3$. Please note the $\log_2$ scale. **(B)** Results are shown as the percentage of IL-6 production induced by IL-17A and/or TGF- β in the absence of inhibitor (levels of IL-6 were: 14.7 ± 7.4 pg/ml for IL-17A, 11.4 ± 6.1 pg/ml for TGF-β, and 48.9 ± 13.7 pg/ml for IL-17A + TGF-β). Bars represent the mean + SEM of three experiments. Significant differences versus control were assessed by paired *t*-test.

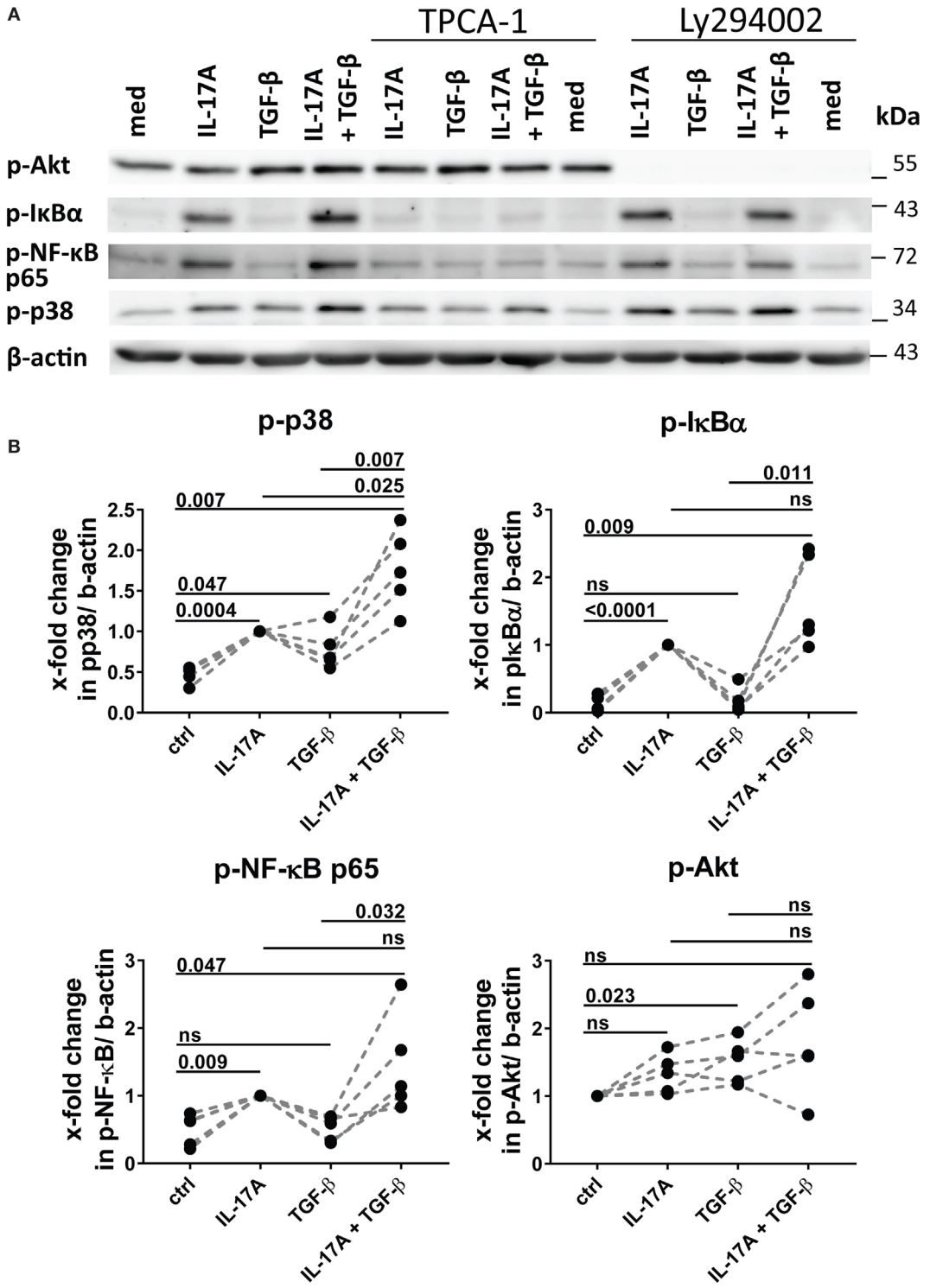

FIGURE 6 | Phosphorylation of MAPK p38 is enhanced by the combined action of IL-17A and TGF-β. **(A)** Western blot (WB) of healthy donors fibroblasts treated with 1 μM TPCA-1 and/or 10 μM Ly294002 for 1 h prior to addition of IL-17A (25 ng/ml) and/or TGF-β (2.5 ng/ml) and cultured for an additional 10 min. Results are representative of three experiments with inhibitors and two additional experiments with cytokines only. **(B)** Quantification of Western blot (WB) analysis was performed with ImageJ software (http://rsbweb.nih.gov/ij) and values were normalized to β-actin, $N = 5$. Results are shown as fold change to IL-17A-treated cells (for p-p38, p-IκBα, and p-NF-κB p65) or to untreated cells (for p-Akt), $N = 5$. Significance assessed by paired *t* test.

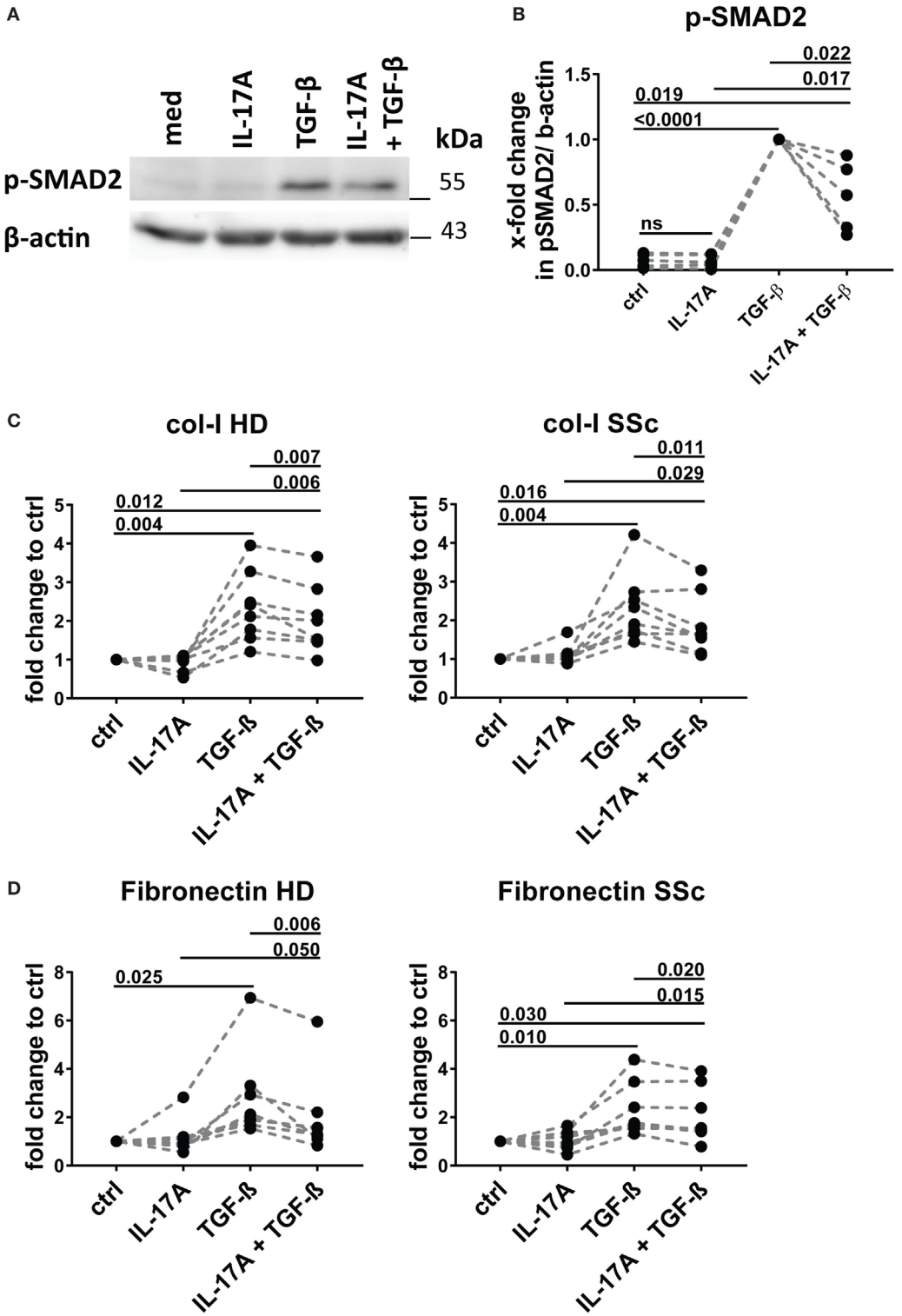

FIGURE 7 | IL-17A inhibits SMAD2 phosphorylation and production of type I collagen and fibronectin induced by TGF-β. Healthy donors (HD) fibroblasts were treated with IL-17A (25 ng/ml) and/or TGF-β (2.5 ng/ml) for 10 min. **(A)**. Western blot (WB) representative of five distinct experiments. **(B)** Quantification of WB analysis. Results are shown as fold change to TGF-β-treated cells, *N* = 5. **(C,D)** Primary human fibroblasts from HD (left panel) and systemic sclerosis (SSc) patients (right panel) in triplicates were treated with IL-17A (25 ng/ml), TGF-β (2.5 ng/ml), or a combination of these cytokines. After 48 h, culture supernatants were collected and type I collagen **(C)** and fibronectin **(D)** levels were assessed by ELISA (*N* = 5). Results are shown as fold change to untreated cells. Basal levels for col-I were 364.8 (±91.0) and 203.8 (±66.5) ng/ml and for fibronectin 972.7 (±273.9) and 1036,4 (±307.6) ng/ml, in HD and SSc, respectively. Significance was assessed by paired *t*-test.

fibroblasts by other cytokines, but we robustly observed a synergistic effect with IL-17A within the dose used.

The synergy of IL-17A and TGF-β on IL-6 production is reported for the first time in this study and observed in both, HD and SSc fibroblasts. While we show the preferential use of NFκB by IL-17A and PI3K/Akt by TGF-β to stimulate the production of IL-6, the combined inhibition of NFκB and PI3K/Akt substantially reduced the production of IL-6 triggered by the joint presence of IL-17A and TGF-β. Interestingly, we report that p38 MAPK signaling pathway was common for IL-17A-, TGF-β-, and combined IL-17A/TGF-β-induced IL-6 production and the phosphorylation of p38 MAPK was significantly higher in the joint presence of IL-17A and TGF-β, when compared to IL-17A alone. Thus, we propose a model where IL-17A synergizes with TGF-β to produce IL-6 by dermal fibroblasts using the common p38 MAPK transduction pathway in addition to the preferentially private use of NFκB and PI3K/Akt by IL-17A and TGF-β, respectively. Of note, inhibition of JNK by SP600125 resulted in higher levels of IL-6, thus suggesting that the expression of IL-6 depends on the fine balance of positive and negative regulators. The signaling studies were performed with HD fibroblasts.

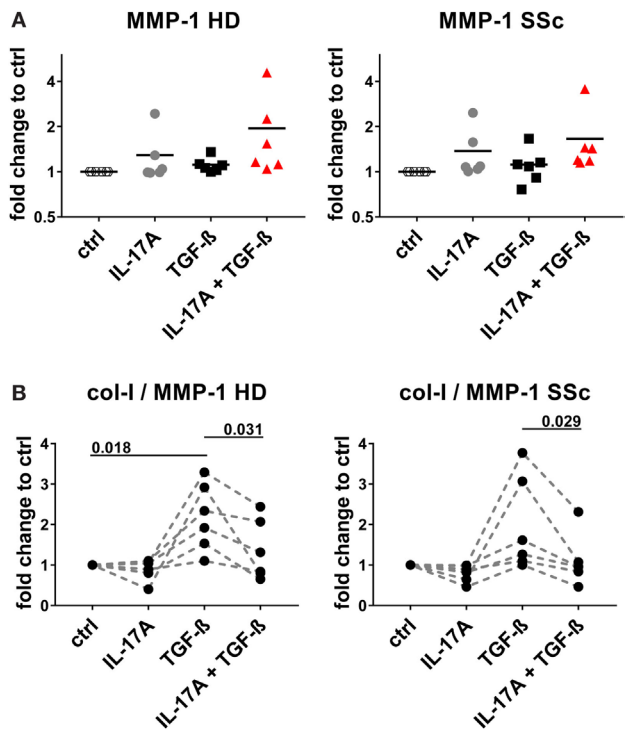

FIGURE 8 | IL-17A decreases the col-I to MMP-1 ratio enhanced by TGF-β. Primary human dermal fibroblasts from healthy donors (HD) (left panel) and systemic sclerosis (SSc) patients (right panel) were cultured in the presence of IL-17A (25 ng/ml), TGF-β (2.5 ng/ml), or their combination for 48 h, in 96-well plates, in triplicates. MMP-1 levels **(A)** were assessed by ELISA in culture supernatants. Results are expressed as fold change compared to spontaneous production in control (ctrl) cultures. Basal levels for MMP-1 were 15.71 (±1.3) and 20.1 (±2.3) ng/ml, in HD and SSc, respectively. **(B)** The ratio of col-I levels from **Figure 7C** to MMP-1 was calculated. Significance was assessed by paired *t*-test.

However, the convergence of these pathways may be at work also in SSc contributing to higher levels of IL-6.

The canonical TGF-β signaling pathway relies on phosphorylation of SMAD family proteins, thus contributing to col-I production (49–51). Our data show that IL-17A decreases TGF-β induced phosphorylation of SMAD2. Consistently with lower SMAD2 phosphorylation levels, both SSc and HD fibroblasts produced reduced levels of col-I and fibronectin in the presence of IL-17A and TGF-β, when compared to TGF-β alone. The mechanisms associated with SMAD2 reduced phosphorylation induced by IL-17A deserve further investigations. However, the inhibitory effect of IL-17A on TGF-β-triggered col-I production supports the data published by Truchetet et al. where IL-17A was shown to decrease TGF-β-induced α-SMA transcriptional activity in fibroblasts (18) and extend the reported IL-17-dependent inhibition of spontaneous col-I production by HD dermal fibroblasts reported by Nakashima et al. (24). However, at variance with Nakashima data, we observed the inhibitory effect of IL-17A on both HD as well as SSc fibroblast. In this respect is important to note that in our experiments the spontaneous col-I production was lower in SSc compared to HD fibroblasts.

Altogether, concerning fibrotic responses, the bulk of data generated *in vitro* with human material indicate that IL-17A has two facets. On the one hand, by itself, it increases inflammation inducing several pro-inflammatory cytokines and MMPs. In addition, it synergizes with TGF-β, as we show here, to further increase the production of IL-6 and MCP-1, which roles in fibrosis have been extensively reviewed (35, 52). On the other hand, it has direct inhibitory effects on TGF-β-triggered col-I production. Furthermore, IL-6 may enhance the activation of

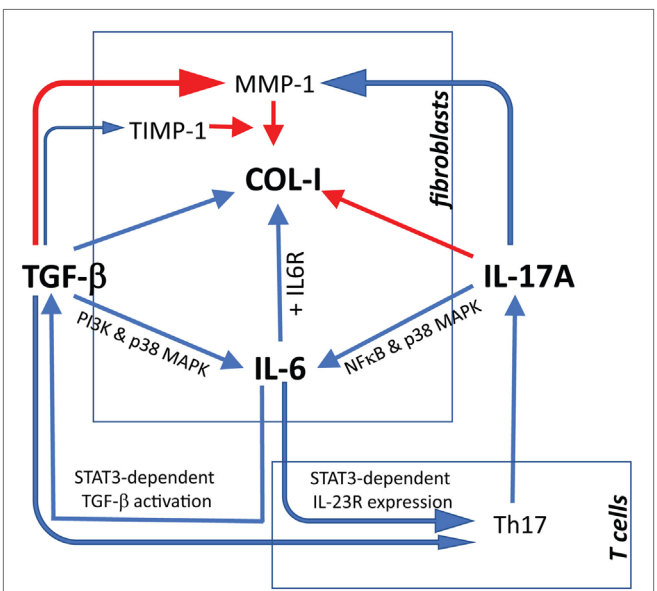

FIGURE 9 | Proposed model linking IL-17A, TGF-β, and IL-6 in the context of extracellular matrix deposition and Th17 cell differentiation. Blue arrows: stimulatory signal; red arrows: inhibitory signal. The relevant references are reported in the discussion. For tissue inhibitor of metalloproteinases 1 (TIMP-1), we refer to Fineschi et al. (45).

TGF-β in a STAT3-dependent mechanisms (53), and enhance Th17 cell proliferation by increasing IL-23 receptor expression on T cells (54). Additionally, TGF-β may play also a role in Th17 differentiation program by enhancing RORγT expression (55). Thus, IL-6 and TGF-β may cooperate to polarize and expand Th17 cells. In turn, IL-17A produced by Th17 cells may simultaneously exert antifibrotic responses by inhibiting collagen and pro-fibrotic responses by enhancing IL-6 production (**Figure 9**). An obvious limitation to this scenario is the minimalistic *in vitro* approach used to generate our data in which fibroblast monolayers are submitted to the influence of a limited number of effector molecules. To overcome such limitation, more complex experimental systems have to be adopted in which fibroblasts should be submitted to stimulation in three dimensional cultures and under the influence of other cell types, which likely play a role in modulating ECM production. This is of outmost importance, since substantial discrepancies have been highlighted when human and *in vivo* murine models are compared for the role of IL-17A in fibrosis (21). It may be argued that no differences were observed in our settings when SSc and HD responses were compared. However, we think that our observations are relevant to SSc since we and other have documented increased levels of the agonist used, namely TGF-β and IL-17A in SSc tissues compared to HD. Differences in the amount of these cytokines may result in stronger responses *in vivo*. The limited number of SSc samples (4 lSSc and 5 dSSc) tested in our experiments did not allow detecting differences between disease subsets.

From a therapeutic standpoint, the interplay between IL-17A, TGF-β, and IL-6 is of major interest, since all these three cytokines are potential targets for therapy in SSc (42, 56–58). On the one hand, the direct antifibrotic role of IL-17A as well as its inhibitory activity on TGF-β-induced collagen production, would suggest that inhibition of this cytokine may have detrimental effects in humans affected by SSc. On the other hand, since IL-6 may directly or indirectly favor fibrosis, the blockade of factors enhancing the production of IL-6 may have favorable effects. In any case, therapeutic trials involving the blockade of any of these cytokines would profit of an in-depth analysis of their potential interactions.

ETHICS STATEMENT

This study was approved by the ethical committee of the institutions involved (06-063, Commission cantonale d'éthique de la recherche, Geneva, Switzerland) and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from each individual.

AUTHOR CONTRIBUTIONS

The author contribution was as followed: conceiving the research and manuscript drafting (AD and CC); performing the experiments (AD, MA, and BR); data analysis (AD, MA, and CC). All authors read and approved the final manuscript.

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Soluble HLA-G Expression Inversely Correlates With Fetal Microchimerism Levels in Peripheral Blood From Women With Scleroderma

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Women with scleroderma (SSc) maintain significantly higher quantities of persisting fetal microchimerism (FMc) from complete or incomplete pregnancies in their peripheral blood compared to healthy women. The non-classical class-I human leukocyte antigen (HLA) molecule HLA-G plays a pivotal role for the implantation and maintenance of pregnancy and has often been investigated in offspring from women with pregnancy complications. However data show that maternal *HLA-G* polymorphisms as well as maternal soluble HLA-G (sHLA-G) expression could influence pregnancy outcome. Here, we aimed to investigate the underlying role of maternal sHLA-G expression and *HLA-G* polymorphisms on the persistence of FMc. We measured sHLA-G levels by enzyme linked immunosorbent assay in plasma samples from 88 healthy women and 74 women with SSc. Male Mc was quantified by DYS14 real-time PCR in blood samples from 58 women who had previously given birth to at least one male child. Furthermore, eight *HLA-G* 5′URR/3′UTR polymorphisms, previously described as influencing HLA-G expression, were performed on DNA samples from 96 healthy women and 106 women with SSc. Peripheral sHLA-G was at lower concentration in plasma from SSc (76.2 ± 48.3 IU/mL) compared to healthy women (117.5 ± 60.1 IU/mL, $p < 0.0001$), independently of clinical subtypes, autoantibody profiles, disease duration, or treatments. Moreover, sHLA-G levels were inversely correlated to FMc quantities (Spearman correlation, $p < 0.01$). Finally, women with SSc had lower sHLA-G independently of the eight *HLA-G* 5′URR/3′UTR polymorphisms, although they were statistically more often homozygous than heterozygous for *HLA-G*

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polymorphism genotypes −716 (G/T), −201 (G/A), 14 bp (ins/del), and +3,142 (G/A) than healthy women. In conclusion, women with SSc display less sHLA-G expression independently of the eight *HLA-G* polymorphisms tested. This decreased production correlates with higher quantities of persisting FMc commonly observed in blood from SSc women. These results shed some lights on the contribution of the maternal HLA-G protein to long-term persistent fetal Mc and initiate new perspectives in this field.

Keywords: human leukocyte antigen-G, microchimerism, scleroderma, systemic sclerosis, pregnancy, fetal

INTRODUCTION

Scleroderma is a rare, invalidating, and complex autoimmune disease, manifested by vascular abnormalities, extensive collagen deposition in the skin, and fibrotic changes in internal organs. SSc is divided into two forms: limited cutaneous disease (lcSSc) characterized by skin involvement below the elbows and knees and diffuse cutaneous disease (dcSSc) for which skin involvement is more extended, with respectively anti-centromere or anti-topoisomerase antibodies (ACA or ATA) as hallmark of each subset (1).

SSc has a strong predilection for women with a peak incidence in women after childbearing years. Moreover, SSc shares some clinical similarities to chronic graft-vs-host disease (cGVHD) (2, 3). Both diseases are characterized by skin, lung, and esophageal involvement and intense fibrosis (2). In cGvHD, donor cells attack the recipient after allogeneic bone marrow stem cell transplantation. This led to the hypothesis that fetal microchimerism (FMc) arising from pregnancy and persisting at long term in the mother may act against the "host" in an "auto/allo" immune reaction and be involved in the development of SSc [for reviews see Ref. (4, 5)].

Although only few studies have demonstrated a role for microchimeric fetal cells in SSc (6–8), many have shown higher levels of fetal Mc in peripheral blood mononuclear cells from women with SSc compared to healthy women (6, 9–12). A few propositions have been raised to explain the possible genetic susceptibility for higher passage of fetal cells during pregnancy and/or maintenance of post-delivery in women with SSc. The host's and/or donor's Human Leukocyte Antigen (HLA) genotype may influence the likelihood of having microchimeric cells in host's blood as previously suggested in SSc and juvenile dermatomyosis (13, 14).

A non-classical class-I HLA molecule, HLA-G, described to play a pivotal role for the implantation and maintenance of pregnancy [for review see Ref. (15)], could influence fetal Mc. As the expression of cell-associated and secreted HLA-G antigens was first and mostly described in placenta on villous cytotrophoblasts at the feto–maternal interface (16, 17), HLA-G has often been investigated in offspring from women with pregnancy complications. Fetal HLA-G is the specific ligand of some receptors (i.e., ILT2, ILT4, and KIR2DL4) present on maternal decidual NK, dendritic cells, and macrophages, which constitute a large part of the immune cells in the uterine compartment, and exerts immunomodulatory functions to secure acceptance of the semiallogenic fetus [for review see Ref. (18)]. Nevertheless, maternal monocytes and dendritic cells express membrane-bound and soluble HLA-G (sHLA-G), respectively (19). The role of maternal HLA-G on the immune tolerance process in pregnancy is poorly understood, although data show that maternal *HLA-G* polymorphisms as well as maternal sHLA-G expression could influence pregnancy outcome (20, 21).

Interestingly, women with pregnancy complications have lower sHLA-G concentration in their plasma (22, 23) and higher fetal Mc passage in their circulation than women with healthy pregnancies (24, 25). Moreover, women with pregnancy complications are at higher risk to develop later autoimmune diseases, such as scleroderma or rheumatoid arthritis (26–28). This has drawn our attention on a potential role for maternal HLA-G in higher FMc levels in women with SSc.

Human leukocyte antigen-G molecule can be expressed as membrane-bound or soluble form through 7 isoforms generated by alternative splicing: HLA-G1 to G7. HLA-G1 to G4 are membrane bound; only HLA-G1 can be shed from the membrane and also released as sHLA-G1 (29). HLA-G5, G6, and G7 are only soluble forms, HLA-G5 being the most expressed. HLA-G protein expression seems to be modulated by several genetic variations within the coding sequence, the 5′ upstream regulatory region (5′URR) and the 3′ untranslated region (3′UTR) of the *HLA-G* gene (30–37). Conflicting results for sHLA-G production were reported for the most studied 3′UTR polymorphism, the 14-bp insertion/deletion polymorphism in the exon 8 (31, 38–40).

Thus, in the current study we test whether 1/women with SSc display lower sHLA-G levels in their plasma than healthy age-matched women, 2/quantities of persistent fetal Mc in their blood inversely correlate with sHLA-G levels, and 3/sHLA-G levels correlate with *HLA-G* polymorphisms/haplotypes. We recruited 96 healthy women and 106 women with SSc, quantified sHLA-G in plasma samples from respectively 88 and 74 women by enzyme linked immunosorbent assay (ELISA) and analyzed 8 polymorphisms in the 5′URR and 3′UTR of the *HLA-G* gene, including the most described 14-bp insertion/deletion to determine UTR1-8 haplotypes. In parallel, using DYS14 real-time PCR assays, we analyzed male microchimerism of fetal origin in peripheral blood samples from 58 women who had given birth to at least one male child.

MATERIALS AND METHODS

Subjects

A total of 106 women with SSc were recruited in four French hospitals (*St. Louis* hospital in Paris; *Claude Huriez* Hospital in Lille; *La Conception* and *Nord* Hospital in Marseille, France). All patients met the requirements of LeRoy for SSc (41), with 48

having diffuse cutaneous disease (dcSSc) and 55 limited cutaneous disease (lcSSc). Three of them were only defined as having SSc with no indication of clinical subtype. Women were majorly Caucasian (80.2%), then African (14.2%), and Asian (5.6%). Their mean age at blood draw was 54.4 years old [range:16–75].

In parallel, 96 healthy women with no family history of autoimmune disease were recruited in the Centre d'Examen de Santé de l'Assurance Maladie (CESAM), Marseille, France. They were majorly Caucasian (94.8%), then African (3.1%), and Asian (2.1%). Their mean age at blood draw was 50.8 years old [range: 36–69]. Questionnaires with detailed information about history of source of male Mc (transfusion, history of pregnancy, and older brother) were filled in for each participant of the study. For one patient, we could not obtain all information.

Ethics Statement

This study has received the approval from the French Ethical Committee Marseille 2 and is registered at the INSERM (Biomedical Research Protocol number RBM-04-10). All participants signed written consent forms according to the Declaration of Helsinki (42).

sHLA-G Measurement

Plasma samples from 88 healthy women and 74 women with SSc were obtained after gradient centrifugation of whole peripheral blood on Ficoll–Hypaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). Plasma were stored at −40°C until tested. Measurement of both shed HLA-G1 and sHLA-G5 isoforms was performed in duplicate on plasma samples from 88 healthy women and 74 women with SSc using the ELISA assay kit (EXBIO/Biovendor, Karásek, Czech Republic; capture antibody: MEM-G/9), defined at the "Wet-Workshop for the Quantification of sHLA-G" in 2004 (43) according to the manufacturer's instructions. It is to note that the current assay does not allow to distinguish which isoform HLA-G1 or G5 is the most expressed. sHLA-G standard was diluted to obtain a calibrator curve within a range from 3.91 to 125 International units/mL (IU/mL) for sHLA-G ELISA. The total protein concentration levels were expressed in IU/mL of plasma.

DNA Sample Handling

DNA from 96 healthy women and 106 women with SSc was isolated from 350 µl of blood samples by commercially available method (Qiagen EZ1). DNA concentrations were quantified by Biodrop instrument according to the manufacturer's protocol and further by beta-globin-specific Q-PCR as previously described (44). DNA samples from 58 women who had given birth to at least 1 male child were further analyzed for microchimerism studies (38 SSc and 20 healthy women). All DNA samples were sent to the Immunogenetic laboratory at the French Blood Transfusion Department (EFS), Marseille, France, for analyzing the 5′URR and 3′UTR polymorphisms (see below).

Quantification of Male Mc of Fetal Origin

Male Mc was quantified by a standardized real-time PCR for a Y-chromosome-specific sequence DYS14 on a Light Cycler® with Light Cycler® Fast Start DNA Master PLUS Reaction kits (Roche, Indianapolis, IN, USA) as previously described (12). Sensitivity of the DYS14 assay was of 1 genome equivalent (1 gEq) of male cell in a background of 20,000 gEq female cells. Each DNA sample from whole peripheral blood was then tested for DYS14 amplification in 10 aliquots of DNA equivalent of 20,000 cells (=132 ng with the conversion of 1 cell = 6.6 pg). The amount of male DNA was expressed as the number of gEq of male cells per million (M) of gEq of female cells (gEq/M).

There was no occurrence of a positive amplification in any of the negative controls (no DNA template). A triplicate of male DNA at 10 or 50 gEq, in a background of 20,000 gEq, was systematically run in each plate and served as inter-plate reference standard and positive control.

Presence of male DNA has been tested in peripheral blood from 58 women (38 women with SSc and 20 healthy women) who had previously given birth to at least one son. Obstetrical and clinical characteristics are detailed in **Tables 1** and **2**, respectively.

HLA-G 5′URR and 3′UTR Genotyping and UTR Haplotype Estimation

A home-made primer extension method, as previously described, was used to simultaneously analyze four SNPs in the 5′URR region (−725C/G/T rs1233334, −716 G/T rs2249863, −201 G/A rs1233333, and −56C/T rs17875397) and four polymorphisms in the 3′UTR region (ins/del exon 8 rs66554220; 3142C/G rs1063320; 3187G/A rs9380142; and 3196C/G rs1610696) (45). *HLA-G* genotypes were analyzed using GeneMapper 4.0 with specific detection parameters. UTR~*HLA-G* haplotypes were estimated using an EM algorithm from the Gene (46) program and confirmed using the EM and ELB algorithms from the Arlequin v3.5.1.2 package. Data were analyzed and interpreted as previously described (45, 47).

Statistical Analyses

Mann–Whitney test was applied to compare sHLA-G dosages (expressed in IU/mL) between patients and controls, independently or according to *HLA-G* genetic status, SSc-associated treatments, age of probands or disease duration.

To quantify the degree to which sHLA-G quantities is related to fetal male DNA quantities, we excluded women with history of blood transfusion, or for whom we did not have transfusion information to not confound natural (arising from pregnancy) and iatrogenic (from transplantation) sources of male Mc. Nevertheless, to test whether male DNA levels arising from pregnancy correlated with sHLA-G levels in plasma, we did not eliminate women who had spontaneous or induced abortions additionally to their son, although we did not know the sex of the fetus. Correlation was assessed with Spearman rank correlation test. *P* values less than 0.05 were considered significant. Statistical analyses were conducted using GraphPad Prism 6 (La Jolla, CA, USA).

Significant deviations from expected values at Hardy–Weinberg equilibrium (HWE) were tested for each polymorphisms using Chi square "goodness of fit" test (http://vassarstats.net). As for two SNPs HWE was not respected (−201 (A/G), −56 (C/T)) in the healthy population, and numbers were small, we did not use the classical χ^2 for evaluating whether women with

TABLE 1 | Obstetrical and clinical characteristics of women with SSc quantified for male DNA and soluble HLA-G (sHLA-G) levels.

SSc women	Age at blood draw (years)	Disease duration at blood draw (years)	# of sons	Age of last son (years)	Spontaneous or induced abortions	Blood transfusions	# of older brothers	Male Mc[a] (gEq/M)	sHLA-G[b] (IU)
SSc01	63	10	1	25	1	Ukn[c]	**0**	**22.5**[d]	84.0
SSc02	60	5	1	35	0	0	**0**	**0.5**	23.6
SSc03	45	2	1	20	0	0	**0**	**12.7**	17.1
SSc04	58	1	1	30	0	0	**1**	**1.6**	49.2
SSc05	73	6	2	Ukn	0	0	0	0	119.7
SSc06	49	5	1	9	1	0	**0**	**4.5**	83.5
SSc07	78	23	1	56	0	0	**0**	**2.6**	83.0
SSc08	64	1	6	Ukn	0	0	1	0.0	62.2
SSc09	55	6	3	33	0	**1**[e]	**0**	**12.5**	25.2
SSc10	45	5	2	Ukn	1	0	**3**	**10.0**	21.3
SSc11	51	4	2	15	1	0	**1**	**4.3**	19.0
SSc12	60	9	1	36	0	0	**1**	**2.7**	116.9
SSc13	62	6	1	37	0	0	1	0.0	52.8
SSc14	47	18	1	16	0	0	**0**	**14.0**	90.8
SSc15	50	5	2	17	0	0	1	0.0	61.3
SSc16	58	5	2	33	0	**4**	**0**	**4.5**	66.2
SSc17	66	9	1	37	3	0	1	0.0	31.0
SSc18	53	3	1	19	1	0	1	0.0	142.0
SSc19	62	23	1	Ukn	1	0	0	0.0	95.9
SSc20	72	6	1	Ukn	2	Ukn	2	0.0	177.2
SSc21	64	5	2	Ukn	0	**1**	1	0.0	176.7
SSc22	35	5	1	10	0	0	2	0.0	123.9
SSc23	48	0	2	20	0	0	3	0.0	87.9
SSc24	43	2	2	Ukn	1	**1**	0	0.0	98.9
SSc25	57	9	2	Ukn	5	**1**	3	0.0	54.2
SSc26	49	12	1	44	Ukn	0	**Ukn**	**9.7**	25.9
SSc27	40	13	1	15	2	0	0	0.0	47.1
SSc28	66	16	2	37	0	0	2	0.0	34.5
SSc29	62	13	1	40	2	Ukn	**0**	**2.3**	67.5
SSc30	61	1	1	32	1	0	**1**	**7.1**	16.3
SSc31	52	2	1	32	0	0	1	0.0	64.2
SSc32	45	22	1	24	0	0	1	0.0	89.2
SSc33	37	0	1	7	0	0	1	0.0	164.0
SSc34	54	17	1	26	0	0	0	0.0	76.3
SSc35	56	12	1	17	1	0	**0**	**53.2**	49.8
SSc36	40	3	1	15	1	0	2	0.0	92.8
SSc37	53	19	2	25	2	0	**0**	**0.5**	37.5
SSc38	75	25	4	33	1	**2**	0	0.0	106.3

[a]*Male microchimerism is expressed in genome equivalent of male cells per million of maternal cells (gEq/M).*

[b]*Quantities of sHLA-G are expressed in International Units per mL of plasma (IU/mL).*

[c]*Unknown data are abbreviated with ukn.*

[d]*Positive values for male Mc are in bold.*

[e]*Women who had an history of transfusion are in bold and were excluded from statistical analysis, as well as women with unknown data for blood transfusion.*

SSc were less often heterozygous for *HLA-G* polymorphisms than healthy women, but rather the Bayesian statistical method (R software version 3.0.2.10), as recommended in such cases (48). *P* values were further corrected for multiple comparison tests by Benjamini–Hochberg correction (49).

Chi-square "χ^2" tests with Benjamini–Hochberg corrections were used to compare UTR haplotype frequencies between healthy women and women with SSc.

RESULTS

Women With SSc Displayed Lower sHLA-G in Their Plasma Compared to Healthy Women

Soluble HLA-G (shed HLA-G1 and sHLA-G5) could be detected by the ELISA technique defined at the "Wet-Workshop for the Quantification of sHLA-G" in all plasma samples (43), with quantities ranging from 8.8 International Units (IU) per mL of plasma to 187.9 IU/mL in samples from women with SSc and from 13.5 to 262.5 IU/mL in samples from healthy women (**Figure 1**).

Women with SSc had significantly lower quantities of sHLA-G than healthy women in their plasma with respectively a mean of 76.2 IU/mL compared to 117.5 IU/mL ($p < 0.0001$).

Lower sHLA-G Was Independent of Clinical Subtypes, Autoantibody Profiles, Disease Duration, Treatments, or *HLA-G* 5′URR/3′UTR Polymorphisms

Quantities of sHLA-G were identical whether women with SSc had diffuse or limited cutaneous SSc (**Figure 2A**), were positive for ATA or anti-centromere antibodies (ACA) or were negative for both autoantibodies (**Figure 2B**). No correlation was observed

TABLE 2 | Obstetrical and clinical characteristics of healthy women quantified for male DNA and soluble HLA-G (sHLA-G) levels.

Healthy women (HW)	Age at blood draw (years)	# of sons	Age of last son (years)	Spontaneous or induced abortions	Blood transfusions	# of older brothers	Male Mc[a] (gEq/M)	sHLA-G[b] (IU)
HW01	37	1	4	0	0	**0**	**0.61**[c]	119.7
HW02	57	2	23	1	0	0	0	26.2
HW03	58	1	22	0	0	2	0	81.2
HW04	58	1	40	3	**1**[d]	**2**	**2.3**	140.7
HW05	42	1	15	2	0	0	0	112.1
HW06	38	1	9	0	0	0	0	13.5
HW07	60	1	32	1	0	0	0	117.8
HW08	54	1	26	0	0	0	0	150.4
HW09	53	1	38	0	0	3	0	37.3
HW10	66	1	35	0	0	0	0	65.0
HW11	47	1	23	0	0	3	0	212.9
HW12	54	2	21	1	0	0	0	157.4
HW13	40	2	9	0	0	0	0	49.3
HW14	54	1	25	0	**2**	1	0	170.2
HW15	46	1	17	1	0	1	0	35.6
HW16	49	1	24	0	0	0	0	94.6
HW17	41	2	13	1	0	**1**	**1.07**	60.0
HW18	50	1	19	1	0	0	0	76.5
HW19	59	3	25	2	0	0	0	62.2
HW20	49	1	22	1	0	1	0	228.0

[a]*Male microchimerism is expressed in genome equivalent of male cells per million of maternal cells (gEq/M).*
[b]*Quantities of sHLA-G are expressed in International Units per mL of plasma (IU/mL).*
[c]*Positive values for male Mc are in bold.*
[d]*Women who had an history of transfusion are in bold and were excluded from statistical analysis.*

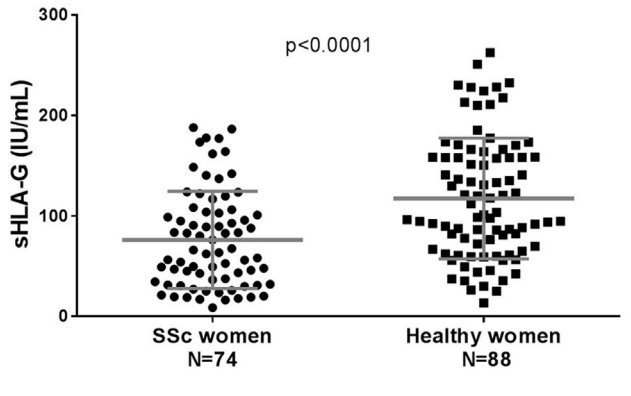

FIGURE 1 | Quantification of soluble HLA-G (sHLA-G) in plasma from healthy and SSc women. Quantification of sHLA-G is expressed in International Units per mL of plasma (IU/mL). Black circles represent values for women with SSc and black squares represent values for healthy women. Means and SDs are given for each scatter plot by gray bars. *P* value is calculated by Mann–Whitney test.

between sHLA-G levels and the age of probands or disease duration (data not shown).

Soluble HLA-G dosages were not different whether women were under immunosuppressive therapies (cyclophosphamide, methotrexate, etc.) with or without anti-inflammatory medications (non-steroidal or corticosteroids, etc.) or had only drugs to treat consequences of the disease, such as vasodilatator therapies (calcium channel blockers, angiotensin converting enzyme inhibitors, etc.), antifibrotic agents (colchicine, etc.), or others (i.e., proton pump inhibitors for gastroesophageal reflux) (**Figure 2C**).

Finally, the significant decrease of sHLA-G expression observed in SSc women compared to healthy women seemed independent from the eight *HLA-G* 5′URR/3′UTR tested polymorphisms as the difference remained significant when patients and controls were separated according to their *HLA-G* genotypes for each polymorphic site (**Figure 3**).

Lower sHLA-G Expression Correlated With Higher Levels of Male Microchimerism

In women with SSc, sHLA-G expression was inversely correlated with levels of fetal Mc in their peripheral blood as illustrated in **Figure 4** [Spearman correlation coefficient $r = -0.46$, 95% confidence interval (−0.71 to −0.10), $p = 0.01$]. Quantities of male DNA in maternal blood seemed to be independent from the number of sons, the number of years since the birth of last son or the disease duration (**Table 1**). Male Mc observed here is more probably from pregnancy than from an older brother, since 69% of women with SSc who had male Mc did not have an older brother (**Table 1**).

Women With SSc Displayed a Lack of Heterozygosity for *HLA-G* Polymorphisms Compared to Healthy Women

A lack of heterozygosity among genotypes was observed in the SSc population with statistical significance for four polymorphisms: −716 (G/T), −716 (G/T), −201 (G/A), exon 8 (Ins/Del), and +3,142 (G/C), even after correction for multiple comparisons (**Table 3**). However, none of the polymorphisms described here displayed allelic frequency differences between women with SSc and healthy women.

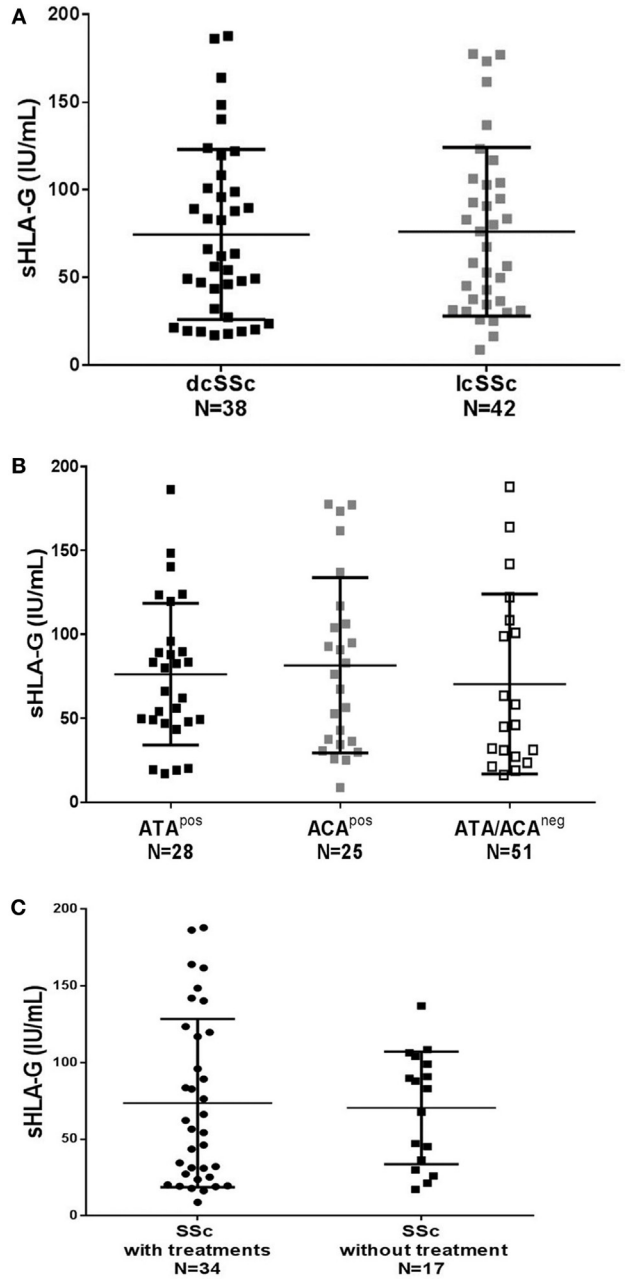

FIGURE 2 | Quantification of soluble HLA-G (sHLA-G) in plasma from women with SSc divided according to **(A)** their clinical status: black squares represent values for women with diffuse cutaneous SSc (dcSSc), gray squares represent values for women with limited cutaneous SSc (lcSSc); **(B)** their autoantibody status: black squares represent values for women with SSc positive for anti-topoisomerase antibodies (ATA^{pos}), gray squares represent values for women with anti-centromere antibodies (ACA^{pos}), and white squares represent values for women negative for both autoantibodies (ATA/ACA^{neg}); and **(C)** their treatments: black circles represent values for women who are under immunosuppressive therapies and/or anti-inflammatory medications, labeled "with treatments"; black squares represent values for women who have only drugs to treat consequences of the disease and labeled "without treatments." Means and SDs are given for each scatter plot by gray bars *P* values are calculated by Mann–Whitney test in **(A,C)** and one way-ANOVA test in **(B)** and are not significant for any of the graphs ($p = 0.85$, $p = 0.49$, and $p = 0.75$, respectively).

As there was significantly more individuals of African and Asian origin in cases than in controls ($P < 0.01$), we performed the same analysis by considering only Caucasian individuals. Results were similar than the ones with all cases and controls (Table S1 in Supplementary Material).

Frequencies of UTR Haplotypes in Healthy Women and Women With SSc

Human leukocyte antigen-G haplotypes were defined by four SNPs in the 5′URR and four polymorphisms in the 3′UTR are named UTR. UTR frequencies between women with SSc and healthy women (**Table 4**), did not display any significant difference, although a tendency for an increase of the UTR2 haplotype in SSc, particularly in dcSSc was observed, none of the *p* values remained significant after correction for multiple tests. Similarly, the small decrease for the UTR5 and UTR7 haplotypes in SSc, particularly in dcSSc, did not remain significant after correction.

DISCUSSION

The HLA-G molecule has tolerogenic functions and its role has been described in transplantation, human reproduction, and more recently in some rheumatologic diseases, including SSc [for review see Ref. (50)]. In the present study, we focus on the initially described role of HLA-G expression on immune tolerance during pregnancy. We hypothesized that the observation described by our group and others, that parous women with SSc maintain higher levels of fetal Mc compared to matched healthy controls, is due to an impaired expression of maternal sHLA-G. Studies on preeclampsia (PE) support our hypothesis as women with pregnancies complicated with PE have lower levels of sHLA-G in their blood in the second and third trimester compared to controls [for review see Ref. (51)]. In parallel to low sHLA-G production, pregnancies with PE are associated with higher passage of circulating fetal DNA and fetal cells in maternal peripheral blood (24, 25).

In the current study, we validate the hypothesis that women with SSc produce less sHLA-G in their plasma than healthy women. Quantities are low and at comparable levels whether women with SSc have a dcSSc or lcSSc and whether they produce ATA, ACA, or none of those antibodies. Notably, treatments associated with SSc do not seem to have an influence on sHLA-G levels, as women who had only drugs treating consequences of the disease (i.e., gastroesophageal reflux, etc.) have levels as low as women under immunosuppressive therapies and/or anti-inflammatory medications. The ELISA used is a sandwich enzyme immunoassay for the quantitative measurement of any soluble forms of HLA-G. Therefore, it measures the shed sHLA-G1 and the secreted HLA-G5 isoform without distinction and we cannot tell at this point which one of the isoform is less present in our measurements.

To our knowledge, only two studies have recently evaluated sHLA-G in sera/plasma samples from patients with SSc (52, 53). The first one showed a tendency for lower levels in patients but failed to find statistical difference. The second one showed a significant increased expression in patients. Contradictory results could come from the relatively small number of patients [$N = 35$ in Ref. (53)] and/or of healthy controls tested in both studies [$N = 32$ and 40, respectively (52, 53)].

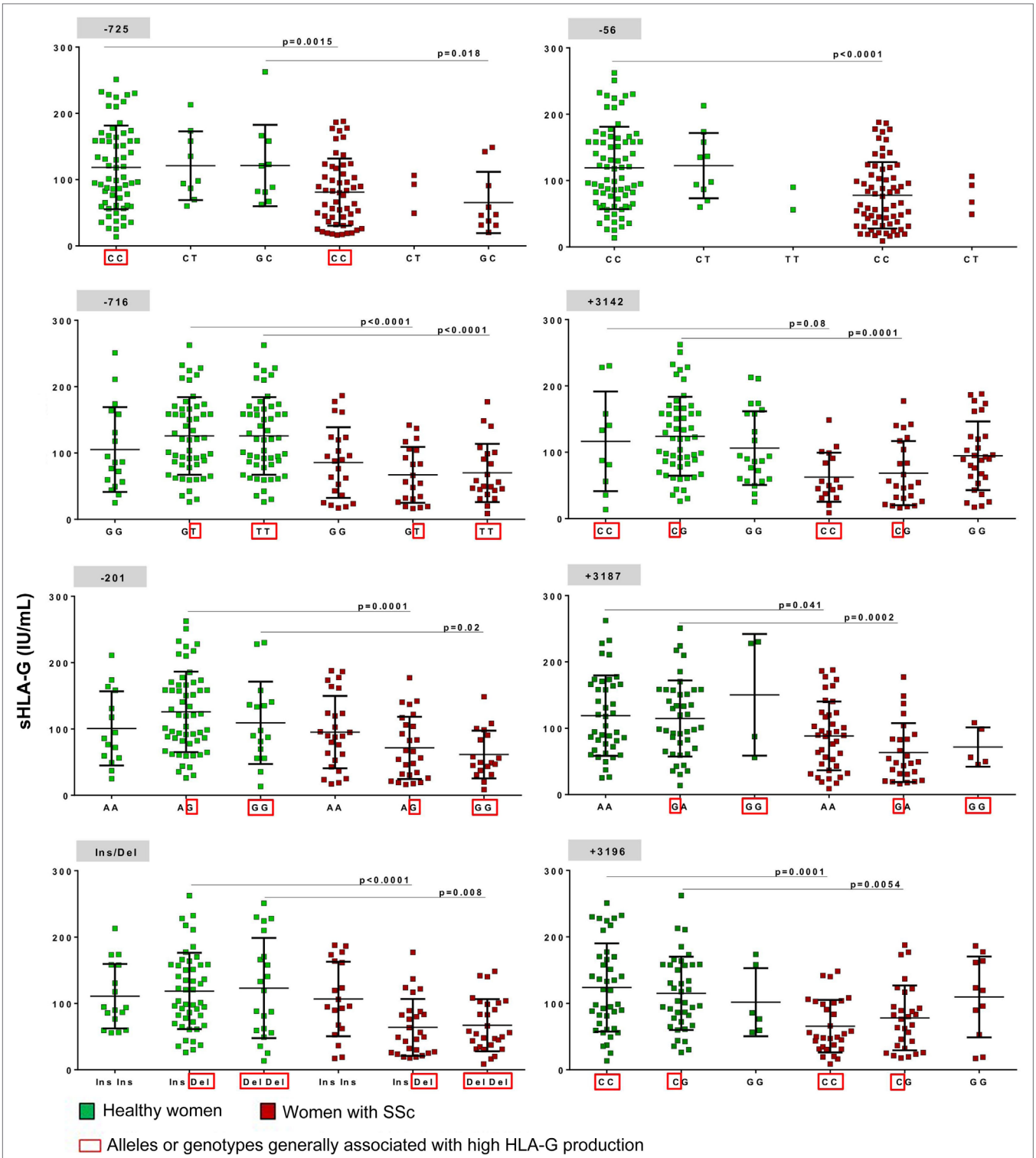

FIGURE 3 | Concentration of soluble HLA-G (sHLA-G) per mL of plasma according to different human leukocyte antigen (HLA)-G genotypes. The eight polymorphisms tested (−725, −716, −201, −56, Ins/Del, +3,142, +3,187, and +3,196) are represented with their three possible genotypes (i.e., GG, GT, and TT for −716 SNP) except for −725 SNP for which six genotypes exist: CC, CT, GC, TT, GG, and GT but the last three were not represented on the graph as only few women had these genotypes; sHLA-G concentration values are represented for each genotypes in green (on the left side) for healthy women and in red (on the right side) for women with SSc. A red square is surrounding alleles or genotypes having been described in most studies with high HLA-G production. Means and SDs are given for each scatter plot by black bars. *P* values are calculated by Mann–Whitney tests.

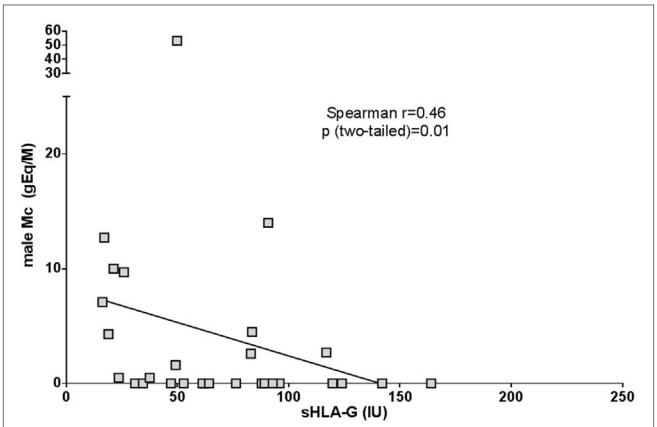

FIGURE 4 | Correlation between male Mc quantities and soluble HLA-G (sHLA-G) levels in women with SSc. On the *x*-axis are represented values for sHLA-G expressed in International Units per mL of plasma (IU/mL). On the *Y*-axis are represented the genome equivalent of male microchimeric cells per million of mother's cells (gEq/M). Correlation is assessed with Spearman rank correlation test.

Although several *HLA-G* polymorphisms have been described as being associated with reduced production of sHLA-G, we could not find any association with *HLA-G* genetic polymorphism and SSc or sHLA-G expression in women with SSc. Our results are concordant with those of Nilsson et al. describing no association with *HLA-G* haplotype or with ins/del polymorphism with increased risk of developing severe preeclampsia/eclampsia in a cohort of more than 900 women (54). Nevertheless, women with PE have low levels of sHLA-G as reported in several studies (20, 55).

As our study is the first to test these polymorphisms in SSc (except Ins/Del), it would be of great value to confirm our data on large cohorts of patients with SSc and on other ethnic groups. Nevertheless, our data suggest that the eight polymorphisms tested in the 5′URR and in the 3′UTR are not sufficient to predict any decreased production of sHLA-G observed in SSc; other SNPs in regulatory regions might contribute to sHLA-G expression modulation. DNA methylation is an important mechanism for regulating gene expression and a few reports suggest that methylation of the *HLA-G* promoter is associated with lower secretion of HLA-G in women with preeclampsia (56, 57). Moreover, our results showing that women with SSc who carry *HLA-G* alleles commonly associated with high sHLA-G levels have low sHLA-G levels, comfort a possible epigenetic regulation of sHLA-G production on 5′ URR or 3′UTR.

Although patients did not have a particular *HLA-G* allele over-represented, they were statistically more often homozygous than heterozygous for *HLA-G* polymorphism genotypes (i.e., Ins/Ins or Del/Del > Ins/Del). High heterozygosity for MHC genes is classically observed in the general healthy population and may be enforced by natural selection for a higher genetic diversity (58). On the opposite homozygotic carriers could be less resistant to broader spectrum of pathogens as they have less antigen presentation possibilities than heterozygotic carriers. A recent study showed that women homozygous for −716 *HLA-G* SNP are at higher risk for spontaneous abortion (59). This confirms the disadvantage of carrying homozygous *HLA-G* polymorphisms for success of reproduction and may be related to the consequence that women with pregnancy complications are at higher risk to develop later SSc.

TABLE 3 | Genotype and allele frequencies of eight human leukocyte antigen-G (HLA-G) polymorphisms in healthy and SSc women.

HLA-G polymorphisms	Genotypes or alleles	Healthy women *N* = 96		SSc women *N* = 106		*P* values[a] cases vs controls
		N	%	*N*	%	
−725	CC	68	70.8	76	71.7	
	CT[b]	10	10.4	8	7.5	
	GC	13	13.5	20	18.9	
	TT	3	3.1	0	0.0	***ns***[c]
	GG	1	1.0	2	1.9	
	GT	1	1.0	0	0.0	
	C	159	82.8	180	84.9	
	G	16	8.3	24	11.3	***ns***
	T	17	8.9	8	3.8	
−716	GG	19	19.8	37	34.9	
	GT	**57**	**59.4**	**37**	**34.9**	***P* = 0.0003**
	TT	20	20.8	32	30.2	***Pc* = 0.002**
	G	95	49.5	116	54.7	***ns***
	T	97	50.5	96	45.3	
−201	AA	16	16.7	35	33.0	
	GA	**60**	**62.5**	**45**	**42.5**	***P* = 0.002**
	GG	20	20.8	26	24.5	***Pc* = 0.006**
	A	92	47.9	115	54.2	***ns***
	G	100	52.1	97	45.8	
−56	CC	82	85.4	98	92.5	
	CT	11	11.5	8	7.5	***ns***
	TT	3	3.1	0	0.0	
	C	175	91.1	204	96.2	***ns***
	T	17	8.9	8	3.8	
Ins/Del	Ins Ins	18	18.8	25	23.6	
	Ins Del	**54**	**56.3**	**42**	**39.6**	***P* = 0.01**
	Del Del	24	25.0	39	36.8	***Pc* = 0.02**
	Ins	90	46.9	92	43.4	***ns***
	Del	102	53.1	120	56.6	
+3,142	CC	13	13.5	24	22.6	
	GC	**57**	**59.4**	**41**	**38.7**	***P* = 0.002**
	GG	26	27.1	41	38.7	***Pc* = 0.008**
	C	83	43.2	89	42.0	***ns***
	G	109	56.8	123	58.0	
+3,187	AA	46	47.9	60	56.6	
	GA	46	47.9	41	38.7	***ns***
	GG	4	4.2	5	4.7	
	A	138	71.9	161	75.9	***ns***
	G	54	28.1	51	24.1	
+3,196	CC	47	49.0	44	41.5	
	CG	43	44.8	48	45.3	***ns***
	GG	6	6.3	14	13.2	
	C	137	71.4	136	64.2	***ns***
	G	55	28.6	76	35.8	

[a]*P values (P) are calculated by Bayesian tests and Pc (P corrected) values correspond to multiple comparison correction by Benjamini–Hochberg test (see Materials and Methods).*
[b]*Genotypes in bold are heterozygous, comparisons are made between heterozygous polymorphisms and homozygous polymorphisms (i.e., for −716 (G/T): GT vs GG + TT).*
[c]*Non significant p values are noted ns.*

TABLE 4 | Frequencies of UTR haplotypes in healthy women and women with SSc.

UTR	Coding allele	5′URR					3′UTR			Haplotype number, frequency (%)			
	Human leukocyte antigen-G	−725	−716	−201	−56	*14 bp*	3,142	3,187	3,196	Healthy women, *N* = 96 *192 hapl.*	SSc women, *N* = 106 *212 hapl.*	dcSSc women, *N* = 48 *96 hapl.*	lcSSc women, *N* = 55 *110 hapl.*
UTR-1	**01:01**	**C**	**T**	**G**	**C**	***Del***	**C**	**G**	**C**	**51 (26.6)**[a]	**50 (23.6)**	**24 (25.0)**	**25 (22.7)**
UTR-2	**All**	**C**	**G**	**A**	**C**	***Ins***	**G**	**A**	**G**	**55 (28.6)**	**76 (35.8)**	**40 (41.7)**[a]	**34 (30.9)**
	01:01									38 (19.8)	57 (26.9)	31 (32.3)	25 (22.7)
	01:05N									2 (1.0)	4 (1.9)	1 (1.0)	2 (1.8)
	01:06									15 (7.8)	15 (7.1)	8 (8.3)	7 (6.4)
UTR-3	**01:04**	**C**	**G**	**A**	**C**	***Del***	**G**	**A**	**C**	**19 (9.9)**	**31 (14.6)**	**11 (11.5)**	**19 (17.3)**
UTR-4	**01:01**	**G**	**T**	**G**	**C**	***Del***	**C**	**A**	**C**	**16 (8.3)**	**24 (11.3)**	**11 (11.5)**	**12 (10.9)**
UTR-5	**01:03**	**T**	**T**	**G**	**T**	***Ins***	**G**	**A**	**C**	**17 (8.9)**	**8 (3.8)**[b]	**2 (2.1)**[c]	**6 (5.5)**
UTR-6	**01:01**	**C**	**T**	**G**	**C**	***Del***	**C**	**A**	**C**	**13 (6.8)**	**14 (6.6)**	**4 (4.2)**	**9 (8.2)**
UTR-7	**01:01**	**C**	**G**	**A**	**C**	***Ins***	**G**	**A**	**C**	**18 (9.4)**	**8 (3.8)**	**3 (3.1)**[d]	**5 (4.5)**
UTR-8	**01:01**	**C**	**G**	**G**	**C**	***Del***	**C**	**G**	**C**	**3 (1.6)**	**1 (0.5)**	**1 (1.0)**	**0 (0)**

The polymorphic sites presented are in 5′URR at position −725, −716, −201, −56, and in 3′UTR, the 14 bp insertion/deletion, +3,142, +3,187, and +3,196. The most frequent human leukocyte antigen-G haplotypes considering the mRNA sequences, named according to Castelli et al. (33), are represented (UTR1–UTR8).

[a]For each subgroup of women tested, the first value represents the number of a given haplotype and the value between brackets indicates the frequency of this haplotype. All P values to compare UTR frequencies from healthy women to UTR frequencies from SSc women are calculated by χ^2 test. Corrections for multiple comparisons are done by Benjamini–Hochbergtest (Pc).

[a]P = 0.02, Pc = not significant.

[b]P = 0.034, Pc = not significant.

[c]P = 0.017, Pc = not significant.

[d]P = 0.040, Pc = not significant.

Besides the role of HLA-G in pregnancy, several studies have indicated a wider immunoregulatory role of this molecule (60). In this context, the expression of HLA-G in inflammatory and rheumatologic diseases is a relatively recent research area. Our results open a new field of investigation for sHLA-G regulation in scleroderma. Finding the pathways explaining low production in women with SSc may also conduct to a better understanding for higher persistence of fetal Mc. HLA-G regulation is likely a key factor for FMc passage and maintenance. Animal models are now required as a proof of concept that maternal sHLA-G molecules play a role on higher traffic of fetal cells during pregnancy.

ETHICS STATEMENT

This study has received the approval from the French Ethical Committee Marseilles 2 and is registered at the INSERM (Biomedical Research Protocol number RBM-04-10). All participants signed written consent forms according to the Declaration of Helsinki (42).

AUTHOR CONTRIBUTIONS

JC, KK, CP, and NL conceived and designed the experiments. JC, KK, SK, DA, MH, and LH performed the experiments. JC, KK, EP, CP, and NL analyzed the data. DF-B, BG, JH, EH, and JR contributed to patient and control recruitments. JC, KK, CP, and NL wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01685/full#supplementary-material.

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The *Nrf2*-Antioxidant Response Element Signaling Pathway Controls Fibrosis and Autoimmunity in Scleroderma

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Systemic sclerosis (SSc) is an autoimmune disease with fibrosis of the skin and internal organs and vascular alterations. Dysregulations in the oxidant/antioxidant balance are known to be a major factor in the pathogenesis of the disease. Indeed, reactive oxygen species (ROS) trigger neoepitopes leading to a breach of immune tolerance and autoimmune responses, activate fibroblasts to proliferate and to produce excess of type I collagen. ROS also alter endothelial cells leading to vascular dysfunction. Glutathione (GSH) is the most potent antioxidant system in eukaryotic cells. Numerous studies have reported a defect in GSH in SSc animal models and humans, but the origin of this defect remains unknown. The transcription factor NRF2 is a key player in the antioxidant defense, as it can induce the transcription of antioxidant and cytoprotective genes, including GSH, through its interaction with the antioxidant response elements. In this work, we investigated whether NRF2 could be implicated in the pathogenesis of SSc, and if this pathway could represent a new therapeutic target in this orphan disease with no curative medicine. Skin biopsies from 11 patients and 10 controls were harvested, and skin fibroblasts were extracted. Experimental SSc was induced both in BALB/c and in *nrf2*$^{-/-}$ mice by daily intradermal injections of hypochloric acid. In addition, diseased BALB/c mice were treated with an *nrf2* agonist, dimethyl fumarate, or placebo. A drop in *nrf2* and target genes mRNA levels was observed in skin fibroblasts of SSc patients compared to controls. Moreover, the *nrf2* pathway is also downregulated in skins and lungs of SSc mice. In addition, we observed that *nrf2*$^{-/-}$ mice have a more severe form of SSc with increased fibrosis and inflammation compared to wild-type SSc mice. Diseased mice treated with the *nrf2* agonist dimethyl fumarate (DMF) exhibited reduced fibrosis and immune activation compared to untreated mice. The *ex vivo* treatment of skin

Abbreviations: ROS, reactive oxygen species; SSc, systemic sclerosis; Nrf2, nuclear factor erythroid 2 (NF-E2)-related factor 2; GSH, glutathione; GCL, glutamate cystein ligase; HO-1, heme oxygenase-1.

fibroblasts from SSc mice with DMF restores GSH intracellular content, decreases ROS production and cell proliferation. These results suggest that the *nrf2* pathway is highly dysregulated in human and SSc mice with deleterious consequences on fibrosis and inflammation and that Nrf2 modulation represents a therapeutic target in SSc.

Keywords: systemic sclerosis, oxidative stress, fibrosis, inflammation, Nrf2

INTRODUCTION

Systemic sclerosis (SSc) is a rare and severe connective tissue disorder characterized by progressive fibrosis of the skin and visceral organs due to excessive collagen deposition, vasculopathy, and autoimmunity. Skin sclerosis and Raynaud's phenomenon are the main clinical features that exhibit a strong impact on quality of life, whereas development of pulmonary fibrosis is life-threatening and intractable (1, 2). Even though the pathogenesis of the disease is unclear, it is well admitted that abnormalities in fibroblasts, endothelial cells, and immune cells lead to the fibrotic, vascular, and autoimmune processes (3). In addition, the role of oxidative stress in SSc has also been highlighted by many studies in animal models and in patients (4–6). Noticeably, skin fibroblasts from SSc patients and mice spontaneously produce high amounts of reactive oxygen species (ROS) that contribute to fibroblasts activation and proliferation as well as collagen synthesis (6, 7). In earlier works, we have developed an animal model of SSc induced by intradermal injections of HOCl, a substance generating ROS *in vivo*, demonstrating the direct role of ROS in the breach of immune tolerance, thus participating to the pathogenesis of the disease (4). Subsequently, the benefit of reducing ROS production by fibroblasts, immune cells, and endothelial cells for the clinical improvement of the disease has been underlined (8–10). Antioxidant defenses now appear to be crucial in SSc development in regulating excessive ROS production, and play a key role in the pathogenesis of the disease (11–13). Among them, the nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) is a key cellular sensor of oxidative stress that can induce the transcription of cytoprotective genes protecting cells from excessive oxidative stress. At physiological levels and in the absence of major cellular stresses, NRF2 is linked to Keap1 in the cytoplasm. When ROS overcomes the endogenous antioxidant capacity, Keap 1 releases NRF2 which then translocates into the nucleus where it binds the antioxidant response elements in association with other transcription factors and accessory proteins. This event causes the transcriptional activation of major key antioxidants and cytoprotective proteins and enzymes responsible, among others, of glutathione (GSH) synthesis. Recent studies have reported a dysregulation in the Nrf2 pathway in a wide area of pathologies including cancers, inflammatory, and fibrotic diseases (14–17). Interestingly, dimethyl fumarate (DMF), a molecule, which at certain concentrations enhances Nrf2 activity, has shown remarkable beneficial effects in two autoimmune diseases: multiple sclerosis and psoriasis (18, 19). Based on the roles that ROS play in SSc, we hypothesized that Nrf2 could be involved in the pathogenesis of SSc and therefore be an interesting target for the treatment of this orphan disease. In the present work, we show that the *nrf2* pathway is dysregulated in patients and in mice with SSc, and report a more severe form of SSc in Nrf2$^{-/-}$ mice along with the therapeutic properties of the *nrf2* agonist DMF.

MATERIALS AND METHODS

Patients

Total mRNA issued from resting cultured fibroblasts derived from 11 ($n = 11$) SSc patients and 10 ($n = 10$) healthy subjects were kindly provided by Pr. Yannick Allanore, Rheumatology Department, Cochin Hospital, Paris, France. All the patients gave their informed consent and their clinical features are represented in **Table 1**.

Mice

Six-week-old female BALB/c mice purchased from Janvier Laboratory (Le Genest Saint Isle, France) were used for the induction of experimental SSc. Nrf2$^{-/-}$ mice and control wild-type mice were a generous gift from S. Kerdine-Römer and were previously described (20). Control mice for all experiments were age-, sex-, and weight-matched. All animals were given human care according to the guidelines of our institution. The project was approved by the approval of the Regional Ethic Committee on Animal Experimentation under the number CEEA34.CN.023.11.

TABLE 1 | Characteristics of systemic sclerosis (SSc) patients and healthy subjects.

Characteristics	Diffuse SSc	Controls
Number of subjects	$n = 11$	$n = 10$
Sex (female/male)	10/1	8/2
Age mean (min–max)	56.3 (26–72)	30 (15–45)
Biospy site	Forearm fibrotic skin	Forearm normal skin
Duration of disease in months mean (min–max)	51 (3–210)	–
Rodnan score mean (min–max)	17.5 (5–29)	–
Autoantibodies	7 anti-topoisomerase I (Scl70) 3 ANA with no specificities	–
Lung fibrosis	6	–
Pulmonary arterial hypertension	2	–
Treatments		
Steroids	6	–
Methotrexate	4	–
Rituximab	2	–
Tocilizumab	1	–
Azathioprine	1	–

Sex, age at time of biopsy, SSc type, disease duration, biopsy site, treatments, Rodnan score, presence/absence of pulmonary arterial hypertension, and autoantibodies were collected for each patient included in this study and reported in this table.

In Vivo Induction of Experimental SSc and Treatments

Two experimental SSc mice models were used. HOCl-induced SSc (HOCl-mice) developed following daily intradermal injections of 200 µl of HOCl-generating reagents into the back of BALB/c mice for 6 weeks, as previously described (21). Bleomycin-induced SSc developed following daily intradermal injections of 100 µl bleomycin (100 µg/ml) in phosphate buffered saline (PBS), for 6 weeks (bleomycin-mice) (22). Control groups received injections of 200 µl sterilized PBS. After 6 weeks, all the animals were sacrificed by cervical dislocation. Lungs were collected and skin biopsies were performed on the back region with a punch (6 mm diameter). Samples were stored at −80°C for western-blot, mRNA quantification, determination of collagen content, or fixed in 10% formalin for histopathological analysis. Experimental *in vivo* mice experiments were performed twice.

DMF Treatment

Mice were given oral 25 mg/kg/day of DMF (Sigma Aldrich, St. Quentin Fallavier, France) by gavage every day for 6 weeks.

Cell Lines and Primary Fibroblasts From Mouse and Humans

Human pulmonary microvascular endothelial cells and human venal endothelial cells (HUVECs) were purchased from Promocell (Heidelberg, Germany) and NIH-3T3 (mouse fibroblasts) were obtained from the American Type Culture Collection (Manassas, VA, USA). Murine control and SSc primary skin fibroblasts were isolated from mouse skin as previously described (23). Human primary skin fibroblasts were isolated from punch biopsies from SSc patients and control subjects. Briefly, 4 mm punch biopsies were collected from patients (forearm fibrotic skin) and immediately diced with scalpels in collagenase for 2 h at 37°C. Cells were then rinsed, filtered, and cultured in complete DMEM in T25 at 37°C. Fibroblasts were observed after 3–5 days and expanded.

Measurement of Intracellular Levels of GSH, and of H_2O_2 Released by Endothelial Cells and Fibroblasts *In Vitro*

Fibroblasts and endothelial cells are seeded in triplicates in 96-well microplates (2×10^4 cells/well) and incubated with complete medium for 24 h at 37°C. Increasing amounts of DMF (0–25 µM) were added to the cells. Levels of H_2O_2 and GSH were assessed by spectrofluorometry (Fusion, PerkinElmer, Wellesley, MA, USA) using 200 µM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) or 50 µM monochlorobimane (both from Molecular Probes, The Netherlands), respectively, in PBS for 30 min at 37°C. Fluorescence intensity was read every hour for 6 h. The numbers of viable cells were evaluated by crystal violet assay as previously described (23). Results were expressed as arbitrary units of fluorescence intensity per million of viable cells.

Reagents

Dimethyl fumarate and all other reagents were purchased from Sigma Aldrich (St. Quentin Fallavier, France).

Assays for Anti-DNA Topoisomerase 1 Autoantibodies and Proinflammatory Cytokines in the Sera

Levels of anti-DNA topoisomerase 1 IgG antibodies (Abs) were detected by enzyme-linked immunosorbent assay (ELISA) using purified calf thymus DNA topoisomerase I bound to the wells of a microtiter plate (Abnova, Germany). A 1:4 mice serum dilution and a 1:1,000 anti-murine Ig HRP (DAKO) secondary antibody dilution were used.

INFγ and IL-13 in the sera were also measured by ELISA using Mouse ELISA Ready-SET-Go (eBioscience-Thermo Scientific), following the manufacturer's instructions.

Assessment of Skin Thickness and Collagen Accumulation in Skin and Lungs

Skin thickness of the shaved back of mice was measured 1 day before sacrificing the mice with a caliper and expressed in millimeters. Fixed lung and skin pieces were embedded in paraffin. A 5-µm-thick tissue section was prepared from the mid-portion of paraffin-embedded tissue and stained with H&E. Slides were examined by standard brightfield microscopy (Nikon Eclipse 80i) (Nikon, Champigny sur Marne, France) by a pathologist who was blinded to the assignment of the animal to an experimental or a control group.

Determination of collagen content in skin samples was performed by the hydroxyproline assay. After digestion of punch biopsies (3 mm) in 6 M HCl for 3 h at 120°C, the pH of the samples was adjusted to 7 with 6 M NaOH. Samples were then mixed with 0.06 M chloramine T and incubated for 20 min at room temperature. Next, 3.15 M perchloric acid and 20% p-dimethylaminobenzaldehyde were added and samples were incubated for an additional 20 min at 60°C. The absorbance was determined at 557 nm.

FACS Analysis of T Lymphocytes and Macrophages Subpopulations

Cell suspensions from mouse spleens were prepared after hypotonic lysis of erythrocytes in potassium acetate solution. Cells were incubated with the appropriate labeled antibody cocktail (ebioscience, ThermoFisher Scientific) at 4°C for 30 min in PBS with 0.1% sodium azide and 5% normal rat serum. Flow cytometry was performed on a FACSCanto flow cytometer (BD Biosciences) using standard techniques. The monoclonal Abs used in this study were anti-CD3-FITC, anti-CD4-BV421, anti-CD8-PE-Cy7, anti-CD69-PE, and anti-B220-APC in a first mix. In a second mix, the mAbs used were anti-B220-BV421, anti-CD11b-PerCP-Cy5.5, anti-Ly6C-PE-Cy7, and anti-CD62L-APC. M1 macrophages were defined as $B220^-CD11b^+Ly6c^+CD62L^+$ and M2 macrophages as $B220^-CD11b^+Ly6c^-CD62L^-$. Data were analyzed with the FlowJo software (Tree Star).

Western-Blot Analysis in Mouse Fibroblasts

Fibroblasts isolated as forementioned were incubated with 50 µl RIPA. Protein extracts (30 µg total proteins) were subjected to 10% polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, blocked with 5% non-fat dry milk in Tris

Buffer Solution-Tween, then incubated overnight at 4°C with an anti-NRF2 antibody (1:500, Santa Cruz, sc-722). The membranes were washed and incubated with an HRP-conjugated secondary antibody (Santa Cruz, Paris, France) for 1 h at room temperature. Immunoreactivities were revealed with ECL (Amersham).

RT-qPCR Analysis

Total murine RNA was extracted from crushed samples using the RNeasy mini kit (Qiagen, France). One-step RT-qPCR was performed using QuantiTect SYBR® Green RT-PCR Kit on a LightCycler 480 II instrument (Roche Applied Science, France). The sequences of the primers are detailed in Table S1 in Supplementary Material. Samples were normalized to mRNA expression of housekeeping genes (HRPT2 for murine RNA and GAPDH for human ones), and results were provided either as relative expression to these housekeeping genes using the formula $2^{-\Delta Ct}$ and as fold increase using the formula $2^{-\Delta\Delta Ct}$. RT-PCR was carried out for 40 cycles, with a denaturing phase of 15 s at 94°C, an annealing phase of 30 s at 60°C, and an extension phase of 30 s at 72°C.

Statistical Analysis

Microsoft Excel 2007 and GraphPad Prism (GraphPad Inc., USA) softwares were used to analyze the data. All values are averages of at least two independent experiments made in triplicates, except when specified. Error bars shown in the figures represent SEM and all results were expressed as arithmetic mean ± SEM. Differences between the experimental groups were analyzed using Mann–Whitney *U* test, statistically significant differences were reported as follows: $^{**}p < 0.01$ or $^{*}p < 0.05$.

RESULTS

The Nrf2 Pathway Is Downregulated in SSc Patients' Fibroblasts

As ROS metabolism is impaired in the skin of SSc patients, we first investigated the Nrf2 pathway in fibroblasts extracted from the skin of patients. We analyzed the mRNA expression levels of *nrf2* and its main target genes involved in the regulation of ROS production [heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL), and thioredoxin (TRX)] by quantitative RT-PCR in fibroblasts extracted from skin biopsies from SSc patients and healthy controls. We showed a highly significant downregulation of *nrf2* ($p = 0.0006$, **Figure 1A**) and *nrf2*-induced genes mRNA levels in patients' skin fibroblasts, HO-1 ($p = 0.001$, **Figure 1B**), GCL

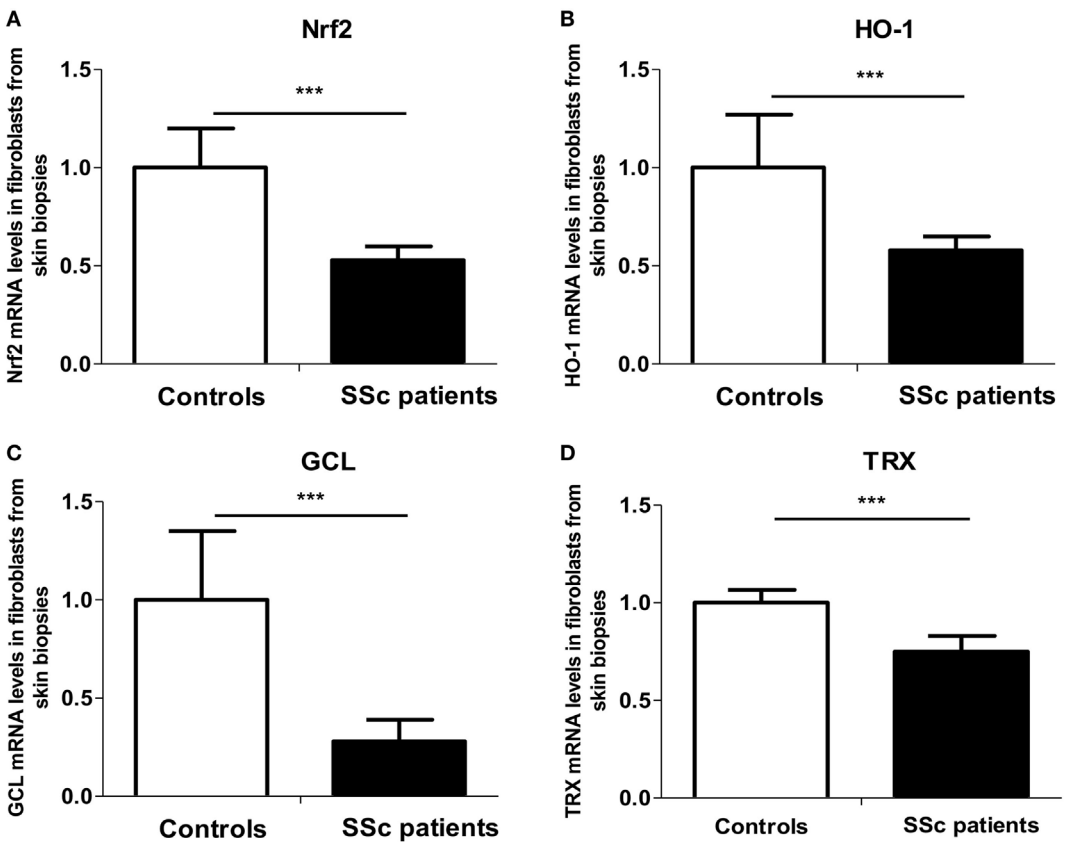

FIGURE 1 | The *nrf2* pathway is downregulated in the skin of systemic sclerosis (SSc) patients. **(A)** *Nrf2* mRNA levels in the fibroblasts from skin of control subjects and SSc patients. **(B)** Heme oxygenase-1 (HO-1) mRNA levels in the fibroblasts from skin of control subjects and SSc patients. **(C)** Glutamate-cystein ligase (GCL) mRNA levels in fibroblasts from skin of control subjects and SSc patients. **(D)** Thioredoxin (TRX) mRNA levels in the fibroblasts from the skin of control subjects and SSc patients. Data from 13 SSc patients and 10 control subjects. Values are mean ± SEM. $^{***}p \leq 0.001$, by Mann–Whitney *U* test.

($p < 0.0001$, **Figure 1C**), and TRX ($p < 0.0001$, **Figure 1D**) compared to healthy individuals.

The Nrf2 Pathway Is Downregulated in the Skin of HOCl-Mice

Glutathione is an essential cofactor of ROS catabolism and its dysregulation can lead to uncontrolled production of ROS, as it is observed in the mouse model of HOCl-induced SSc (8, 24). As previously described (8), fibroblasts from HOCl-mice display a severe reduction in the levels of reduced GSH of more than 40% compared to those from control PBS mice ($p = 0.003$, **Figure 2A**).

Since Nrf2 controls the transcription of GCL, a key enzyme in the synthesis of GSH, we therefore, in this study examined the protein expression levels of NRF2 in the skin of HOCl-mice. We observed a reduction in the NRF2 protein levels in HOCl-fibroblasts compared to PBS-fibroblasts ($p = 0.0002$ **Figures 2B,C**). This result correlated with the mRNA levels of *nrf2* that were also strongly diminished in diseased skin fibroblasts from HOCl-mice compared to those from PBS mice ($p = 0.008$, **Figure 2D**). Furthermore, the mRNA expression levels of GCL and HO-1 were downregulated in diseased HOCl-fibroblasts compared to control PBS-fibroblasts ($p = 0.02$ and $p = 0.004$, respectively, **Figures 2E,F**). No difference in TRX mRNA expression levels between normal and disease fibroblasts was, however, observed; but expression levels of TXNIP, a natural inhibitor of TRX, were dramatically increased in HOCl-SSc mice compared to controls (data not shown).

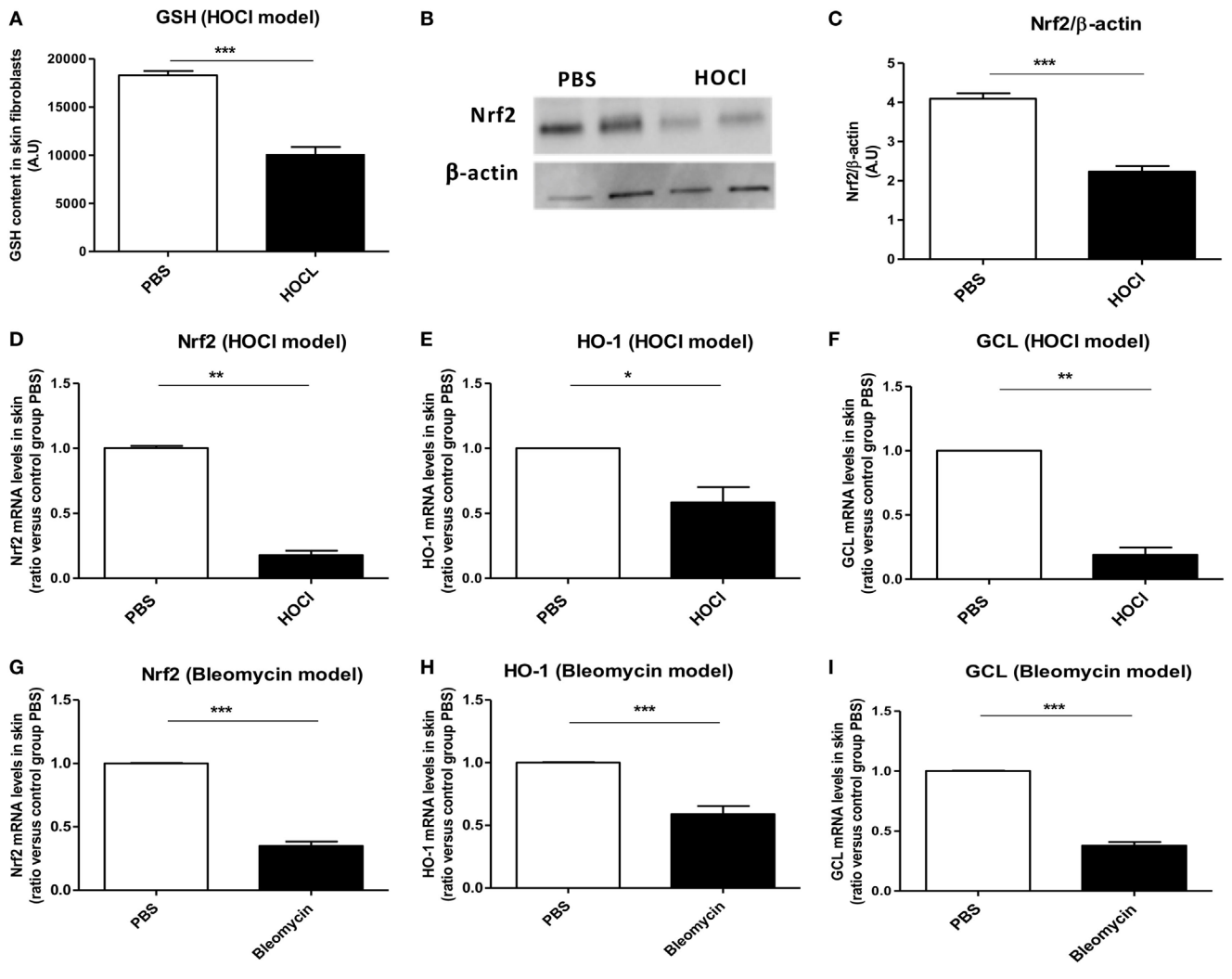

FIGURE 2 | The *Nrf2* pathway is downregulated in the skin of mice with SSc. **(A)** GSH content in skin fibroblasts from PBS (controls) and HOCl-mice (SSc mice) (A.U per cells). **(B)** Protein expression levels of NRF2 in PBS and HOCl in skin extracts by western-blot (two mice representative of seven). Photographs were taken with a Fujifilm LAS-3000. **(C)** Intensity ratio of Nrf2 and β-actin expression in skin in PBS- and HOCl-mice. **(D–F)** The Nrf2 pathway is downregulated in the skin of mice with ROS-induced SSc. mRNA levels of *Nrf2* **(D)**, HO-1 **(E)**, and GCL **(F)** mRNA measured in skin extracts. **(G–I)** The Nrf2 pathway is downregulated in the skin of mice with bleomycin-induced SSc. Levels of NRF2 **(G)**, HO-1 **(H)**, and GCL **(I)** mRNA measured in skin extracts. Abbreviations: AU, arbitrary units; SSc, systemic sclerosis; GSH, glutathione; PBS, phosphate buffered saline; ROS, reactive oxygen species; HO-1, heme oxygenase-1. Values are mean ± SEM ($n = 8$ mice per group). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$, by Mann–Whitney *U* test.

The same phenomenon was observed in bleomycin-treated SSc mice with a very significant downregulation in *nrf2* and related genes mRNA levels compared to PBS mice ($p = 0.0004$, $p = 0.0002$, $p < 0.0001$, respectively, **Figures 2G–I**).

Nrf2$^{-/-}$ HOCl-Mice Display Severe Dysregulations in the Redox Balance

Skin fibroblasts from HOCl-treated sclerodermic animals have a clear decrease in intracellular GSH content compared to PBS-treated control mice ($p = 0.0002$). As expected, skin fibroblasts from *nrf2*$^{-/-}$ mice show a severe drop in GSH content compared to wild-type mice ($p < 0.0001$). Moreover, intracellular GSH levels are significantly reduced in fibroblasts from HOCl-treated *nrf2*$^{-/-}$ mice compared to fibroblasts from HOCl-treated WT mice ($p = 0.01$ **Figure 3A**). These dramatic modulations in the intracellular GSH content have important consequences on the production of the highly ROS H_2O_2 by fibroblasts. Indeed, measurements of H_2O_2 in skin fibroblasts from HOCl-treated *nrf2*$^{-/-}$ mice showed a significant increase of spontaneous H_2O_2 production compared to fibroblasts extracted from HOCl-treated WT mice ($p = 0.04$ **Figure 3B**).

Invalidation of the *nrf2* Gene Exacerbated the Symptoms of SSc in the Mice

We examined the clinical effects of the absence of *nrf2* in the development of SSc. Nrf2$^{-/-}$ mice were exposed to daily injections of HOCl

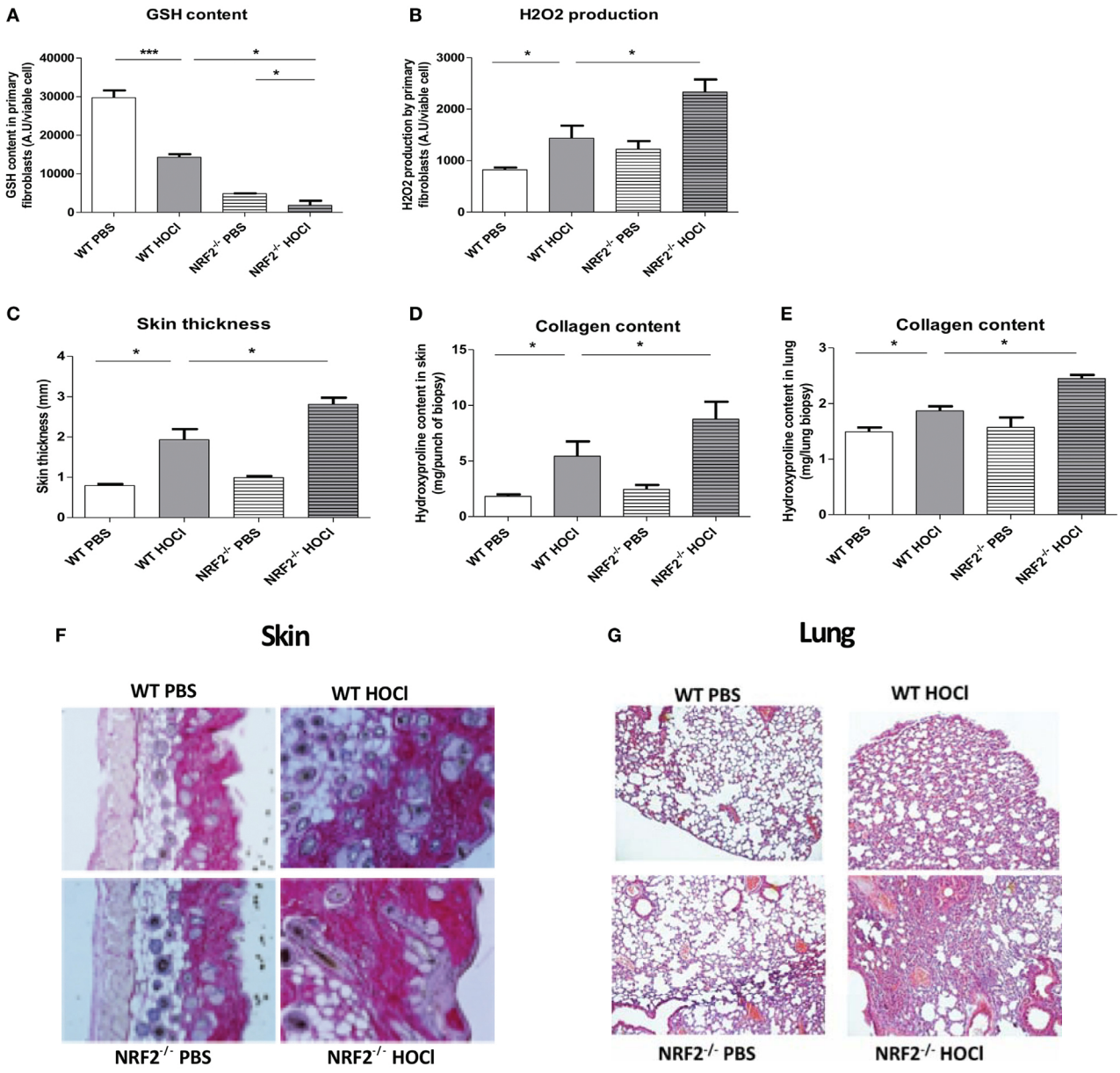

FIGURE 3 | Continued

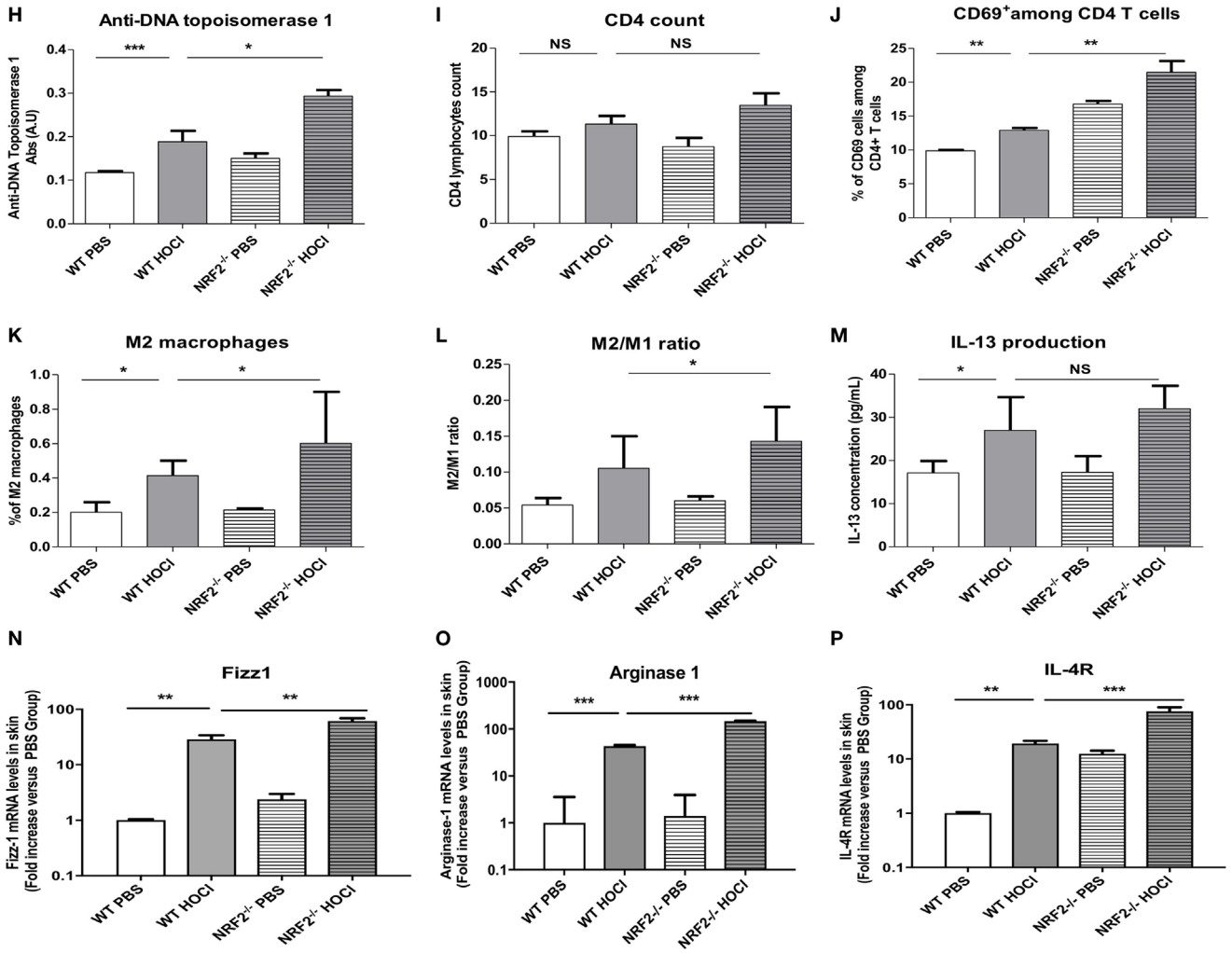

FIGURE 3 | *Nrf2*$^{-/-}$ mice exposed daily to HOCl display a severe phenotype of SSc. WT or *Nrf2*$^{-/-}$ mice were injected daily with HOCl for 6 weeks. **(A,B)** Measurements of oxidative stress markers in the skin of PBS and HOCl-mice with WT and *Nrf2*$^{-/-}$ background. **(A)** GSH content in primary skin fibroblasts (AU per viable cells). **(B)** H_2O_2 production by primary skin fibroblasts (AU per viable cells). Fibrosis markers in PBS and HOCl-mice with WT and *Nrf2*$^{-/-}$ background. **(C)** Skin thickness in millimeters. **(D)** Collagen content in the skin (Hydroxyproline, mg per punch biospy). **(E)** Collagen content in lung (Hydroxyproline, mg per lung biopsy). **(F)** Skin biopsies stained with Hematoxylin and eosin (H&E). Representative sections of 5 µm. **(G)** Lung biopsies stained with Hematoxylin and eosin (H&E). Photographs were taken with a Nikon Eclipse 80i microscope. Original magnification ×50. **(H–M)** Immunological parameters measured in PBS and HOCl-mice with WT and *Nrf2* background. **(H–K)** Anti-DNA topoisomerase 1 antibodies levels in the sera (A.U). **(L)** Number of CD4 T lymphocytes. **(M)** Percentages of activated T cells (CD4$^+$CD69$^+$ lymphocytes). **(N)** Percentages of M2 macrophages (B220$^-$CD11b$^+$Ly6c$^-$CD62L$^-$). **(O)** M2-macrophages (B220$^-$CD11b$^+$Ly6c$^-$CD62L$^-$)/M1-macrophages (B220$^-$CD11b$^+$Ly6c$^+$CD62L$^+$) ratio. **(P)** IL-13 concentration measured in splenic T-cells supernatants by ELISA (pg/ml) **(I–M)**. Abbreviations: AU, arbitrary units; SSc, systemic sclerosis; PBS, phosphate buffered saline; GSH, glutathione; ELISA, enzyme-linked immunosorbent assay. Values in **(A–K)** are mean ± SEM (n = 8 mice per group). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$, by Mann–Whitney U test.

to induce SSc. They showed a more severe form of SSc compared to wild-type SSc mice as demonstrated by the increase of dermal thickness ($p = 0.02$, **Figure 3C**) and collagen type I content in the skin and lungs ($p = 0.02$, **Figures 3D,E**) in comparison to wild-type SSc mice. Histopathological studies of skin and lung section stained with picro-sirius red showed an excess of collagen accumulation in the skin and in the lungs of diseased *nrf2*$^{-/-}$ mice compared to WT HOCl-mice, as shown in **Figure 3F** (skin) and **Figure 3G** (lung).

Altogether, these results strongly suggested a protective role of NRF2 and related proteins in the development of fibrosis in SSc mice.

Nrf2 Invalidation Worsens Inflammation and Autoimmunity in SSc Mice

Systemic sclerosis is a systemic autoimmune disease with chronic activation of adaptive immunity by nuclear auto-antigens such as DNA topoisomerase 1 and production of autoantibodies. Anti-DNA topoisomerase 1 Abs were detected in the sera from both WT-HOCl- and *nrf2*$^{-/-}$ HOCl-mice. A significant increase in these autoantibodies was observed in the sera from *nrf2*$^{-/-}$ HOCl-mice compared to WT-HOCl-mice ($p = 0.02$ **Figure 3H**).

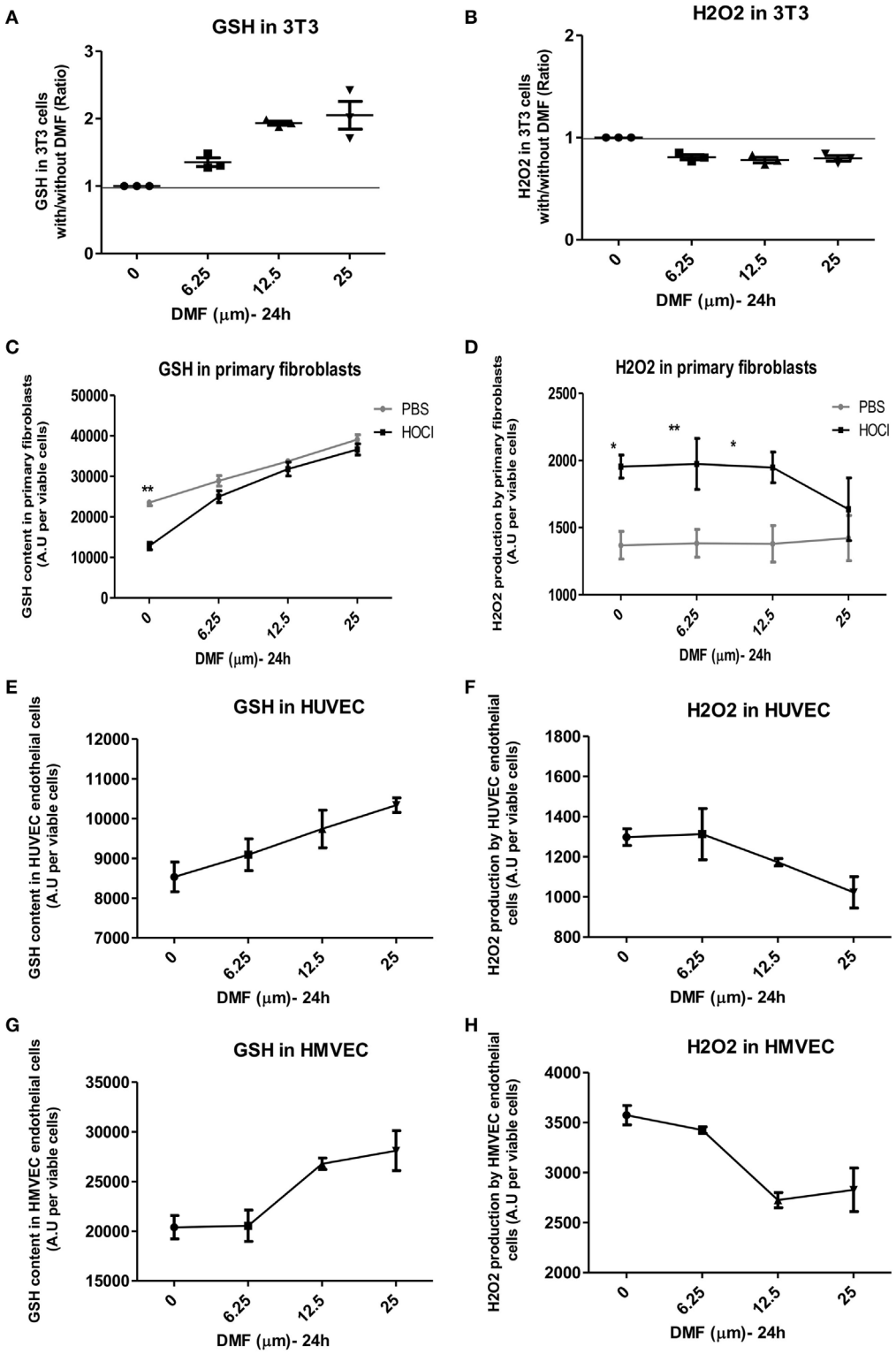

FIGURE 4 | Dimethyl fumarate (DMF) has antioxidant properties *in vitro*. Cells were incubated *in vitro* with increasing concentrations of DMF for 24 h. Glutathione (GSH) content and H_2O_2 production were measured by spectrofluororometry in triplicates. **(A,B)** Effects of DMF on GSH content **(A)** and H_2O_2 production **(B)** in 3T3-fibroblasts, expressed as ratio with/without DMF. **(C,D)** Effects of DMF on GSH content **(C)** and H_2O_2 production **(D)** in phosphate buffered saline (PBS)- and HOCl-primary skin fibroblasts (from three mice per group). **(E,F)** Effects of DMF on GSH content **(E)** and H_2O_2 production **(F)** in human venal endothelial cells (HUVEC), expressed as ratio with/without DMF. **(G,H)** Effects of DMF on GSH content **(G)** and H_2O_2 production **(H)** in HMVEC endothelial cells, expressed as ratio with/without DMF. Values are mean ± SEM. $^*p \leq 0.05$; $^{**}p \leq 0.01$; by Mann–Whitney *U* test.

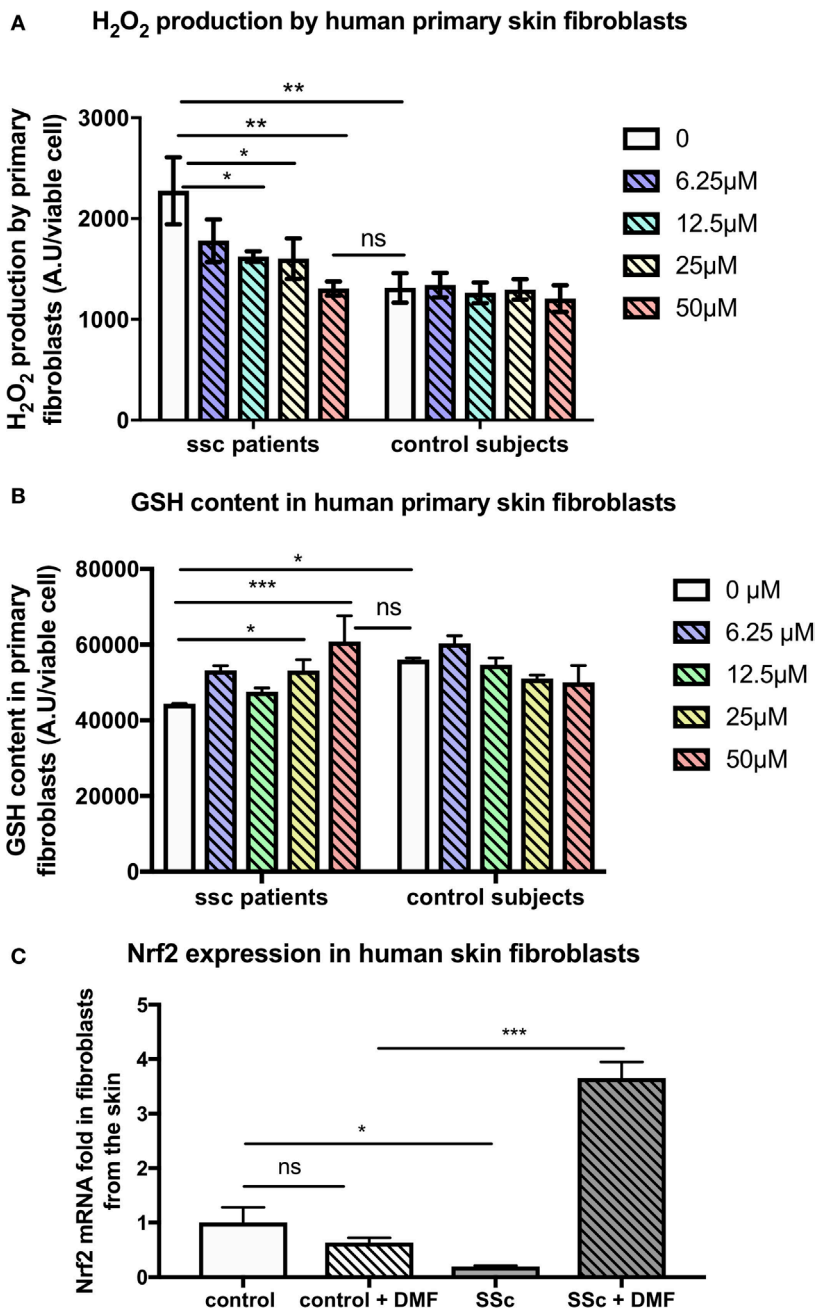

FIGURE 5 | Skin fibroblasts from systemic sclerosis (SSc) patients display reduced H_2O_2 production and increased *nrf2* expression upon dimethyl fumarate (DMF) *in vitro* treatment. Human primary skin fibroblasts from SSc patients ($n = 2$, forearm skin biopsies) and control subjects ($n = 2$) were treated with increasing doses of DMF for 24 h. **(A)** H_2O_2 production of human skin fibroblasts treated with increasing doses of DMF (0–50 μM). **(B)** Glutathione (GSH) content in human skin fibroblasts treated with increasing doses of DMF (0–50 μM). **(C)** Expression of *nrf2* in skin fibroblasts upon *in vitro* treatment with 50 μM of DMF. Values are mean ± SEM. $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$, by Mann–Whitney *U* test

Analysis of spleen cell population showed an accumulation of activated $CD4^+$ $CD69^+$ T cells in $nrf2^{-/-}$ HOCl-mice compared to WT HOCl-mice ($p = 0.01$ **Figures 3I,J**).

In addition, we observed a polarization toward the M2 profibrotic "resolver" macrophage phenotype in WT HOCl-mice, that was even more pronounced in $nrf2^{-/-}$ HOCl-mice (**Figures 3K,L**, $p = 0.044$ and $p = 0.049$ for $nrf2^{-/-}$ HOCl-mice versus WT HOCl-mice). Likewise, we observed an upregulation of the expression of M2 macrophages markers Fizz1, Arginase 1, and IL4R in the skin of $nrf2^{-/-}$ HOCl-mice compared to WT HOCl-mice (**Figures 3N–P**, $p = 0.0054$, $p < 0.0001$, $p = 0.0031$). IL-13 can regulate the production of Abs by B cells and the polarization of macrophages toward the M2 phenotype (25). IL-13 also has profibrotic properties that can be crucial in the pathogenesis of

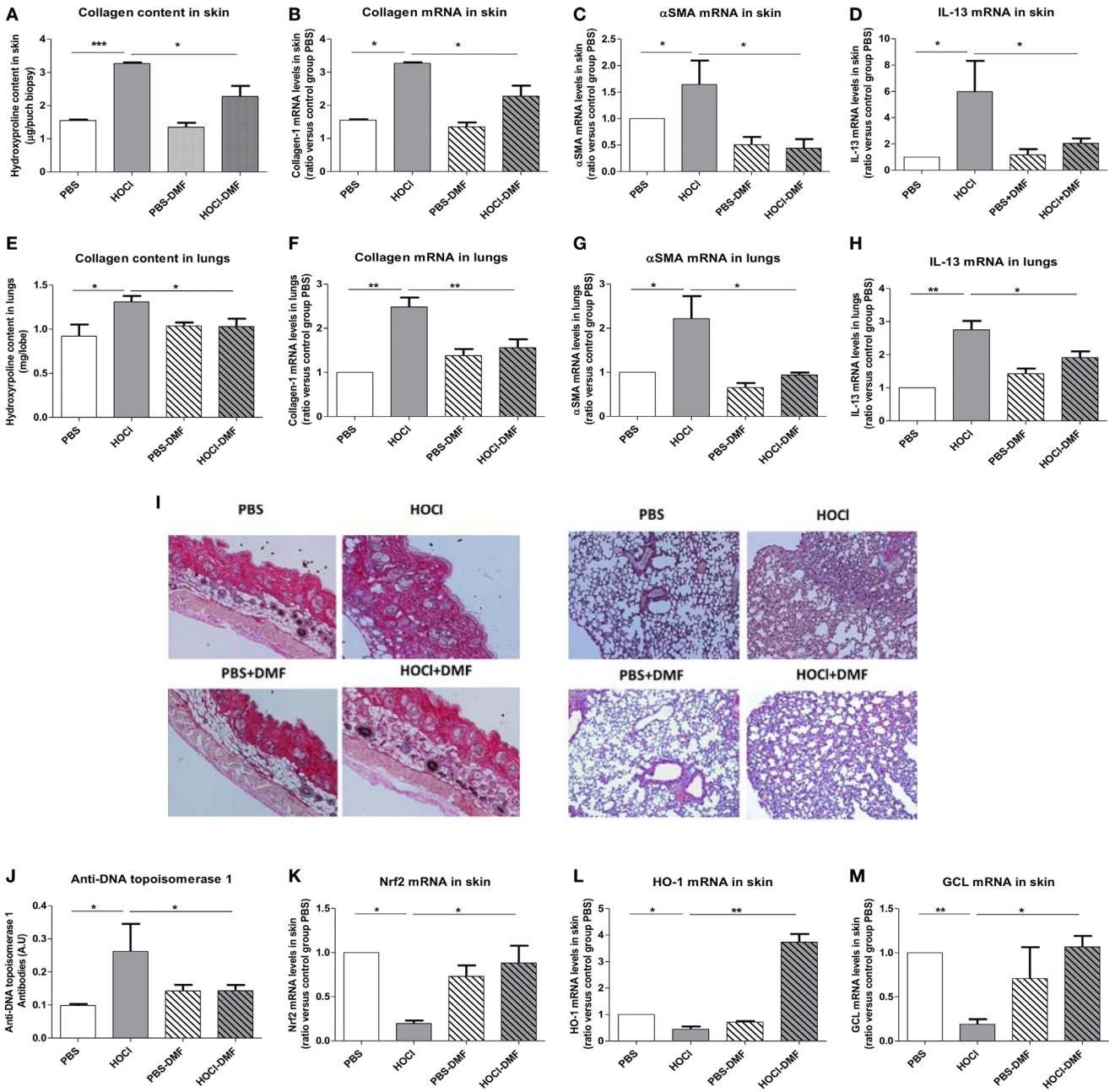

FIGURE 6 | Effects of *in vivo* treatment with DMF on HOCl-induced SSc. BALB/c mice were intradermally injected with HOCl or phosphate buffered saline (PBS) and simultaneously treated with DMF or vehicle alone for 6 weeks. **(A–D)** *In vivo* treatment with DMF reduces skin fibrosis in mice with HOCl-induced SSc. **(A)** Collagen type I content in skin (Hydroxyproline, mg/punch biopsy). **(B)** Collagen type I mRNA levels in skin (ratio versus control group PBS). **(C)** Alpha-SMA mRNA levels in skin (ratio versus control group PBS). **(D)** IL-13 mRNA levels in skin (ratio versus control group PBS). **(E–H)** DMF reduces lung fibrosis in mice with HOCl-induced SSc. **(E)** Collagen type I content in lungs (Hydroxyproline, mg/lobe). **(F)** Collagen type I mRNA levels in lungs (ratio versus control group PBS). **(G)** Alpha-SMA mRNA levels in lungs (ratio versus control group PBS). **(H)** IL-13 mRNA levels in lungs (ratio versus control group PBS). **(I)** Skin and lung biopsies stained with Hematoxylin and eosin (H&E). Representative sections of 5 µm. Photographs were taken with a Nikon Eclipse 80i microscope. Original magnification ×50. **(J)** Anti-DNA topoisomerase 1 antibodies levels in the sera (A.U). **(K–M)** *In vivo* treatment with DMF upregulates the Nrf2 pathway in the skin. Levels of Nrf2 **(K)**, HO-1 **(L)**, and GCL **(M)** mRNAs in the skin (ratio versus control group PBS). Abbreviations: AU, arbitrary units; DMF, dimethyl fumarate; SSc, systemic sclerosis; HO-1, heme oxygenase-1. Values in **(A–M)** are mean ± SEM ($n = 8$ mice per group). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$, by Mann–Whitney *U* test.

SSc and is increased in the skin of WT-HOCl mice compared to untreated control animals. We observed a slight, but not significant, increase in IL-13 in *nrf2*$^{-/-}$ HOCl-mice compared to WT-HOCl mice ($p = 0.32$, **Figure 3M**).

Altogether, these data comfort the predominant role of *nrf2* in the redox balance and the immune dysregulation in SSc HOCl-mice.

The Nrf2 Agonist DMF Display Antioxidant Properties in Fibroblasts and Endothelial Cells

Antioxidant effects of DMF, a fumaric acid ester that display an agonist activity on *nrf2*, were evaluated *in vitro* in fibroblastic and endothelial cell lines and in murine and human primary diseased fibroblasts. A range of DMF doses were first tested *in vitro* on these different cell types (data not shown).

NIH-3T3-fibroblasts were treated *in vitro* with increasing amounts of DMF for 24 h. A dose-dependent elevation in GSH was observed: a dose of 6.25 μM DMF allowed an increase in GSH content by 40% and a concomitant decrease in H_2O_2 content by 20% (**Figures 4A,B**).

Primary skin fibroblasts from PBS- and HOCl-mice were also treated *in vitro* with increasing amounts of DMF. Fibroblasts from HOCl-treated mice showed a significant drop in GSH content compared to normal control fibroblasts (**Figure 4C**, $p < 0.0001$). A significant increase in GSH content was observed in primary fibroblasts from both PBS- and HOCl-mice upon DMF treatment, but this effect was amplified in fibroblasts from HOCl-mice as the content of GSH in those cells reached those of control PBS-fibroblasts and was no more significantly different at 6.25 and 12.5 μM DMF (**Figure 4C**) as was the levels of H_2O_2 between the two types of cells at 25 μM DMF (**Figure 4D**).

Endothelial cells represent a key target in SSc as many vascular abnormalities related to endothelial dysfunctions have been described (26). A dose-dependent increase in GSH content along with a decrease in H_2O_2 levels was observed in the endothelial cell lines HUVEC and HMVEC that was optimal at the dose of 25 μM DMF (**Figures 4E–H**). These results confirm the antioxidant effect of DMF in both fibroblasts and endothelial cells.

In addition, our findings were confirmed on human fibroblasts from SSc patients and control subjects. Indeed, DMF also exerted beneficial antioxidant effects on primary skin fibroblasts from SSc patients. DMF dose-dependently decreased the levels of H_2O_2 produced by skin fibroblasts from SSc patients and restored H_2O_2 production to the levels of production of control fibroblasts (**Figure 5A**). *In vitro* treatment with DMF also restored the GSH content in these cells (**Figure 5B**). Finally, *in vitro* treatment with 50 μM DMF of these human SSc skin fibroblasts significantly induced the expression of *nrf2* (**Figure 5C**).

Treatment of HOCl-Mice With DMF Prevents the Development of SSc

We explored the clinical *in vivo* effects of DMF in mice with SSc. Mice exposed to daily injections of HOCl developed increased skin and lung fibrosis with elevated collagen contents that were significantly reduced by *in vivo* treatment with DMF ($p = 0.03$ and 0.045, **Figures 6A,E**). Quantification of mRNA levels of collagen-1 (skin $p = 0.045$, lung $p = 0.005$, **Figures 6B,F**) and α-SMA (skin $p = 0.04$, lung $p = 0.08$, **Figures 6C,G**) confirmed the beneficial effect of DMF. Staining of skin and lung biopsies with Hematoxylin and Eosin also showed a reduction in fibrosis in both organs in diseased-mice treated with DMF compared to untreated diseased-mice (**Figure 6I**).

The development of fibrosis in the skin and lungs of HOCl-mice correlated with the elevation of the profibrotic cytokine IL-13 in these organs. Treatment with DMF decreased the levels of IL-13 in both skin and lungs from HOCl-mice ($p = 0.02$ in skin and $p = 0.041$ in lungs versus HOCl-mice; **Figures 6D,H**).

As mentioned above, mice exposed daily to HOCl develop an autoimmune reaction characterized by the presence of anti-DNA topoisomerase 1 autoantibodies. Treatment with DMF allowed a reduction in the development of these autoantibodies in mice with HOCl-induced SSc ($p = 0.047$ versus untreated HOCl-mice, **Figure 6J**).

Altogether, these data report a beneficial role of DMF in the treatment of SSc in mice, as the molecule ameliorates both fibrotic and inflammatory phenomenons *in vivo*.

We tested the clinical effects of DMF administered with the same dose and schedule in another mouse model of SSc induced by daily administration of bleomycin for 6 weeks. Consistent with the results in the HOCl model of SSc, histopathological studies of skin sections stained with picro-sirius red showed that the excess of collagen accumulation in the skin of bleomycin-injected mice returns to normal upon DMF treatment. This observation was confirmed by the significant reduction in the expression of the fibrotic markers α-SMA ($p = 0.001$) and type-1 collagen ($p = 0.05$) in the skin of bleomycin-injected mice treated with DMF compared to untreated animals (Figure S1 in Supplementary Material).

Treatment With DMF Induces an Nrf2 Signature in Skin Fibroblasts From Mice With HOCl-Induced SSc

We investigated whether the amelioration of the clinical symptoms of SSc was related to a reduction in oxidative stress markers in mice. *In vivo* DMF treatment of HOCl-mice allowed an elevation of the transcription levels of *nrf2* in the skin along with an increase in its target genes GCL and HO-1 ($p = 0.013$, $p = 0.019$, and $p = 0.002$, respectively, versus untreated HOCl-mice, **Figures 6K–M**).

These data confirm that the clinical amelioration of the symptoms of SSc observed in mice following DMF treatment are related to the enhanced transcription of Nrf2 and its downstream target genes HO-1 and GCL triggering a cytoprotective and antioxidant response.

DISCUSSION

It is now well established that *nrf2* plays important roles in the cellular adaptive defense responses to oxidative stress, leading to

an efficient expression of detoxifying enzymes and antioxidant molecules. In this study, we report a defect in the *nrf2* pathway in patients and mice with SSc, an autoimmune disease with fibrosis and vascular dysfunctions. We also bring new evidence for a direct role of this defect in the induction of the disease using murine models of SSc, *nrf2* knockout mice, and *in vivo* treatment with DMF, an agonist of *nrf2*.

Our cohort of SSc patients displayed a downregulation of the *nrf2* pathway (i.e., *nrf2* and *nrf2*-target genes GCL, HO-1, and TRX) in fibroblasts from fibrotic skin. We observed the same results in fibrotic skin and lungs from mice with HOCl-induced SSc. These results provide new insights on the role of intrinsic ROS dysregulation in SSc fibroblasts and strengthen the link between *nrf2* and fibrosis, in accordance with the recent findings from Wei et al. (27). Indeed, the drop in antioxidant levels and the overproduction of ROS have previously been described in scleroderma and assigned with a direct role in the pathogenesis of SSc as ROS can trigger the proliferation of fibroblasts and collagen synthesis (28, 29). However, so far, the intrinsic mechanism responsible for the redox imbalance in SSc fibroblasts remains unclear. The drop in NRF2 levels in the skin of SSc patients may be a major factor at the origin of this imbalance between oxidant and antioxidant molecules in scleroderma. HOCl-mice, as well as SSc patients, display an important depletion in GSH in skin fibroblasts that is responsible for the uncontrolled ROS production and proliferation of these cells leading to fibrosis (7). Our present data bring new insights to explain this phenomenon as the downregulation of NRF2 in SSc fibroblasts can directly lead to the lack of GSH through the drop in GCL expression, its rate-limiting enzyme (30, 31). NRF2 levels have indeed been shown to directly regulate the levels of GSH in many cellular types both in normal and pathologic conditions such as cancer (32, 33). Thus, in SSc fibroblasts, the downregulation of NRF2 will lead to a drop in GSH concentration and to an uncontrolled ROS production. Furthermore, elevated ROS levels will induce an increased phosphorylation of the MAPK pathway proteins (34) leading to fibroblasts proliferation, along with an activation of the Smad pathway, which subsequently leads to collagen production (35).

We next showed that the SSc features were exacerbated in *nrf2* KO-mice when inducing SSc by daily exposure to HOCl as demonstrated by the major defects in antioxidative defenses, increased fibrosis of skin and lungs and immune activation with inflammation and autoimmunity. Recently, Wei et al. also reported that *nrf2* KO-mice displayed an exacerbated phenotype of bleomycin-induced SSc (27). *Nrf2*-KO-mice are more prompt to develop fibrotic and inflammatory responses, and the daily exposure to HOCl emphasized this phenomenon (20, 36). Indeed, in endometriosis, a gynecological disease with dysregulated proliferation of endometrial stromal cells closely resembling that of the dysregulated proliferation of fibroblasts in SSc, wild-type mice bearing endometrial implants from *nrf2*-KO-horn display an aggravated phenotype, with elevated volume of the implants and higher amounts of collagen and inflammatory markers compared to those with implants from wild-type animals (37). SSc is a systemic autoimmune disease with chronic inflammation and activation of adaptive immunity by nuclear auto-antigens such as DNA topoisomerase 1 and production of autoantibodies. Anti-DNA topoisomerase 1 Abs were detected in the sera from both WT-HOCl- and *nrf2*$^{-/-}$ HOCl-mice but at a significantly higher titer in the sera from *nrf2*$^{-/-}$ HOCl-mice compared to WT-HOCl-mice. Few data are available regarding *nrf2*-KO-mice in the context of autoimmunity. It has, however, been shown that autoimmunity in SSc may be dependent on the formation of ROS-induced oxidized neoepitopes that may induce the breach of tolerance against DNA topoisomerase I and autoimmunity (4). Thus, further increasing ROS by *nrf2* invalidation could be responsible for the overproduction of oxidized neoepitopes and autoantibodies.

In SSc pathogenesis, macrophages polarization plays a key role linking immune activation with fibrosis (38). The preponderance of M2 "resolver" macrophages over M1 both in the spleen and in the skin of *nrf2*-KO mice could also contribute to the enhanced phenotype of the disease in these animals. The exacerbated immune response observed in HOCl-*nrf2* KO-mice has also been reported in the model of experimental autoimmune encephalomyelitis where *nrf2* KO-mice show a more severe form of the disease compared to wild-type mice (39).

To confirm the pathogenic role of the *nrf2* defect in SSc, we tested the impact of its pharmacological activation with DMF, a potent and FDA-approved Nrf2 pharmacological activator.

In fibroblastic and endothelial cell lines, the two cell types particularly involved in ROS-mediated SSc pathogenesis, DMF shows potent antioxidative properties as demonstrated by the dose-dependent increase in the reduced GSH content of those cells and their reduced H_2O_2 production. We observed this effect on cell lines (3T3, HUVEC, and HMVEC), but also on primary murine and human cells from skin biopsies (**Figures 4** and **5**). At a dose of 50 μM, DMF strongly induced the expression of the *nrf2* gene in fibroblasts from SSc patients. Such an antioxidative effect was already observed by Hoffmann et al. who demonstrated that DMF was able to restore the GSH pool even in the context of total GSH depletion (40) as observed in fibroblasts from humans or mice with SSc. Increasing GSH was associated with a decreased production of ROS and a decreased proliferation of these cells.

The *in vivo* use of DMF in HOCl-induced SSc was associated with the induction of Nrf2 and the expression of its downstream antioxidant defense genes expression (HO-1 and GCL) along with a reduced skin and lung fibrosis and immune activation in DMF-treated HOCl-SSc mice compared to untreated animals. DMF restored to normal the content of collagen in the lung whereas its effect on the skin was much milder. Skin fibroblasts may need a higher dose of DMF than lung-fibroblasts to decrease more potently their proliferation and production of matrix. The Nrf2-activating properties of DMF have been widely studied but few data are available regarding its direct anti-fibrotic properties (41, 42). In human fibroblasts, DMF can promote the degradation of β-catenin, a transcriptional factor activating the Wnt pathway, implicated in pulmonary fibrosis (43). Wnt is a key mediator of fibrosis in HOCl-induced SSc, and besides its antioxidant properties, DMF could block directly the development of fibrosis through its regulating effect on Wnt (44). Toyama et al. have also recently

studied the effect of DMF in SSc fibroblasts (43). Consistent with our results, they demonstrate that *in vitro* treatment with DMF can block the TGF-β-induced profibrotic response in fibroblasts *via* the inhibition of PI3K/Akt pathway and the transcriptional regulators TAZ and YAP (43).

In our work, the levels of anti-DNA topoisomerase 1 were significantly decreased in the sera of DMF-treated SSc mice compared to untreated SSc mice. This result is consistent with the *in vivo* elevation of antioxidant defenses induced by DMF treatment that blocks chronic ROS production and consequently the release of oxidized antigens that play a key role in the breach of immune tolerance leading to the development of the autoimmune reaction in SSc. DMF also displays direct anti-inflammatory properties including inhibition of NFκB and STAT3 pathways that are known to contribute to the inflammation in the HOCl-induced model of SSc (44). A recent paper has provided mechanistic insights into the immune-modulatory effects of DMF by showing that DMF can inhibit the aerobic glycolysis in activated immune cells (45).

In summary, we confirm the profound defect of the Nrf2 antioxidant pathway in skin fibroblasts of SSc patients and mice, together with the aggravation of the disease in *nrf2*-KO SSc mice. We propose a critical regulatory role for Nrf2 in the homeostasis of oxidative stress in SSc. Recently published results regarding the role of Nrf2 in other models of SSc and the use of DMF in pulmonary arterial hypertension models strongly confirm our observations and, along with ours, brings major data to strongly support the use of DMF to ameliorate the clinical symptoms of SSc, as the molecule has already been approved by the FDA in multiple sclerosis and psoriasis (27, 42, 43).

ETHICS STATEMENT

Animal (mice): this study was carried out in accordance with the recommendations of the Regional Ethic Committee on Animal Experimentation under the number CEEA34.CN.023.11. The protocol was approved by the Regional Ethic Committee on Animal Experimentation. Human samples: the samples were collected by the Rheumatology Department of Cochin Hospital. This study was carried out in accordance with the recommendations of the Cochin Hospital Ethic Committee. The protocol was approved by the Cochin Hospital Ethic Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: NK, FB, SM, and YA. Performed the experiments: NK, SM, MJ, CN, CC, CCh, MA-D, and SC. Analyzed the data: NK, SM, FB, SK-R, and YA. Contributed reagents/materials/analysis tools: NK, FB, NS, OC, AC, SC, SK-R, and YA. Wrote the paper: NK, FB, and SM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01896/full#supplementary-material.

FIGURE S1 | *In vivo* treatment with dimethyl fumarate (DMF) reduces bleomycin-induced fibrosis. BALB/c mice were injected subcutaneously with bleomycin or phosphate buffered saline (PBS) and simultaneously treated with DMF ("Bleo DMF") or vehicle alone ("Bleo") for 6 weeks. **(A,B)** Alpha-SMA and collagen type 1 mRNA levels in the skin of PBS- and bleomycin-mice. **(C)** Skin biopsies stained with picro-sirius red. Representative sections of 5 µm. Photographs were taken with a Nikon Eclipse 80i microscope. Original magnification ×50. Values in **(A,B)** are mean ± SEM (n = 7 mice per group). *$p \leq 0.05$; **$p \leq 0.01$, by Mann–Whitney U test.

TABLE S1 | Primers used for quantitative RT-PCR in this study.

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CORRECTION
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Corrigendum: The *Nrf2*-Antioxidant Response Element Signaling Pathway Controls Fibrosis and Autoimmunity in Scleroderma

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A Corrigendum on

The *Nrf2*-Antioxidant Response Element Signaling Pathway Controls Fibrosis and Autoimmunity in Scleroderma

By Kavian N, Mehlal S, Jeljeli M, Saidu NEB, Nicco C, Cerles O, Chouzenoux S, Cauvet A, Camus C, Ait-Djoudi M, Chéreau C, Kerdine-Römer S, Allanore Y and Batteux F (2018). Front. Immunol. 9:1896. doi: 10.3389/fimmu.2018.01896

In the original article, there was a mistake in the legend for **Figure 3G** and **6I** as published. Skin and lung biopsies were stained with Haematoxylin and Eosin, and not "with picro-sirius red and Haematoxylin and Eosin" as stated in the original legend. The correct legends appear below. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

In the original article, there was a mistake in the photos used in **Figures 3G** and **6I** as published. The authors noticed that some of the lung histology photos used in **Figures 3** and **6** as illustrations only were not correct due to an inadvertent mishandling of the names list and photo files. The corrected **Figures 3** and **6** appear below. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way, as these photos were used as illustrations only, and not as a source of quantifiable data. The original article has been updated.

In the original article, there was an error in the **Methods** section: "A 5-µm-thick tissue section was prepared from the mid-portion of paraffin-embedded tissue and stained with H&E or picro-sirius red."

A correction has been made in the text of the **Methods** section, **Assessment of Skin Thickness and Collagen Accumulation in Skin and Lungs**, paragraph 1:

"A 5-µm-thick tissue section was prepared from the mid-portion of paraffin-embedded tissue and stained with H&E."

In the original article, there was an error in the **Results** section: "Staining of skin and lung biopsies with picro-sirius red also showed a reduction in fibrosis in both organs in diseased-mice treated with DMF compared to untreated diseased-mice (**Figure 6I**)."

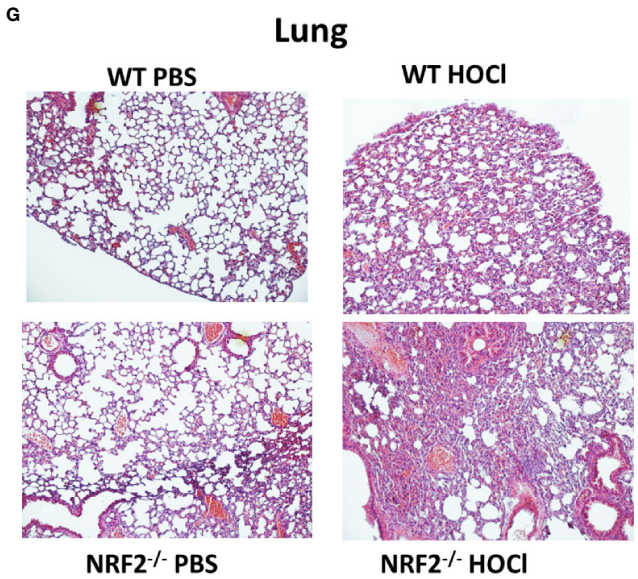

FIGURE 3G | **(F, G)** Skin and Lung biopsies stained with Hematoxylin and eosin (H&E).

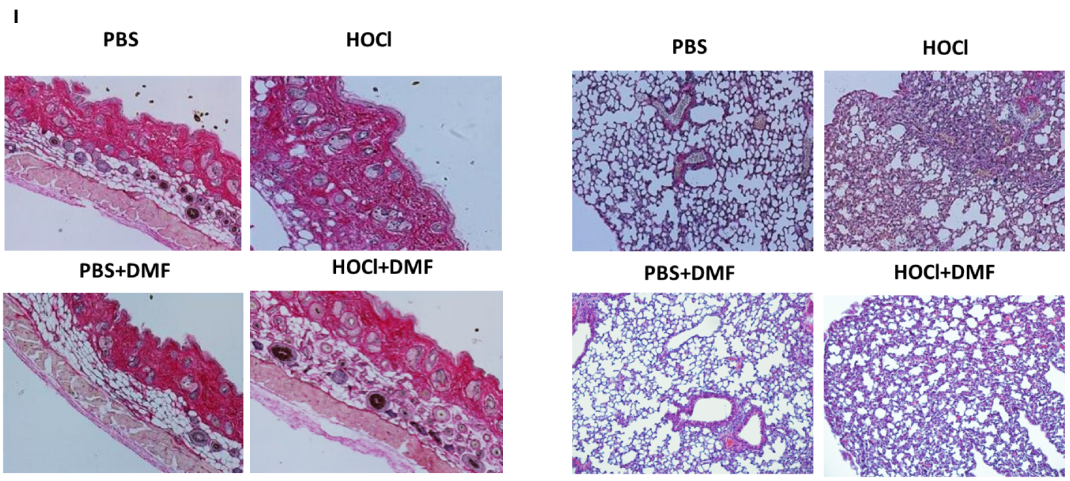

FIGURE 6I | **(I)** Skin and lung biopsies stained with Hematoxylin and eosin (H&E).

A correction has been made in the text of the **Results** section, **Treatment of HOCl-mice with DMF prevents the development of SSc**, paragraph 1:

"Staining of skin and lung biopsies with Hematoxylin and Eosin also showed a reduction in fibrosis in both organs in diseased-mice treated with DMF compared to untreated diseased-mice (**Figure 6I**)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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MINI REVIEW
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Vascular Leaking, a Pivotal and Early Pathogenetic Event in Systemic Sclerosis: Should the Door Be Closed?

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The early phase of systemic sclerosis (SSc) presents edema as one of the main features: this is clinically evident in the digital swelling (puffy fingers) as well as in the edematous skin infiltration of the early active diffuse subset. Other organs could be affected by this same disease process, such as the lung (with the appearance of ground glass opacities) and the heart (with edematous changes on cardiac magnetic resonance imaging). The genesis of tissue edema is tightly linked to pathological changes in the endothelium: various reports demonstrated the effect of transforming growth factor β, vascular endothelial growth factor and hypoxia-reperfusion damage with reactive oxygen species generation in altering vascular permeability and extravasation, in particular in SSc. This condition has an alteration in the glycocalyx thickness, reducing the protection of the vessel wall and causing non-fibrotic interstitial edema, a marker of vascular leak. Moreover, changes in the junctional adhesion molecule family and other adhesion molecules, such as ICAM and VCAM, are associated with an increased myeloid cells' extravasation in the skin and increased myofibroblasts transformation with further vascular leak and cellular migration. This mini-review examines current knowledge on determinants of vascular leak in SSc, shedding light on the role of vascular protection. This could enhance further studies in the light of drug development for early treatment, suggesting that the control of vascular leakage should be considered in the same way that vasodilation and inflammation reduction, as potential therapeutic targets.

Keywords: systemic sclerosis, edema, capillary leak, extravasation, vasculopathy, endothelial dysfunction, permeability

Systemic sclerosis (SSc) is characterized, in its early phase, by the prominent interplay between the microvasculature and the immune system (1). The injury to the endothelium and the vessel wall, the activation and perivascular homing of inflammatory cells and the contemporary loss of the vascular tone control are a major triad contributing to the initiation and maintenance of vascular leak (2). The aim of this review is to examine the characteristics and mechanisms of vascular leaking in SSc.

ANATOMY

The vessel structure depends on its size and function: while larger arteries, arterioles, veins and venules have an endothelial layer plus varying amounts of surrounding muscular cells, capillaries and post-capillary venules have an inner surface coat overlying the endothelium called the glycocalyx, a negatively charged glycosaminoglycan (GAG) layer, and are usually surrounded by pericytes (3). The endothelium represents a barrier to extravasation, preventing circulating cells and macromolecules from crossing the lipid membrane. Endothelial cells (ECs) are anchored via integrins to a basement membrane (BM) which can be fenestrated or continuous. Central nervous system, connective tissue, heart and muscle have a continuous endothelium: ECs are linked together with tight intercellular junctions, adherent junction (VE-cadherin and catenin) and tight protein and glycoprotein junctions (occludins, claudins, and junctional adhesion molecules -JAMs- family members) controlling cell trafficking and protein and fluids passage (4).

In specific conditions, an intercellular vascular leak can be a physiological reparative event, such as during neovascularization following wound healing. This is consecutively characterized by BM degradation, pericyte detachment, endothelial thinning and increase in lumen size, mostly at a post-capillary venule level (5).

VASCULAR MEDIATORS AND PHYSIOLOGICAL PERMEABILITY

Independent of BM structure, various angiogenic and lymphangiogenic mediators derived by several inflammatory effector cells (such as mast cells, eosinophils, basophils, macrophages, etc.) can regulate physiological vascular permeability and extravasation, such as the vascular endothelial growth factor (VEGF) (6). VEGF isoforms signal through different members of the VEGF receptors family, which are expressed on several cells including ECs. VEGF is a mitogen and a vasodilator stimulating vascular permeability (7, 8), affecting perivascular pericytes and concomitantly increasing cellular migration (9). Moreover, VEGF-A induces VE-cadherin phosphorylation thus impairing endothelial barrier integrity and increasing vascular permeability (10). Angiopoietin (Ang) system represents a complementary pathway in the regulation of vascular endothelial barrier function (11). In humans, Ang1 and Ang2 are, respectively, a full agonist and a partial agonist of the Tie2 receptor on ECs: the former inhibits endothelial permeability, the latter induces it (12, 13).

In addition, transforming growth factor beta (TGFβ), a potent inhibitor of ECs proliferation and migration, induces pericyte differentiation, production of BM and induces VEGF inhibiting Ang1 expression in pericytes and fibroblasts. Therefore, TGFβ can exacerbate vascular leak in certain pathological conditions (14).

Capillary permeability may be significantly increased by hypoxia-reperfusion injury. In a pig-heart model, in fact, reperfusion caused damage to the glycocalyx, with increased serum levels of heparan-sulfate derived from GAGs shedding, influenced by oxygen-derived free radicals and xanthine-oxidoreductase activity (15).

PATHOLOGICAL CAPILLARY LEAKING

Capillary leak may take place in several diseases due to local pathological conditions and the following are prototypes arising from different origins:

1) Anti-angiogenic and potential permeability inhibitors have been demonstrated in neoplasms (16),
2) Hypoxia-induced VEGF production can determine vascular leak and local edema in ischemic diseases such as stroke and myocardial infarction (17),
3) Pathological or mechanical endothelial stretch induces increased vascular permeability in pulmonary diseases including asthma (18), acute and ventilator-induced lung injury (19),
4) Hypoxia-induced VEGF production has been demonstrated in ocular conditions such as diabetic retinopathy or age-related macular degeneration (20).

Similarly, systemic inflammatory pathologies such as sepsis, pancreatitis and major traumas may induce a capillary leak. These are characterized by an increase in pro-inflammatory mediators (e.g., G-CSF, IL-6, IL-8, sTNFR1), followed by an increase of endothelial protective proteins, like Ang1, and of heparin binding proteins derived from glycocalyx damage (21, 22).

A distinct pathological entity called Systemic Capillary Leak Syndrome is characterized by arterial hypotension, hemoconcentration and low albumin levels with hypotensive shock and anasarca. In these patients, pro-inflammatory and endothelial mediators are significantly increased (23) and prompt treatment is required (24).

VASCULAR LEAKING IN SSc

In SSc, endothelial injury is a pivotal pathological event (25) which may have twofold explanations. Firstly, several unknown toxic stimuli (including ischemia-reperfusion) may induce a state of persistent endothelial activation resulting in apoptosis and detachment. Altered vasculature causes the direct activation of the alternative complement and coagulation cascades, leading to abnormal platelet activation (26), which amplifies and

maintains vascular permeability and promotes the formation of intravascular fibrin deposits contributing to intimal proliferation, luminal narrowing and vessel obstruction (26). Secondly, there is an ineffective ability to respond to all these insults by promoting vascular repair (27).

In the pathogenetic progression, the vascular leak could be due to the modification of endothelial glycocalyx, vesiculo-vacuolar organelles, extracellular matrix (ECM), BM, intercellular junctions, cytoskeletal proteins and/or vascular pericytes. Using electron microscopy, SSc skin capillaries revealed intercellular gaps, vacuolization and destruction of ECs, reduplication of the basal lamina, perivascular cellular infiltrates and fibroblasts and pericytes with an enlarged rough endoplasmic reticulum accompanied by perivascular fibrosis (28–30).

In early SSc, vasculopathy is paralleled by an increased production of pro-angiogenic factors (e.g., VEGF, endothelin-1) (31, 32) and ECs defective response. These early events lead to vascular tone dysfunction, reduced capillary blood flow and chronic tissue hypoxia, further exacerbated by ECM accumulation and fibrosis (32). Biomarkers of vascular leak in SSc suggest intracellular signaling cascades impact on endothelial cytoskeletal and junctional proteins. The localization and function of junctional proteins and vesicular bodies can be significantly influenced by vasoactive substances, inflammatory mediators, and mechano-transduction. Damaged cells and inflammatory cells produce signaling mediators, such as histamine, TGFβ and VEGF, that can directly increase vascular permeability (2, 8, 33, 34). While perivascular cellular infiltrates and EC damage seemingly precede fibrosis (28), once hyperpermeability or leak occurs, the ECM is important for the propagation of fibrosis through its direct influence on fibroblast-myofibroblast transition and endothelial-to-mesenchymal transition (EndoMT) and the generation of profibrotic myofibroblasts (35).

Taken together, these events lead to a microcirculatory endothelial dysfunction, characterized by inflammatory immune cells surrounding microvessels (36), and to an organ damage, both independent from the rate of fibrosis (37). The role of microvascular endothelium is pivotal in triggering the activation of tissue cells, such as dendritic cells and macrophages, through the presentation of self-antigens, and myofibroblasts, through the release of TGFβ and other cytokines and growth factors (26).

IMMUNE CELLS AND VASCULAR LEAKING IN SSc

Genetic factors are implicated in SSc development and are related to immunity and inflammation, thus suggesting a crucial role of the autoimmune dysregulation in all the phases of the disease (1), including vascular leak onset (26, 27, 36–43).

The response of the innate immune system against pathogens or non-specific damage is achieved through the activation of the so-called pattern recognition receptors, in particular the Toll-like receptors (TLRs). TLRs are evolutionary conserved receptors that, upon binding to their ligands, trigger the inflammatory response and induce several cellular changes (44). In addition to microbial antigens, TLRs can recognize endogenous molecules, contributing to the pathogenesis of many autoimmune diseases, including SSc (45, 46). The damage-associated molecular patterns (DAMPs), released from endogenous cells upon necrosis or tissue injury, generated by stressed cells, or resulting from mechanical or biochemical fragmentation of extracellular molecules, serve as an alarm "signal" for cells via TLRs (47). The mechanism regulating DAMPs release and interaction with the microenvironment is still poorly understood. The EC stress and injury trigger the inflammatory response mainly through the activation of NF-kB. Aberrant TLR signaling may be involved at the onset and during progression of SSc, contributing significantly to tissue inflammation and aberrant wound healing process (47).

Evidence suggests that the perivascular infiltrate participates in vessel wall remodeling (29). In early SSc, precursor cells such as monocytes, recruited from the bone marrow, migrate in the tissues (47) with T-cells and macrophages, to form the perivascular infiltrate between collagen fibers (**Figures 1A,a1,C**). Macrophages can generate different form of cells roughly categorized as classically activated (M1) and alternatively activated (M2) macrophages (48): M1 macrophages are effector phagocytes with an enhanced microbicidal or tumoricidal capacity, whereas M2-polarized macrophages are activated mostly by IL-4, IL-13, and IL-10, as well as by CCL2 and IL-6 (49). Commonly found during the peak of the profibrotic immune response, M2 macrophages have been proposed as inducers of wound healing, tissue fibrosis and remodeling in SSc. First, M2 macrophages suppress M1 responses and then promote collagen synthesis and profibrotic cytokine release, and Th2 effector responses. Consequently, if inflammation and tissue damage fail to resolve, a persistent fibrotic state may arise (50).

In the early edematous phase of SSc, the perivascular infiltrate is dominated by T-cells, with $CD4^+$ T-cells predominating over $CD8^+$ T-cells. The T-cell infiltrate is characterized by γδT-cells, mostly expressing the V1 chain ($V\delta1^+$cells), which are a "non-conventional" T-cell population able to recognize non-peptidic antigens independent of major histocompatibility (MHC) molecule (51). These cells represent another potential mechanism that can contribute to the initial immune damage of SSc (52). The role of the γδT-cells is not completely understood. On one hand a defective regulatory function of γδT-cells may play a role in the breakdown of tolerance contributing to the early stages of the disease (53). $V\delta1^+$-cells represent the majority of γδT-cells found in SSc skin lesions (54), peripheral circulation and bronchoalveolar lavage fluid suggesting an effector rather than regulatory function (55). In SSc, $V\delta1^+$ γδT-cells are recruited by chemokines secreted by local cells (i.e., fibroblasts), starting immune-mediated endothelial damage. This is demonstrated by the presence of the activation marker CD49d mediating γδT-cells adherence to the ECs through the binding of the vascular cell adhesion molecule-1 (VCAM-1) (56). CD49d-VCAM-1 interactions are implicated in endothelial injury and cytotoxicity of activated γδT-cells (55).

In SSc vasculopathy evolution, the contact of leukocytes with ECs and fibroblasts is of paramount importance (57, 58).

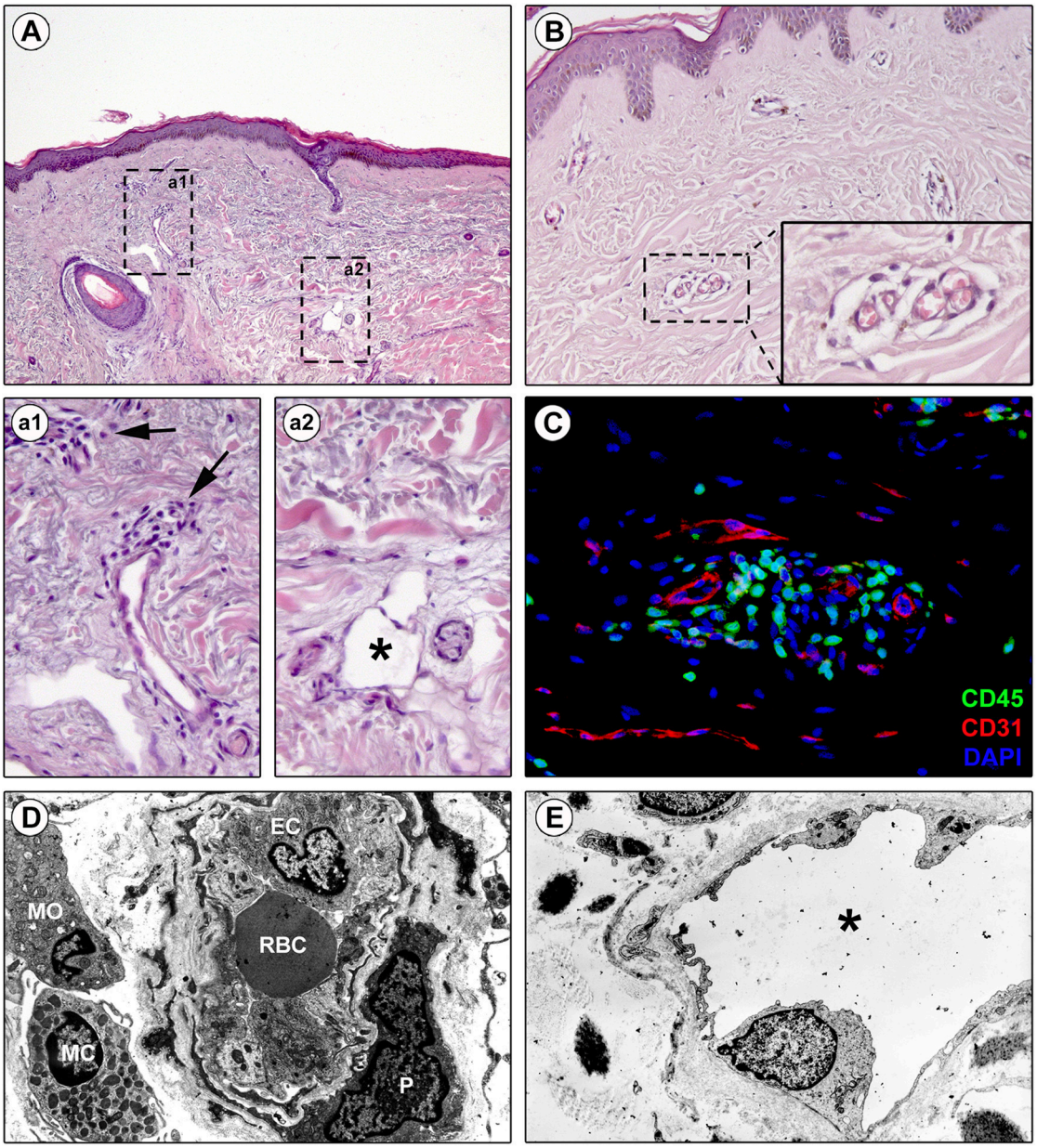

FIGURE 1 | Perivascular edema and inflammatory cell infiltration are prominent in skin of early diffuse cutaneous systemic sclerosis (SSc). Representative microphotographs of paraffin-embedded skin sections from patients with early diffuse cutaneous SSc subjected to hematoxylin and eosin staining **(A, a1, a2, B)** or double immunofluorescence staining for CD45/leukocyte common antigen (green) and CD31/pan-endothelial cell marker (red) with 4′,6-diamidino-2-phenylindole (DAPI, blue) counterstain for nuclei **(C)** are shown. **(a1)** and **(a2)** represent higher magnifications of the boxed areas in **(A)**. **(a1)** Infiltrating inflammatory cells are observed around small dermal blood vessels (arrows). **(a2)** A dermal lymphatic vessel with an enlarged lumen (asterisk) is surrounded by edematous extracellular matrix. **(B)** Edema is prominent around blood capillaries. The inset depicts a higher magnification view of the boxed area from the respective panel. **(C)** CD45-positive inflammatory cells are widely found in the perivascular area. **(D,E)** Representative transmission electron microscopy microphotographs of ultrathin skin sections from patients with early diffuse cutaneous SSc. **(D)** A blood capillary displays hypertrophic endothelial cells and is surrounded by edema and inflammatory cells. **(E)** A lymphatic vessel surrounded by edema shows an enlarged lumen (asterisk). EC, endothelial cell; MC, mast cell; MO, mononuclear cell; P, pericyte; RBC, red blood cell.

The expression and function of several cell adhesion molecules regulate the maintenance of trans-endothelial leukocyte migration. In affected SSc skin, specific EC activation markers are upregulated, including E-selectin, P-selectin, intercellular adhesion molecule (ICAM), JAMs, platelet EC adhesion molecule (PECAM), and vascular cell adhesion molecule (VCAM), particularly on ECs and fibroblasts in proximity to the perivascular infiltrate (55). Several other markers of EC

activation, although expressed to a lesser extent, are also elevated in the sera of SSc or certain SSc subtypes (i.e., von Willebrand factor, fractalkine, β1/β2/β4 integrins, etc.) (58).

The complex role of the immune system in the pathogenesis of SSc is further highlighted by the detection of substantial differences in cytokine production in SSc, with differences according to cutaneous and internal organ involvement (59, 60).

Recently, the role of the adaptive immune system in SSc pathogenesis has been highlighted. In early SSc, Th1 and Th17 cells are predominant and pro-inflammatory cytokine release drives inflammation, whereas Th2 cells predominate in the later stages, participating through the release of profibrotic cytokines (60).

Mast cells (MCs) and basophils have also been implicated in the pathogenesis of systemic autoimmune diseases (SADs) (61, 62). It is becoming evident that MCs and basophils can be activated by a plethora of stimuli relevant in SADs (e.g., viral and bacterial products, complement, cytokines, etc.) and can also modulate innate and adaptive immune responses (18). MCs are implicated in different fibrotic conditions (renal fibrosis and pulmonary fibrosis) (60) and have also been identified in SSc dermis (47). Mast cells, due to their ability to inhibit Treg cells and enhance generation of Th17 cells, are potential antagonists of a proper development and function of Treg cells (1). Interestingly, peripheral blood basophils are upregulated in patients with SSc (63).

B-cells are yet another potential mediator of vascular injury. In fact, highly specific autoantibodies (anti-topoisomerase I, anticentromere and anti-RNA polymerase III antibodies) appear years before the clinical disease (64). In SSc, the immunoregulatory involvement of B-cells is demonstrated by the finding that anti-EC antibodies induce apoptosis. In SSc sera, a subset of autoantibodies against heterogeneous antigens on ECs (referred to as AECA) are able to induce microvascular EC apoptosis through antibody-dependent natural killer cell cytotoxicity (47).

Finally, the involvement of the immune system seems to be responsible not only for tissue inflammation but also as an inducer of fibroblast-myofibroblast transition (65).

CUTANEOUS BLOOD CAPILLARY LEAK AS PATHOGENIC INITIATOR OF LYMPHATIC MICROANGIOPATHY IN SSc

Clinical and histological findings have shown that lesional SSc skin exhibits lymphatic microcirculation abnormalities that may be involved in the early edematous phase, when digital painless swelling is a clinical hallmark (66–68). Of note, early blood capillary leak may occur during the initiation of the cascade of lymphatic microangiopathy (66). Indeed, dermal blood capillary leaking causes greater amount of fluid and macromolecules in the interstitium, with maximal increase in lymph flow provoking micro-lymphatic insufficiency and consequent accumulation of protein-rich interstitial fluid, clinically manifest as edema (**Figures 1A,a2,E**) (66). The consequent inflammatory response and fibrotic process then perpetuate micro-lymphatic injury in a vicious circle similar to that occurring in chronic venous insufficiency. The investigation of the cutaneous capillary lymphatic system in SSc using fluorescence micro-lymphography revealed an augmented dye expansion into the superficial network of lymphatic capillaries and dermal backflow, postulated to be the consequence of hampered drainage of interstitial fluid into the deeper lymphatic collector vessels (66). Moreover, disease duration correlated with the loss of the micro-lymphatic network at the dorsum of the fingers (66). This evidence was further corroborated by histological studies reporting that the numbers of either lymphatic capillary or pre-collector vessels are decreased in SSc skin, with the reduction of the latter being more pronounced than that of capillaries (67, 68). In addition, such a loss of cutaneous micro-lymphatics appears closely linked to the progression of dermal fibrosis and development of fingertip ulcers (67–69).

CLINICAL EVIDENCE AND QUANTIFICATION OF VASCULAR LEAK IN SSc

In SSc, capillaries not only undergo morphologic changes that suggest vascular leak (29), but also display functional deficits (70), resulting in a hyperpermeable state (**Figures 1B–D**). Recovery of the normal vasculature requires resolution of vascular leak (2). The inability of SSc microvessel to recover from vascular leak increases the edema from lymphatic microangiopathy (see above). In fact, the most typical SSc clinical presentation is vascular dysfunction, as manifested by Raynaud's phenomenon, followed by the onset of edematous puffy hands (71). As such, this early stage of hyperpermeability prior to the onset of fibrosis would be an ideal target for therapeutic intervention, thus highlighting the importance of its characterization. While the skin is the primary organ that has been studied in SSc, autopsy data suggests that all organs' microvasculature is affected (72). Indeed, vascular leaking can be also hypothesized in early lung involvement (with ground glass opacities on high resolution computed tomography) as well in the myocardial edema detected on cardiac magnetic resonance (73).

Non-invasive investigational studies support a progressive hyperpermeability of microvasculature in SSc. Nailfold videocapillaroscopy (NVC) is one of the major components in the 2013 classification of SSc (74), demonstrating progressive loss of the peripheral vascular network, dilatation of capillaries, deficient vascular repair and inadequate angiogenesis (75). While NVC is efficient for measuring morphological microvascular changes (microangiopathy), other methods, such as Laser Doppler, thermography or Near Infrared Spectrometry (76), are essential for the assessment of functional blood flow abnormalities. Results published so far do not clearly define the mechanistic changes of glycocalyx, ECM, BM, vesiculo-vacuolar organelles, cellular junctions, cytoskeletal proteins, and/or vascular pericytes abnormalities. Detailed longitudinal studies are in progress to define a unified vascular phenotype in SSc (77).

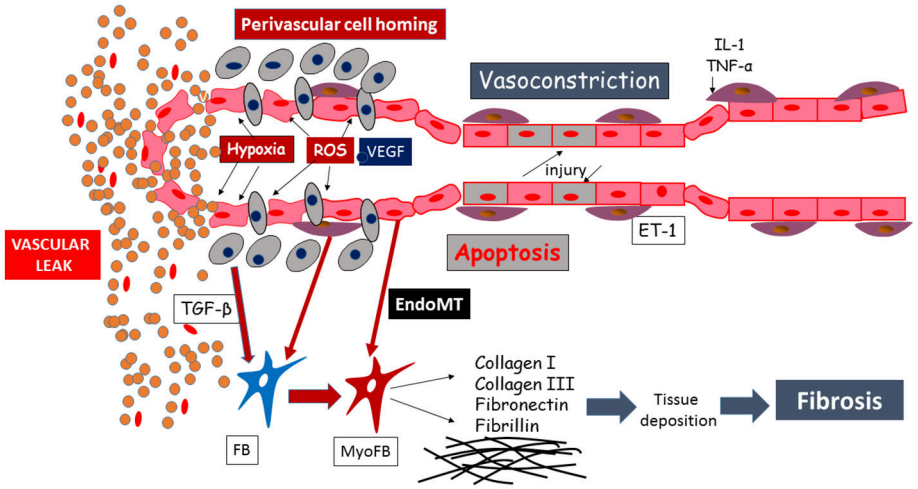

FIGURE 2 | Schematic representation of the mechanisms leading to endothelial injury and capillary sufferance, evolving into vascular leaking.

Evaluation of sublingual microcirculation can detect both morphological and functional capillary impairment and allows measurement of the glycocalyx layer. A pilot study found correlation of abnormal sublingual microcirculation, evaluated by intravital microscopy using Sidestream Dark Field (SDF) with a CapiScope HVCS handheld video capillary microscope (KK Technology, Honiton, UK), suggesting a decrease in glycocalyx thickness. Correlation between NVC capillary density and sublingual capillary density measured by SDF was discovered, supporting the concept of diffuse multi-organ microcirculation abnormalities in SSc (78). In another study of sublingual microcirculation, intravital microscopy demonstrated that the perfused boundary region, a marker of the barrier properties of endothelial glycocalyx, was higher in 40 SSc patients compared with 10 controls, suggestive of dysfunctional glycocalyx (79).

CONCLUSIONS AND THERAPEUTIC PERSPECTIVES

Degradation of the endothelial barrier occurs in response to perivascular inflammation and to reactive oxygen species (ROS) generated by the ischemia-reperfusion injury, taking place in SSc patients' microvasculature (**Figure 2**). This phenomenon is a noxious trigger to the endothelial barrier leading to opening of the endothelial junctions, inflammatory cells homing, sustained hyperpermeability and continuous vascular leak (**Figure 2**). In early SSc, vascular and lymphatic angiopathy leads to leaking into tissue and interstitial edema generation, clinically visible as puffy fingers and detectable in NVC as a fluffy appearance of giant capillaries. It is clear that this phenomenon is at its zenith in the early edematous disease phase, is tightly linked to significant perivascular homing of inflammatory cells and progressively disappears with disease progression (32). From the pathogenetic point of view, vascular leaking testifies to the beginning of a process concentrated in the vessel wall involving ECs, inflammatory cells as well as the other tissue components. A pivotal role in these processes may be played by endothelin-1, which was shown to drive vascular fragility and endothelial dysfunction in animal models (80, 81), regulate adhesion molecules expression (82) and cellular migration (83), and to promote EndoMT (84). This was also indirectly proven by the amelioration induced by endothelin-1 receptor antagonists, manifested both clinically (85) and in NVC (86).

Therefore, the early SSc phase may be the ideal target to achieve the paradigm "to close the door," i.e., prevent leaking into the tissues, mainly with a twofold strategy. The first to induce disease remission with immune suppression by blocking the vascular leaking, and in particular cellular trafficking, and therefore the progression of the disease. The second, to achieve vasoactive protection, restoring endothelial function and block remodeling of the vessel wall. This combination regimen may impact on disease evolution avoiding the progression to fibrosis. The induction therapy dealing with early vascular leaking needs to be carefully tested, including the possibility of using early intense immunosuppression followed by a lower dose "maintenance" treatment, assisted by vasoactive treatment and, in case of already manifested fibrotic changes, anti-fibrotic compounds (87).

In the future, increasing attention to vascular leaking is warranted to better understand early SSc and to evaluate a new strategic targeted therapy.

AUTHOR CONTRIBUTIONS

CB, MM-C, and SB-R conceived the study and contributed to the draft of the manuscript. TF, MM, FWR, DEF, ADP, FR, and SG contributed to the draft of the manuscript.

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REVIEW
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Mesenchymal Stromal Cells Based Therapy in Systemic Sclerosis: Rational and Challenges

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Systemic Sclerosis (SSc) is a rare chronic disease, related to autoimmune connective tissue diseases such as Systemic Lupus Erythematosus and Sjögren's Syndrome. Although its clinical heterogeneity, main features of the disease are: extensive tissue fibrosis with increase matrix deposition in skin and internal organ, microvascular alterations and activation of the immune system with autoantibodies against various cellular antigens. In the diffuse cutaneous scleroderma subtype, the disease is rapidly progressive with a poor prognosis, leading to failure of almost any internal organ, especially lung which is the leading cause of death. Primary trigger is unknown but may involve an immune process against mesenchymal cells in a genetically receptive host. Pathophysiology reveals a pivotal role of fibrosis and inflammation alterations implicating different cell subtypes, cytokines and growth factors, autoantibodies and reactive oxygen species. Despite improvement, the overall survival of SSc patients is still lower than that of other inflammatory diseases. Recommended drugs are agents capable of modulating fibrotic and inflammatory pathways. Cellular therapy has recently emerged as a credible option. Besides autologous hematopoietic stem cell transplantation which demonstrated remarkable improvement, mesenchymal stromal cells (MSCs) represent promising therapeutic candidates. Indeed, these cells possess anti-inflammatory, antiproliferative, antifibrotic, and immunomodulary properties especially by secreting a large panel of bioactive molecules, addressing the most important key points of the SSc. In addition, these cells are very sensitive to their environment and are able to modulate their activity according to the pathophysiological context in which they are located. Autologous or allogeneic MSCs from various sources have been tested in many trials in different auto-immune diseases such as multiple sclerosis, Crohn's disease or systemic lupus erythematosus. They are characterized by a broad availability and no or low acute toxicity. However, few randomized prospective clinical trials were published and their production under ATMP regulatory procedures is complex and time-consuming. Many aspects have still to be addressed to ascertain their potential as well as the potential of their derived products in the management of SSc, probably in association with other therapies.

Keywords: cell therapy, good manufacturing procedures, immunomodulation, mesenchymal stromal cells, systemic sclerosis

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INTRODUCTION

The word "scleroderma" originates from two Greek words: "sclero" meaning hard, and "derma" meaning skin due to skin hardening being one of the most visible manifestations of the disease. Scleroderma (also known as systemic sclerosis or SSc) is a multisystem connective tissue disease classified as autoimmune due to the presence of autoantibodies detected in nearly all patients (1, 2). SSc is a rheumatic disease with heterogeneous clinical manifestations and variable course, but, a characteristic clinical picture includes skin fibrosis, Raynaud's phenomenon, vascular disturbances, joint pain, digital ulcers, and telangiectasia. Internal organ changes such as gastrointestinal tract, lung, heart, and kidney dysfunction precede or simultaneously occur with cutaneous changes (1). Functional impairment of the hands caused by sclerodactily, flexion contractures of the fingers and stiff hands have a significant impact on quality of life of those overcome by SSc. This was the case with a prolific painter Paul Klee who after the diagnosis was forced to discontinue playing violin and his ability to paint was greatly restricted (3). Fatal health complications associated with SSc took Paul Klee's life in 1940. Since that time the understanding of SSc pathophysiology has evolved significantly. Many promising targets aimed at alleviating patient suffering and halting progression of painful and often lethal outcomes of this disease continue to be identified. Immunomodulation and stem cell-based therapy are emerging as an effective strategy in the treatment of SSc patients with poor prognosis (4). Pro-angiogenic, anti-inflammatory, and immunomodulatory properties of multipotent mesenchymal stromal cells (MSCs) make them ideal candidates for targeted cell-based therapy capable of restoration of immune functionality or in other words, immune "reboot" in autoimmune diseases. In this review we discuss therapeutic potential and rationale for application of mesenchymal stromal cells in the treatment of SSc.

CLINICAL ASPECTS

Epidemiology

Although epidemiological data related to the incidence and prevalence of SSc vary depending on geographical region and period of observation (5), worldwide prevalence is estimated to range from 50 to 300 cases per 1 million persons, whereas the incidence is predicted to range from 2.3 to 22.8 cases per 1 million persons per year (1). SSc prevalence and incidence estimates are consistently higher in the USA and Australia (prevalence is 276–443 per million; incidence is 14–21 per million per year) compared to Europe and Japan (prevalence is <150 per million; incidence is <10 per million per year). The highest prevalence of SSc is amongst a Choctaw Native American group in Oklahoma with an estimated prevalence of 660 cases per million suggesting that genetic factors and predisposition of certain ethnic and racial backgrounds may play a role in SSc etiology (5). There exists a gender disparity when it comes to SSc prevalence—a common factor shared by more than 70 other autoimmune disorders. When it comes to SSc disease expression women have a much higher susceptibility than men with a ratio ranging from 3:1 to 14:1 (1).

Clinical Features

There are several subsets of SSc. According to LeRoy and colleagues (6), SSc can clinically be differentiate into 3 main phenotypes: (i) limited cutaneous systemic sclerosis (lcSSc), (ii) diffuse cutaneous systemic sclerosis (dcSSc), and (iii) SSc sine scleroderma. In lcSSc, skin involvement is limited to areas distal to the knees and elbows, and often just to the wrist and ankles. Skin changes on the face and neck may also be evident. There is typically a long antecedent history of Raynaud's phenomenon, often severe and associated with recurrent digital ulceration. Other manifestations include oesophageal dysmotility, gastro-oesophageal reflux, cutaneous telangiectasia, which is generally seen on the palms and around the mouth, and subcutaneous calcinosis. The term lcSSc is preferred to CREST syndrome as it does not ignore the important internal organ manifestations of mid-gut disease (small bowel bacterial overgrowth), pulmonary fibrosis and pulmonary arterial hypertension. Anticentromere antibodies are the hallmark antibodies in this condition, although other antibodies may also be present. The formal classification of dcSSc is determined by the presence of skin sclerosis proximal to the knees and elbows, and usually affecting the trunk. The classical presentation is an abrupt onset of inflammatory change in the skin and other structures. Pain and swelling of the extremities often occur and expansion of tissues around the wrist often results in bilateral carpal tunnel syndrome. Tendon friction rubs can be felt across joints. Affected skin is often intensely pruritic with a loss of specialized skin structures leading to changes in perspiration and hair growth. Raynaud's phenomenon develops simultaneously with other features or once the disease is established. Oesophageal involvement is almost universal and severe internal organ complications tend to occur earlier in the course of disease compared with lcSSc. Lung fibrosis or hypertensive renal crises are relatively frequent, and some specific antibodies can be predictive of these. The autoantibodies classically associated with hypertensive scleroderma renal crisis are Scl-70, antifibrillarin, and anti-RNA polymerases I and III. In addition to these are the less-commonly occurring anti-Pm-Scl and anti-nRNP autoantibodies, which have also been reported to occur in patients with connective tissue diseases other than SSc. The natural history of this condition is heterogeneous and skin sclerosis can remit after several years despite progression of internal organ disease. Finally, SSc sine scleroderma is observed in patients with typical vascular features and serological changes associated with SSc together with visceral complications, such as lung fibrosis, hypertensive renal crisis or severe bowel involvement, but without any evidence of skin fibrosis. This clinical profile is termed SSc sine scleroderma and probably accounts for<1% of cases, although it may well be underdiagnosed (7).

Outcome and Prognostic Factor

SSc is associated with high morbidity and mortality. Five and ten-years survival rates, although improving, are in the order of 68 and 50% respectively (8). This is highlighted in a recent

study showing an age and sex adjusted standardized mortality ratio of 4.06 for newly diagnosed SSc patients, with 22.4 and 26.0 years of loss of life in women and men, respectively (9). Disease-related causes, in particular pulmonary fibrosis, pulmonary arterial hypertension, cardiac complications and renal crisis, accounted for the majority of deaths in SSc (10). Malignancy, sepsis, cerebrovascular disease, and ischemic heart disease are the most common non–SSc-related causes of death. Predictors of early mortality included male gender, older age at disease onset, diffuse disease subtype, pulmonary arterial hypertension, and renal crisis (9).

Disease Classification

The diagnosis of SSc is based on recognition of specific features and physician judgment. In 2013, American College of Rheumatology (ACR)–European League Against Rheumatism (EULAR) established a new classification criteria for SSc incorporating important elements (proximal scleroderma, sclerodactyly, digital pits, pulmonary fibrosis, Raynaud's phenomenon, and scleroderma specific autoantibodies) (11). The new criteria placed more emphasis on the vasculopathic manifestations of the disease and accounted for other clinical features including puffy fingers—another pivotal sign of SSc. The new criteria is more specific, especially when it comes to identifying early stages, mild or limited form of the disease. Classification criteria is used to identify homogenous groups of patients for inclusion into studies and can be used to identify the disease stage and progression. Patients with a total score of ≥ 9 are classified as having SSc.

RATIONAL OF MSC-BASED THERAPY OF SSC

Physiopathology

SSc is characterized by a triad of vascular damage, aberrant inflammatory response and tissue fibrosis (12). Fibrosis gradually replaces healthy tissue and ultimately disrupts the architecture of the affected area causing debilitating symptoms. In fibrotic tissue, normal architecture is replaced with collagen rich, largely acellular, stiff connective tissue resulting in loss of functional integrity consequently leading to severe dysfunction and, in some cases, failure of vital internal organs including the heart and lungs–fatal complications of SSc (13). Pathogenesis of SS-associated fibrosis has been the subject of extensive research, and, although gaps in knowledge still exist, common features of fibrosis are becoming clear regardless of the organ or tissue it affects. Common features of SSc fibrosis include: (i) increased presence and persistence of differentiated fibroblasts (myofibroblasts); (ii) excessive deposition of extracellular matrix components, caused by overproduction of collagen and other glycoproteins and (iii) increased tissue contraction. SSc is characterized by vascular injury which is an early event preceded by fibrosis. A common consensus is that, a primary vascular and immune event cause fibroblast activation, which further activates innate immune signaling resulting in a vicious cycle of fibrogenesis (12, 13). Vascular injury and reduced blood supply lead to progressive tissue hypoxia, which further stimulates collagen synthesis and contributes to the progression of fibrosis in SSc (12). Activated inflammatory cells secrete cytokines causing fibroblast differentiation into myofibroblasts, which produce excessive collagen, contract and induce pathological changes in the connective tissue. Chronic inflammation and persistent presence of pro-inflammatory cytokines and growth factors further drive excessive accumulation of extracellular matrix (13).

Biological specimen of skin, lungs (including bronchoalveolar lavage fluid), and other organs affected by SSc, serve as study tools, which assist in unrevealing possible mechanisms involved in SSc pathogenesis. It has been suggested that an initial event in the form of genetic mutation or environmental trigger, induces autoimmunity and autoantibody production, which in turn activates innate immune cells (such as resident macrophages) and the secretion of innate immune cytokines leading to chronic inflammation (13). At the same time, adaptive immune response is activated and T_H1 cells are mobilized. T_H1 cells are known to be responsible for the secretion of inflammatory cytokines and growth factors, whereas T_H2 cells are predominantly profibrotic (12). Hence, it has been concluded that autoimmunity and inflammation activate fibroblasts and result in pathological fibrogenesis (13). This hypothesis was formed based on histological and molecular analysis of SSc specimen which demonstrate the presence of mononuclear-cell infiltrate (1) including bone marrow-derived $CD4^+$ T cells, macrophages, activated B cells, dendritic, mast cells, and other markers of inflammation (14). Interestingly, an overwhelming presence of $CD4^+$ T cells, type 2 T helper (T_H2) cells known to secrete IL-4 and IL-13 and to a lesser extent T_H1 cells, which primarily secrete anti-fibrotic interferon γ (IFNγ) was detected as part of the cellular infiltrate in SSc biopsy samples of skin (12). Similarly, macrophages have been identified as important players of SSc-associated inflammation. M2–also known as alternatively activated macrophages were detected in skin and lung biopsies, whereas soluble levels of CD163 (known M2 marker) in sera of patients with SSc were significantly elevated compared to controls further suggesting a role for this immune cell in disease pathogenesis (12).

Overview of Current Therapeutic Approaches

Chronic inflammation and autoimmunity are cardinal pathogenic events associated with SSc and hence, logically, therapeutic targeting of either of these processes is likely to be beneficial. Although current therapeutic approaches include general immunosuppression and complication-specific therapies, immunomodulatory regimens with myelosuppression or myeloablation followed by autologous haematopoietic stem cell (HSCs) transplantation (4, 15–17) have been evaluated as a therapeutic strategy in systemic sclerosis (**Table 1**).

The rationale behind autologous stem cell therapy is that after profound depletion of immune cells, including autoreactive T and B cells, a new and naive immune system originating from the stem cell graft will re-establish immune tolerance. Apart from

TABLE 1 | The updated EULAR recommendations for treatment of systemic sclerosis, according to the organ involvment, including strength of the recommendation.

Organ involvement	Recommendation	Strength of recommendation
I. SSc-RP	To reduce the frequency and severity of SSc-RP attacks: - First line therapy: dihydropyridine-type calcium antagonists (nifedipine) or PDE-5 inhibitors - Second line therapy: intraveinous prostanoids (iloprost) - Other: fluoxetine	A A C
II. Digital ulcers on patients with SSC	In treatment of digital ulcers in patient with SSc: - intravenous iloprost - PDE-5 inhibitors To prevent development of new digital ulcers in SSc: - PDE-5 inhibitor To reduce the number of new digital ulcers in SSc, especially in patients with multiple digital ulcers despite use of calcium channel blockers, PDE-5 inhibitors or iloprost therapy: - Bosentan	A A A
III. SSC-PAH	To treat SSc-related PAH and CTD-PA: - ERA (ambrisentan, bosentan and macitentan) - PDE-5 inhibitors (sildénafil, tadalafil) - Riociguat - Intravenous epoprostenol (severe SSC-PAH with class III and IV dyspnea) - Prostacyclin analogs	B A B
IV. Skin and lung disease	To treat skin manifestations of early diffuse SSc: - Mehtotrexate Treatment of SSc-ILD, in particular for patients with SSc with progressive ILD: - cyclophosphamide Treatment of selected patients with rapidly progressive SS and risk of organ failure: - HSCT should be considered	A A A
V. SRC	- ACE inhibitors	C
VI. SSc-related gastro intestinal disease	To treat SSc-related GERD and prévention of oesophageal ulcers and stricture - PPI To manage SSc-related symptomatic motility disturbance (dysphagia, GERD, early satiety, bloating, pseudo-obstruction…) - Prokinetic drugs To treat symptomatic small intestine bacterial overgrowth: - intermittent or rotating antibiotics	B C D

CTD, connective tissue disease; ERA, endothelin receptor antagonists; GERD, gastro-oesophageal reflux disease; HSCT, haematopoietic stem cell transplantation; PAH, pulmonary arterial hypertension; PDE-5, phosphodiesterase type 5; PPI, proton pump inhibitor; RCTs, randomized contrpolled trials; SRC, scleroderma rénal crisis; SSC, systemic sclerosis; SSC-RP, Raynaud's phenomenon in patients with SSC; ILD: intersitial lung disease

the presence of inflammatory infiltrate and markers of chronic inflammation in diseased specimen another observation supports the rational for profound immunomodulation as a therapeutic approach in SSc. This includes clinical overlap of SSc with other autoimmune rheumatic diseases where immunomodulation in the form of stem cell therapy reset immunological clock, and was associated with sustained disease remission (18) further adding to the evidence and supporting the concept of cell-based targeted therapies in the treatment of SSc.

To date, a number of stem cell transplantation studies were shown to be effective in international multicenter clinical trials demonstrating sustained improved clinical effects especially when it comes to late stage disease diffused cutaneous SSc (dcSSc) (15, 17, 19). Autologous stem cell therapy offers the patient an opportunity to "reboot" the immune system and is currently the only curative measure that is able to induce sustained clinical improvement and long-term drug-free remission compared to short-lived (and often ineffective) results of "debulking of inflammation" with cytotoxic and immunosuppressive regimens (17, 20). Mesenchymal Stromal Cells (MSCs) with their immunomodulatory and anti-inflammatory properties may provide an alternative to HSC with a potential to provide long-term benefits in patients with scleroderma (18, 21). Further along we will discuss biological properties of MSCs and elaborate of their potential use for cell-based therapy in the context of SSc.

MSCs are well known as very well tolerated and hence, it is hoped that the use of MSC-based therapy may be used as an adjuvant treatment or may indeed replace immunosuppressive drug therapy. The feasibility of cell therapy, regardless of the cell origin and mode of administration, in adult patients has already been demonstrated and MSCs are considered safe. Indeed, to date, reviews of reported adverse events in clinical trials failed to demonstrate side effects, especially when it comes to toxicity, infection, death or tumorigenicity (22, 23).

Mesenchymal Stromal Cells: Origin and Properties

The beginnings of cell therapy using so-called MSCs go back several decades. However, we are still far from fully understanding the complex nature of these cells. Early MSC characterization studies appeared at the time when Bone Marrow (BM) transplantation was born. The first human BM transplantation took place in 1957, which resulted in hematopoietic reconstitution following an accidental irradiation (24). Subsequently, the first heterotopic BM transplantation was able to highlight the osteogenic potential of medullary cells (24). During the 1960s, the characterization of various cellular constituents of BM gradually begun. Alexander Friedenstein discovered the existence of fibroblastic multipotent progenitors isolated by culture plastic adhesion from BM but also from spleen (25). He also demonstrated the ability of these culture-isolated fibroblast cells to recreate a hematopoietic environment *in vivo* after heterotopic grafting (26). These founding experiments also provided the first clues to the existence of a memory of the original tissue. These cells of similar appearance favoring lymphopoiesis or myelopoiesis according to their medullary or splenic origin. Arnold Caplan later introduced the term mesenchymal stem cell in the early 1990s and showed that these cells were able to generate cartilage, tendons and muscle *in vitro* (27). Finally, in the 2000s, the lack of convincing data to assert the stemness of MSCs, as defined by Loeffer and Potten in 1990 (28), caused the International Society for Cellular Therapy (ISCT) to make an amendment to existing terminology, hence thereafter these cells were termed Mesenchymal Stromal Cells. This allowed to keep the same acronym and to highlight their trophic capacities (29). More recently, it has been shown that MSCs can be isolated from different mesodermal support tissues as well as perinatal tissues (30).

The *in vitro* differentiation capabilities of MSCs were the first to attract the attention of clinicians. This initially led to suggest their use in repair of musculoskeletal defects (27, 31). Gnecchi's team in 2005 used MSCs after myocardial infarction. A significant reduction in the size of infarcted area and apoptotic cell index were recorded as early as 72 h after MSCs injection. It was suggested that as the myocardium assessment was carried out shortly after the treatment with MSCs the likelihood of cardiomyogenic differentiation of MSCs is unlikely. It was then hypothesized that this protective action was related to the secretion of paracrine factors by MSCs. To test this hypothesis, the group produced conditioned media from MSCs cultures and injected this media into occluded coronary arteries of rats. Beneficial effects of cardioprotection have been observed with the use of conditioned media (32, 33).

Other studies based on BM transplantation trials have been performed to treat hematopoietic disorders. Indeed, MSCs derived from the medullary microenvironment participate in the regulation of self-renewal and differentiation of HSCs. In the 2000s, injection of autologous MSCs after myeloaplasia and autologous HSCs transplantation was shown to lead to an earlier resolution of aplasia (34). Moreover, it has been shown by several teams that the co-graft of MSCs and HSCs from the same donor allowed for better engraftment of HSCs while decreasing the risk of graft-vs.-host reaction (GvHD) (35, 36). Finally, the study carried out by the team of Le Blanc et al. on patients suffering from GVHD has shown that injections of haploidentic MSCs could have an immunosuppressive effect *in vivo* (37). All these studies led to the idea that the efficacy of MSCs was probably more related to the secretion of factors regulating endogenous cell activity, than by differentiation to replace damaged cells.

There exist multiple modes of communication used by MSCs. These include secretion of a wide range of bioactive molecules (cytokines/chemokines/growth factors), direct cellular communication through the expression of different membrane markers, mitochondrial transfers and production of extracellular vesicles (EVs) containing proteins, mRNA, miRNA together with mitochondrial fragments. EVs is a collective term for different types of membrane-surrounded structures with overlapping composition, density, and sizes (ranging from 20 to 1,000 nm in diameter), including exosomes, ectosomes, microvesicle particles and apoptotic bodies in accordance with the recommendations of the International Society for Extracellular Vesicles (ISEV) (38). These EVs are released from most types of cells and serving as a means of communication. EVs released from different cells have been implicated in a host of normal cell functions including immune modulation, tissue repair, reproduction, or cancer progression (39). Moreover, EVs released into body fluids have been shown to serve as biomarkers for disease states. Their content, their mode of release or uptake vary between cell types and have also been shown to play a role in the elimination of unwanted cellular components or drugs (39). Recent studies demonstrated that EVs represent a powerful component of the MSCs secretome which could play an important role in tissue regeneration (38).

Finally, the identification of factors that can influence the effectiveness of MSCs has led to a better understanding of interactions between MSCs and their "targets." This also highlighted their great sensitivity to their environment. The modulation of the environment can be chemical, physical or dependent on matrix support and cellular interactions. Therefore, it is important to characterize pathophysiological context in which MSC could be useful and optimize their therapeutic benefit by modulating cellular culture environment before their administration. This forms the basis of cellular "priming" concept. The concept of priming has a twofold aim, firstly, to prepare MSCs to the environment, in which they will be administrated and, secondly, to modulate their behavior to counteract or promote a desired physiological response. Current research aims to understand "innate" and "acquired" components that influence MSCs activity. It must be kept in mind that the concept of cell memory, which enables a cell to "remember" it's tissue or organ origin is as essential as the priming conditions applied before the cell is delivered to the target site.

MSCs and Immune System

SSc is an autoimmune disease with altered cellular immunity, including T and B lymphocyte functional disturbances (40). Moreover, recent data showed an aberrant dendritic cell function (41) and that although the number of natural Tregs is increased

during SSs, their ability to suppress CD4+ effector T is impaired (42). Autologous HSCT for severe autoimmune disease has demonstrated remarkable improvement in many patients. Even if the emergence of new biologic agents reduced the need of cellular therapy for several pathologies such as rheumatoid arthritis and multiple sclerosis, it still remains an option for SSs (20). However, on 35 patients receiving allogeneic HSCT for autoimmune disease 50% showed remission (43). In addition, this strategy also involves the risk of developing GvHD.

MSC therapy in humans in the context of bone marrow graft enhancement (34) but also in the context of acute GvHD (37) was already described as a potentially effective therapy. Following these pioneer studies, many authors highlighted the capacity of MSCs to inhibit the mixed lymphocyte reaction independent of HLA restriction in 2003 and showing anti-proliferative, anti-inflammatory and immunomodulatory properties of MSCs on all actors of the immune system (20). Indeed, it was described that MSCs could favor monocyte polarization to anti-inflammatory M2 macrophages which increase the production of IL-10 and decrease the production TNF-alpha and IL-12 (44). However, MSCs are very sensitive to their environment and we already demonstrated *in vitro* that they can be reversed from having a suppressive to supportive phenotype when exposed with defective immune cells. We further showed that this immune activating effect may be due to MSC pre-stimulation (45). Therefore, before any clinical application, the plasticity of MSCs should be carefully considered and explored in the pathophysiological context in which they will be used.

Only few studies have investigated the immunoregulatory activity of MSCs in the context of SSs. Recent report indicated that SSs-MSCs showed impaired proliferation, differentiation, secretion of cytokines and immune modulation (21). However, two *in vitro* studies have reported that SSc-MSCs, although senescent, might display the same immunosuppressive properties *in vitro* as their healthy counterparts. These observations were realized by using co-culture models between MSCs and peripheral blood mononuclear cells (PBMCs) (46). Moreover, SSc-MSCs could favor Tregs population (46).

Recent studies showed that systemic infusion of BM-MSCs induced transient T cell apoptosis via the FAS ligand (FASL)-dependent FAS pathway. Then, the apoptotic T cells triggered macrophages to produce high levels of TGF-β, which in turn led to the upregulation of CD4+CD25+Foxp3+ regulatory T cells and could ameliorate the disease phenotype in fibrillin-1 mutated SSc mice. This mechanism is not observed after the administration of FASL-/- BM-MSCs. Moreover, they demonstrated that BM-MSCs recruited T-cells for FASL-mediated apoptosis through the secretion of Monocyte Chemotactic Protein 1 (MCP-1) (47). Another study also demonstrates that allogeneic MSCs could attenuate a mice model of cutaneous sclerodermatous GVHD by selectively blocking immune cell migration and down-regulating chemokines and chemokine receptors (48).

MSCs and Vascular System

SSc is characterized by a widespread vasculopathy, defective angiogenesis and progressive fibrosis of the skin and internal organs (49). This vasculopathy is related to endothelial cell (EC) dysfunction which interferes with the cell survival (activation and apoptosis), angiogenesis and vasculogenesis and also by their interactions with various other cells (50, 51). Indeed, despite marked tissue hypoxia, there is no evidence of compensatory angiogenesis in SSc (52). In the process of vasculogenesis, endothelial progenitor cells (EPCs) are mobilized from the BM to the site of neovascularization and differentiate into mature endothelial cells. In SSc, reduced number of EPCs has been detected (52). It was thought that MSCs are an alternative source of EPCs because they display some features of mature endothelial cells, such as the expression of von Willebrand factor (vWF), vascular endothelial growth factor receptor 1 (VEGFR-1), VEGFR-2, VE-cadherin, and vascular cell adhesion molecule 1 (VCAM-1) (52). It was shown that SSs MSCs expressed less VEGFR-2, CXCR4, VEGFR-2/CXCR4 cells than MSCs from healthy controls and early senescence was detected (52).

Furthermore, it has already been described that MSCs could play a crucial role in the modulation of angiogenesis in several models like hindlimb ischemia (53, 54). They were able to produce cytokines and growth factors which could protect endothelial cells from apoptosis and to promote angiogenesis (55). Therefore, several authors investigated the possible paracrine therapeutic role of MSCs in the pathogenesis of SSc. Guidicci et al. showed that BM-derived MSCs from patients suffering from early severe and rapidly progressive diffuse SSc (SSc-MSCs) overexpress bioactive mediators and pro-angiogenic growth factors in contrast with BM-MSCs issued from healthy donors. SSc-MSCs seems to be influenced by the local microenvironment (stimulation by vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ) or stromal cell-derived factor-1 (SDF-1) and upregulate the release of these factors in response to these exogenous stimuli. They also showed that SSc-MSC-conditioned medium had a greater pro-angiogenic effect on dermal microvascular endothelial cell (MVECs) *in vitro* than healthy MSCs donors (49).

Cipriani et al. also showed that environmental cues associated with SSc seem to induce an upregulation of α-SMA and SM22α gene expression on perivascular BM-MSCs and a downregulation of their proliferative activity. Moreover, by using BM-MSCs and healthy human MVECs coculture system the group observed that BM-MSCs isolated from patients with SSs (SSs BM-MSC) like BM-MSCs from healthy patients (H BM-MSC) were able to improve endothelial cell tube formation in stressed condition. Finally, it was shown that co-culture of SSc BM-MSC with healthy MVECs reverts the expression of contractile gene apparatus (α-SMA and SM22α). The authors concluded that SSc BM-MSCs display a more mature and myofibroblast-like phenotype and that their coculture with endothelial cells re-programs these cells toward a pro-angiogenic behavior (56).

In a case report in 2010, the team of Guidicci used intravenous infusion of expanded autologous MSCs in a patient with critical limb ischemia due to SSc. Angiography showed that MSCs enhanced revascularization of patient's extremities. Moreover,

histological skin analysis revealed cell clusters with tube-like structures and an increase expression of angiogenic factors. These data suggested that MSCs could promote vascular network recovery in case of severe peripheral vascular disease in a patient with SSc (49).

MSCs and Fibrosis

Microvascular damage that causes tissue hypoxia is pivotal in the pathogenesis of SSc and it preceding fibrosis. Fibrosis may be considered as the main characteristic of SSc and affecting not only the skin but also all internal organs (57). Fibrosis is characterized by an excessive production of collagen and thickening of the skin and connective tissue caused by a fibroblast dysfunction (58). A large number of soluble paracrine mediators have been implicated in fibrosis and notably, the transforming growth factor (TGF)- β signaling pathway which enhance the pro-fibrogenic cellular programs (59). TGF-β, which is mainly produced by fibroblasts and T helper type 2 lymphocytes, is the major cytokine involved in collagen production leading to fibrosis. TGF-β is regulated by TGF-β receptors (TBRI and TBRII) expression level (60). Moreover, activated fibroblasts are the key effector cells in SSc. Platelet-derived growth factor (PDGF), a potent mitogen for cells of mesenchymal origin, has been implicated in the activation of fibroblasts in SSc. Therefore, inhibition of PDGF signaling could be an attractive therapeutic approach for SSc (61). It had already been demonstrated that BM-MSCs could have a benefit on fibrosis development in different organs including: lung (62), kidney (63), heart (32) or skin (64).

Administration of allogeneic vs. xenogeneic MSCs was evaluated in a mouse model of HOCl-induced diffuse SSc. Additionally, this team evaluate two distinct human MSCs sources: BM and adipose tissue. They showed that xenogeneic human BM-MSCs were as effective as allogeneic or syngeneic BM-MSCs. They decreased skin thickness, expression of Col1, Col3, α-SMA transcripts and collagen content in skin and lungs. Moreover, human Adipose-derived stem cells (ADSCs) were significantly more efficient to reduce skin fibrosis, which was related to a stronger reduction of TNF-α, IL1-β, and enhanced ratio of MMP1/TIMP1 in skin and lung tissues than BM-MSCs (65). Finally, they demonstrated that this anti-fibrotic effect was not associated with MSCs migration to injured skin or with their long-term survival. Indeed, Human MSCs were undetectable 7 days after infusion, whereas they observed a plateau of clinical fibrosis and a decrease of clinical symptoms 21 days after infusion. Finally, this team presumed that the progressive loss of effect might be due primarily to the early disappearance of cells rather than a putative progressive trans-differentiation of MSCs into endothelial cells (65). Moreover, in 2017 Chen et al. showed that in a murine model of bleomycin-induced cutaneous scleroderma, subcutaneous administration of ADSCs significantly attenuated bleomycin-induced dermal fibrosis, reduced skin thickness and total content of hydroxyproline. This team also explained that pathophysiology of SSc involves a complex interplay of inflammation, fibrosis, and vasculopathy. VEGF is a central regulatory factor for the formation of new vessels that controls angiogenesis and has protective effects in SSc patients. TGF-β1 is known to be a fibrosis stimulus factor in SSc. In their mouse model, the ADSCs treatment group showed significant lower levels of TGF-β1 and higher levels of VEGF than the control group (66). It is also already known that inhibiting oxidative stress protect against fibrosis. The thioredoxin (Trx) system is one of the principal intracellular redox systems and regulate its function. Jiang et al. investigated the therapeutic potential of BM-MSCs overexpressing Trx-1 to treat SSc skin after transplantation into a bleomycin-induced murine model exposed to 48 h of hypoxia. They showed that that Trx-1-overexpressing BM-MSCs inhibited hypoxia-induced apoptosis and fibrosis and also promoted the formation of tubular-like structures by endothelial progenitor cells. They suggested that the mechanism of inhibition of fibrosis could involve downregulation of TGF- β (51). These data also suggested that MSC priming could enhance their efficacy in SSc pathophysiology. Finally, another team showed that MSCs isolated from SSs patients showed significant increase in mRNA levels and membrane expression of TGF-β receptor types II (TBRII). Moreover, in response to TGF-β activation, SSs-MSCs showed a significant increase in collagen 1α synthesis and an upregulation of Smad-3 phosphorylation (60). These properties are summarized in **Figure 1** and **Table 2**.

MSC-BASED THERAPY IN SSC

Regulatory Issues

In the EU, products containing living cells and/or products of gene therapy or tissue engineering efforts are regulated by the advanced therapy medicinal products (ATMP) regulatory path (70). The 2007 legislation replaced previous legislations, the EU 2001/83 and 2004/76, by creating an EU legislation regarding medicinal drugs and establishing procedures for authorization and monitoring. A Committee for Advanced Therapies (CAT) was created in charge of the classification of these products. ATMPs are defined as having properties for treating or preventing diseases in patients, or products that may be used in or administered to them with a view to restoring, correcting or modifying physiological functions by exerting principally an immunological, pharmacological, or metabolic action. The 2007 legislation also confirmed the innovative aspect of ATMPs and provided a legal definition of tissue engineered products and combined ATMPs. This directive, by defining the legal status of ATMP, determines its legal framework of manufacturing, development and market authorization. Gene therapy medicinal products and somatic cell therapy medicinal products were defined previously in the EU 2001/83 but the 2007 legislation precise that if a product can be related to the gene therapy definition as well as to the somatic cell therapy or tissue engineered products, then it will be considered as gene therapy product. In the same line, if a product meets both definition of tissue engineered product and somatic cell therapy, then it will be considered as tissue engineered product. Somatic cell therapy medicinal products are cells or tissues that have been subject to substantial manipulation, so that biological characteristics, physiological functions or structural properties relevant for the intended regeneration, repair or replacement are achieved. If the function in recipient and donor is different, the

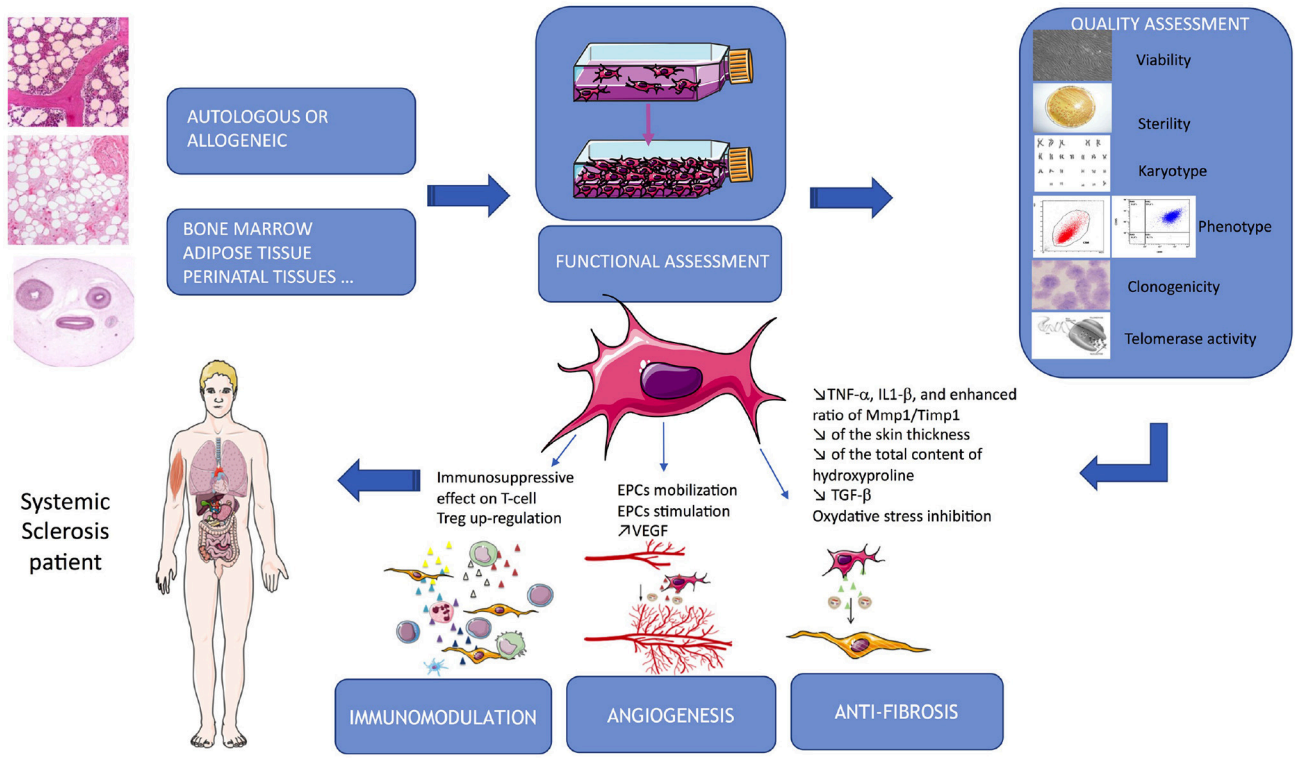

FIGURE 1 | Summarize of MSCs-based therapy in SSc. MSCs are issued from different autologous or allogeneic tissues, their properties used in SSc target immunomodulation, angiogenesis and fibrosis. Quality assessment is a critical part of the process and required several tests allowing their delivery to the patients.

TABLE 2 | Summarize of characteristic of SSc-MSCs and therapeutic targeting the main features of the disease.

	Immune system	Vascular system	Fibrosis	References
SSc-MSCs characteristics	↘ T-cell proliferation ↗ Of functionally $CD4^{+}CD25^{bright}FoxP3^{+}CD69^{+}$ cells ↗ Antioxidant capacity, notably the expression of SOD2 antioxidant gene **In contrast to other reports:** ↘ Capacities of proliferation ↘ Capacities of differentiation ↘ Secretion of cytokines ↘ Capacities of immune modulation	↗ Secretion of bioactive mediators and pro-angiogenic growth factors ↗ Tube formation by endothelial cells (enhanced under hypoxic conditions). SSc-MSCs coculture with endothelial cells → re-programs these cells toward a pro-angiogenic behavior	↗ mRNA protein expression of TGF-β receptor types II ↗ Collagen-1α synthesis ↗ Regulation of Smad-3 phosphorylation	(21) (46) (49) (56) (60) (67) (68) (69)
	SSc-MSCs = literature discrepancy	**SSc-MSCs = pro-angiogenic**	**SSc-MSCs = pro-fibrotic**	
Therapeutic MSCs	↗ $CD3^{+}$ T cell apoptosis ↗ Treg population Blocking of immune cell migration and down-regulating chemokines and chemokine receptors	↗ revascularization ↗ number of clusters with tube-like structures ↗ expression of angiogenic factors ↗ level of VEGF	↘ Skin thickness ↘ Expression of Collagen-1α, Collagen-3, α -SMA transcripts and collagen content in skin and lungs ↘ skin fibrosis, which was related to: ↘ TNF- β1, IL1- β ↗ Ratio of MMP1/TIMP1 in skin and lung tissues ↘ Levels of TGF-β1	(46, 47) (48) (49) (58) (65) (66)
	MSCs = immunosuppressive	**MSCs = pro-angiogenic**	**MSCs = anti-fibrotic**	

cells are no longer considered as cell therapy but ATMPs. Finally, combined advanced therapy medicinal product are advanced therapy medicinal product that incorporate one or more active implantable medical devices and viable cells or tissues, or non-viable cells or tissues which must be liable to act upon the human body with action that can be considered as primary to that of the devices referred to. At the end of the day, ATMPs are classified into 4 groups: gene therapy product, somatic cell therapy medicinal product, tissue engineered cell product and combined advanced therapy medicinal product. However, the scope of this Regulation is restricted to advanced therapy medicinal products which are intended to be placed on the market in EU and either prepared industrially or manufactured by a method involving an industrial process; Advanced therapy medicinal products which are prepared on a non-routine basis according to specific quality standards, and used within the same EU country in a hospital under the exclusive professional responsibility of a medical practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient, are excluded from the scope of this Regulation. Although local regulations, these products are considered as ATMPs, there only difference is that they are manufactured for a single patient.

Managing the Variability

Due to their variability and plasticity, the nonclinical and clinical studies need to be carried out with well-defined and characterized MSCs. Clinical MSC-based batches have to be produced following a robust and standardized process and controlled for their quality and safety to ensure substantial and reproducible results. Several points may arise from each step of the production process of MSCs. Below main issues will be addressed.

Donor Related Variability: Autologous vs. Allogeneic

The question of using autologous or allogeneic MSCs in cell therapy trials often arises. Some groups suggested that MSCs survival may be increased with autologous than with allogeneic cells that should be easier rejected in immunocompetent patients. Even if several studies found that *in vitro* SSs-MSC immunomodulatory properties were similar to that of healthy donors (46, 67) other reported that SSs-MSCs exhibited impaired capacities of proliferation, differentiation, secretion of cytokines and immune modulation (21). Another study showed that BM-MSCs issued from SSs environment exhibit abnormal functional activities, such as increased expression of TGF-β and vascular endothelial growth factor (VEGF), and impairment of endothelial cell (60). Moreover, an impairment of MSCs endothelial cell differentiation capacities (52) and an overexpression of their secretion of proangiogenic factors was already described (49). Therefore, several data seemed to indicate that autologous approaches could be inappropriate because of functional alterations in MSCs from patients, whereas others described similar potential between healthy and SS-MSCs. This discrepancy in the literature indicated that this question has to be carefully considered for clinical use in SSc patients and highlight the need of further studies. Recent clinical trials using allogeneic MSCs illustrated that it could be an interesting option (71–73). Because of the age of the scleroderma patients, their poor health and thus the high probability that the MSCs proliferative power may be impacted, allogeneic donors should be preferred. But in this case of allogeneic use, French regulatory authorities now ask for the development of a Human Leucocyte Antigen (HLA) cross-match test between donor's MSCs and recipient's plasma, as MHC (Major Histocompatibility Complex) mismatch should be responsible for the lack of therapeutic effect.

Due to their heterogeneity within a population and their inter-donor variability, MSCs cultures for clinical use are hard to standardize (in case of autologous use). Our recent data of clinical BM-MSCs expansion (healthy individuals, $n = 5$) reveal a variability in bone-marrow richness of mononuclear cells between donors and a great variability in terms of cell proliferative potential. For instance, gap could be up to a factor 5.6 between the best donor and the worst (data not published). Previous studies suggest a decline of MSCs in bone-marrow with age when comparing groups with a bigger age difference. Caplan's team evaluated, thanks to CFU-F assays, that marrow of newborns, teens, 30, 50, and 80 years old donors would contain respectively about 0.01, 0.001, 0.0004, 0.00025, and 0.00005% of MSCs (74). In addition, exposure to environmental damage and stress over a long time seems to be responsible of negative effects on physical and biological properties of MSCs including proliferation, clonogenicity, differentiation, immunoregulation, paracrine secretion, life-span and senescence (75, 76). In consequence, donor's age is a criterion that should be taken into consideration when proceeding to a clinical trial.

MSCs Sources

Despite the variety of source tissues and the common characteristics that all MSCs share (morphology, proliferation, clonogenicity, differentiation potential, plastic adherence, and a common surface marker profile), they can be more or less easily isolated according to the tissue. For example, ADSCs are described as cells more widely available (because of the high abundance of fat in the body), with a higher yield at isolation, easier to expand in culture and causing less donor-site morbidity than other sources. In addition, cells of different origin can differ by their paracrine activity potency and thus, by their functional properties. For example, Bortolotti et al. have shown that murine BM-MSCs had a better therapeutic potential than murine ADSCs in a preclinical model of critical limb ischemia (77). In this study, both cell types were able to reduce necrosis and inflammation, and to stimulate muscle regeneration, but at a different level. MSCs from bone-marrow expressed higher matrix-remodeling and proangiogenic factors implied in the retention, recruitment and migration of cells involved in vessel remodeling. In a contradictory study, Kim et al. (78) found that human ASCs had a higher therapeutic potential than human BM-MSCs in a similar model explained by a better proangiogenic action. The differences between these studies is the origin of the cells and also the isolation protocol. This highlights that in addition to tissue origin, isolations protocols could also influence the therapeutic properties of the cells. Indeed, cell

biology, composition and viability can be disrupted by separation methods that vary greatly between production sites. In the case of scleroderma, Maria et al. (65) compared human ASCs and human MSCs in a murine model of diffuse SSc and underscored a significantly better effect with ASCs. They have especially found a higher secretion of matrix remodeling and anti-inflammatory factors by these latter, enabling improvement on skin thickness. Regarding fibrosis, both cells were able to reduce it in this preclinical model. These results indicate that tissue choice is important and depends on the pathology to treat and the cell functions of interest.

ADSCs are emerging as an alternative stem cell source for cell-based therapies as well as adipose-derived stromal vascular fraction containing ADSCs. A recent review classified a total of 41 papers describing the factors which modified ADSCs viability and function. These factors including age, gender, body mass index, donor site preference, diabetes mellitus or exposure to previous therapy, although this was not uniformly seen across all studies (79). All these factors, like in case of SSc patients, interact and future studies using ADSCs need to take them into consideration. Then, the same team characterized ADSCs from a cohort of six SS patients in comparison to six healthy age- and sex-matched controls. They indicated that the proliferation and migration capacity of ADSC issued from SS patients is reduced (68). In contrast, Capelli et al., showed that ADSCs from SSs patients had similar surface phenotype and multilineage differentiation capabilities. They did not observe any difference between ADSCs from SS patients and healthy donors neither in PBMC proliferation inhibition assays nor in ADSCs/Endothelial Cells cocultures. Moreover, this effect was enhanced under hypoxic conditions in all of the cocultures. They conclude that autologous ADSC grafting may represent a possible therapeutic option for SS (80). Finally, the previously described study of Maria et al. comparing the efficacy of xenogeneic BM-MSCs with ADSCs in a mouse model of HOCl-induced diffuse SS showed that ADSCs were significantly more efficient to reduce skin fibrosis than BM-MSCs (65). Other sources also emerged recently in clinical trials such as umbilical cord (UC-MSCs), in a recent trial, the association of plasmapheresis and allogeneic UC-MSCs transplantation led to a clinical benefit for the lung of SsS patients (73).

Medium and Conditions of Culture

Duration of expansion as well as culture conditions have a significant impacts on MSC proliferation, functional properties as well as on potential cytogenetic abnormalities. As a consequence, MSCs environment during expansion, including medium composition, needs to be thoroughly evaluated in the clinical process development and population doubling level (PDL) number must be limited to maintain maximal cell potential and safety *in vivo*.

Like isolation, no standardized expansion protocols yet exist between production facilities. MSCs seeding density can differ but it is now accepted that this criterion can have a significant impact on cell quality and yield. Our team noticed a significant difference in term of expansion rate between BM mononuclear cells seeded at 50,000 vs. 200,000/cm^2. Marrow seeded at a lower concentration produces a better yield of MSCs, potentially due to a lesser concentration of red blood cells that can have a harmful effect on MSCs (data not published). Therefore, seeding concentration from primary tissue and also when passaging the cells need to be optimized early in the process development.

Presently, fetal bovine serum (FBS) is still widely used in MSCs productions. When associated with growth factors, in particular FGF-2, or cytokines such as PDGF-BB or EGF, it allows a great cell proliferation *in vitro*. With the combination of ascorbic acid, FGF-2 and PDGF-BB, MSCs are able to increase cell doublings when compared to each factor alone. However, surprisingly, some studies have shown that these factors could induce a loss of the differentiation potential of MSCs especially when cultured for a long time (81). Recent data suggest that the addition of heparan sulfate glycosaminoglycan in the media could promote selection of MSCs with increased self-renewal and with an enhanced survival *in vitro* and *in vivo*. In addition to lot to lot variability, FBS is derived from animals and represents a potential contamination risk. Consequently, the use of human platelet lysate (HPL) or serum-free medium (SFM) has been progressively preferred. In our lab, we demonstrated that, whatever the passage, there is a better proliferation of the cells when they are seeded in a medium containing 5–8% HPL in comparison with a medium containing 10% FBS and FGF-2. SFM is a chemically defined media that presents the advantage to allow a reproducibility between batches, but it also has drawbacks because of its high cost and the confidentiality of its composition. The choice of medium is very important and need to be tested to ensure that it doesn't negatively affect MSCs potential at least *in vitro*.

Priming

Many authors described the impact of SSc pathological context on MSCs, underlining their great sensitivity to their environment. However, it has been described that co-culture of SS-MSCs with endothelial cells from healthy donor could re-programs these cells toward a pro-angiogenic behavior (46). In this line, Fonteneau et al., showed that serum-mediated oxidative stress from SSc patients affects MSCs function. However, even if some functional properties of MSCs were affected upon culture with patient serum, MSCs can adapt to the oxidative environment and exert their therapeutic effect (69).

MSCs paracrine activity as well as exosomes release can be notably modulated by various extracellular signals form the microenvironment including soluble factors, gas, extra-cellular matrix and mechanical stimulation (82). Thereby, this again supports the idea that culture conditions need to be optimized to elicit a particular response and the expected therapeutic effect.

Among soluble signaling factors, TNF-alpha and IFN-gamma, for example, have been shown to induce secretion of CXCL9, CXCL10, IL-6, HGF, VEGF, and TGF-β by MSCs, which then become immunosuppressive by suppressing T cell proliferation. TGF-β is also able to induce MSCs secretion of multiple immunosuppressive factors. MSCs can also change their paracrine signaling in response to a serum-free medium

or in response to gas modification. It was demonstrated that serum-starvation associated to 1% O_2 culture induced the MSCs secretion of extravesicles (EVs) that contain more PDGF and EGF and that are able to induce angiogenesis via the NFkB pathway in a dose dependent manner (83). Hypoxia during culture might also provide pH and medium stabilization allowing a prevention of senescence, an improved genomic stability in BM-MSCs after genotoxic stress as well as the retention of their immunophenotype (84). Concerning effects of low oxygen on proliferation rate and differentiation potential, results are contradictory and seem to be linked to the applied percentage of O_2. Indeed, Holzwarth et al. have in particular shown an impaired osteogenic differentiation at 1% O_2 that was restored at 3% O_2 (85). Physical micro-environment can influence MSCs as shown in 3D experiments developing spheroidal aggregates within which, cells shape, polarity and interactions are modified. MSCs in spheroids were shown to secrete high amount of PGE2 and to inhibit pro-inflammatory factors secretion by stimulated macrophages (86). At last, mechanical forces such as shear stress, tension and compression can modify MSC comportment and secretome.

Fresh vs. Cryopreserved Cells

The use of fresh cells is logistically difficult because it implies to harvest the required number of cells (dose/weight concentration) at the injection scheduled date and to wait few weeks for cell expansion but it remains entirely possible for many diseases including scleroderma. The interest of cryopreservation is the possibility to entirely evaluate the final product in terms of quality and safety prior to injection and to store and thaw MSC as needed with a stock available at any time. In addition, this allows the possibility to select the best donors (based on potency assays results *in vitro*).

Like for cell culture process, no standardized method is described for MSC cryopreservation. As suboptimal protocols can deeply cause cells damage and compromise their survival and stability, freezing protocol need to be optimized to retain the cells characteristics (87). To maximize cell recovery, cells should be harvested at the right time, that is to say in the exponential phase of growth and not at confluence, and cryopreservation medium composition needs to be optimized. It generally contains between 5 and 10% dimethyl sulfoxide (DMSO) solution but as it is potentially toxic, its replacement by sugars such as lactose, sucrose, trehalose, and raffinose is actually tested. As a base solution, balanced salt solutions as well as culture medium supplemented with FBS or, better, human albumin can be used. The freezing step should be done with a device applying a cooling rate of about 1°C per min, and cells viability and functionalities need to be checked post-thaw.

Many groups using diverse protocols for cryopreservation of MSCs from various origin have reported no negative effects on expansion, phenotype profile, differentiation and immunomodulating potential. As MSCs seem more robust than other cell types, this mode of conservation when optimized, can allow a good MSC survival, conservation of their characteristics and a lack of malignant transformation (88).

At contrary, some groups reported that cryopreservation could damage cells in particular by increasing necrosis and apoptosis and could impair immunosuppressive activities of MSC. Moll et al. showed that after a freezing step, thawed MSCs exhibit increased triggering of Instant Blood Mediated Inflammatory Reaction (IBMIR) as well as activation of the complement cascades leading to a lysis of the cells when exposed to normal human serum. They also demonstrated reduced immunomodulatory properties *in vitro* with a worse suppression of PHA-stimulated MLRs and an impaired efficacy in GVHD clinical trials (89).

MSCs Production: New Approaches

Conventional cell culture flasks or factories can be used to assure MSCs expansion for a single patient in the case of hospital exemption (autologous or allogeneic cells). For allogeneic MSCs production and banking, on the other hand, hyperstacks and especially bioreactors begin to have significant interest since they allow expansion of cells at a large scale in a closed environment. These devices can monitor temperature, dissolved oxygen, pH level and agitation, and allow a reproducible and more standardized process (87). Different types of bioreactors compatibles with GMP manufacturing are actually commercialized, proposing various technologies depending on the type of cells and the desired final product (i.e cells or conditioned medium). For MSCs, dynamic suspension culture using microcarrier beads in a stirred-tank bioreactor is often used.

As described previously MSC could exert their therapeutic effect through the secretion of a large panel of bioactive molecules but also by the transfer of extracellular vesicles (EVs) containing proteins, RNA or MicroRNAs (miRNAs). miRNAs are small non-coding RNAs that function as post-transcriptional regulators of gene expression (90). A study of Chen et al. showed that MSCs could transfer miR-151-5p to the recipient BM-MSCs in SS mice. They also showed that the delivery of miR-151-5p could rescue osteopenia, impaired bone marrow MSCs, tight skin, and immune disorders in SS mice (66). Therefore, EVs/miRNA transfer may play a significant role in the MSCs therapies.

Safety Controls

To ensure patient safety, cytogenetic tests have to be considered when using MSCs in therapeutics. The conventional test used in clinical production is a Giemsa-banding (G-banding) karyotype analysis. ISCT recommends not to inject cells if at least 2 out of 20 metaphases have an identical abnormality—a recommendation based on the 2009 edition of the International System for Human Cytogenetics Nomenclature (91). However, this test has its limits and does not necessarily warrant a high standard of quality required in clinical use of MSCs. One of the limits of G-banding karyotype analysis is that it considers mitotic cells that have been arrested in the metaphase portion of the cell cycle, which accounts for <0.1% of tested cells with the majority being in interphase. In addition, this test identifies only abnormalities greater than 5-10 mega-bases, whereas structural abnormalities such as inversions, deletions and duplications within the same

chromosome are not detected. The result of this test is therefore far from being a representation of the overall cell population obtained after culture. Importantly, if G-banding karyotype analysis is positive, it is not possible to make a connection with a selective proliferation advantage and a transformation of the cells with abnormalities. Indeed, MSCs can acquire random and spontaneous genetic aberrations during their *ex vivo* expansion, mainly aneuploidies, but these chromosomal alterations are non-recurrent and interpreted as being related to senescence (92, 93). Based on current literature, no evidence exists suggesting that MSCs with karyotypic abnormalities at the time of injection are deleterious to the patient. When MSCs from bone marrow presenting aneuploidy were injected into immunodeficiency mice, they did not produce tumors after 8 weeks (94). In addition, injected MCS have a limited lifespan, don't persist *in vivo* and their transformation, a very rare event, has never been reported in humans after almost 20 years of clinical use. It would appear that, unlike other species, human MSCs exhibit resistance to spontaneous transformation (95). Investigations conducted following the occurrence of karyotypic abnormalities (aneuploidy) in MSC cultures, found that there are no elements to promote a risk of transformation: no selective clone advantage, complete proliferation arrest between 35 and 52 population doublings, no expression of telomerase (hTERT) and, interestingly, no modification of expression of genes involved in transformation (p53 and p21) was observed and a decrease of c-myc during culture. These results indicate no sign in favor of genetic transformation or a potential tumorigenicity (94). Another team (96), for its part, has shown that human MSCs are genetically stable until passage 4 (increased incidence of abnormalities during later passages) and that they can be cultured for long-term *in vitro* without losing their immunomodulatory capacity or multipotency, even when they had karyotypic abnormalities.

Finally, techniques that are more sensitive than karyotype have shown large number of cellular mosaics (somatic mutations) accumulated in normal adult tissues, particularly in elderly individual (97), highlighting the complexity of genetic analysis interpretations. The hTERT assay for telomerase expression seems therefore more appropriate for the quality control of MSCs from a safety point of view, which is more predictive of a risk of tumorigenicity of the cells. Indeed, activation of the enzyme telomerase is considered one of the classic markers of cancer, since 90% of cancer cells and 70% of human tumor lines have overexpression of telomerase and thus replicative immortality.

To ensure safety and minimize the risk of cell transformation, a cytogenetic assay should be performed in addition to telomerase expression studies, which would ideally consist of G-banding karyotyping in addition to more sensitive spectral karyotyping (SKY) or CGH array designed to detect abnormalities $\leq$50kb (91).

Stability and Route of Administration

MSCs final product "expiration" is often defined as being between 4 and 6 h at 4°C. But some stability data suggest that conditioned MSCs demonstrate a loss of clonogenicity and anti-inflammatory potential *in vitro* after 4 h, even if viability remains good. To enhance cell stability, the final product can be stored at 4°C in a specialized hypothermic storage media such as HypoThermosol prior to injection even if this solution seems to pause the cell metabolism and that a recovery delay after rewarming would be appreciated (98). If freeze-thawed MSCs are used, a period of cell recovery post-thaw would allow optimize cell function. Indeed, while freshly thawed MSCs have showed significantly reduced viability as well as a decreased ability to suppress T-cell proliferation *in vitro* compared to actively growing MSCs, thawed MSCs that were cultured for 24 h regained these immunomodulatory–linked functions (98).

The MSC delivery is also an important criterion to discuss before a clinical use. MSCs can be delivered systemically even though it has been suggested that the majority of cells are trapped by the lungs after infusion and are eliminated thereafter. Consequently, the choice of the site of injection will influence the cell fate and capacity to migrate to injured tissues. However, Maria et al. (65) described a therapeutic effect of MSCs on the skin of SSc mice while no injected cells were found in this organ (half being found in the lung after 48 h and total clearance being observed after 7 days) suggesting that the observed effect couldn't be associated with the MSCs migration to the injured skin.

Locally MSCs injections into tissues (intramuscular or other way) are also used in clinic and provide promising results. Since MSCs are fragile and tend to be removed rapidly from the recipient's body, some groups are working on the encapsulation of MSCs to allow a higher cell protection and a slow delivery of the therapeutic product (99).

Perspectives

MSC-based therapy represents a potential hope for the patients inflicted by an advanced form of SSc. MSCs require a high level of competence and extreme care which should be taken at each stage from cell isolation to clinical trial evaluation. Indubitably, the understanding of MSCs biology has grown profoundly over the last decade. The ability of MSCs to positively influence processes such as immunosuppression, angiogenesis and inflammation generated a lot of interest and enthusiasm from clinicians and researchers alike. However, fundamental questions related to their biological properties especially when it comes to their mode of action *in vivo* are yet to be elucidated. Development of novel highly efficient *in vitro* functional tests and potency assays are a prerequisite to clinically effective and reproducible treatment. Moreover, clinical use of MSCs warrants stringent testing, which is already imposed by legislations and relevant regulatory authorities. An ideal test would assist in selection of cells of the highest quality, which will in turn boost the therapeutic potential and efficacy of cell therapy *in vivo*. EMA, in their 2011 guidelines, recommend a semi-quantitative functional assay such as cellular migration, immunosuppression which may involve phenotyping and profile studies in the form of membrane marker expression or cytokine synthesis. Although some tests have shown efficacy both *in vitro* and *in vivo* (100), systematic correlation between results obtained *in vitro* and

in vivo are still lacking. It is apparent that many questions remain unanswered, however what is becoming clear is that MSCs-based therapy should considered as a safe and potentially efficient therapeutic option in the management of advanced stage of SSc.

AUTHOR CONTRIBUTIONS

CM wrote the manuscript with input from all authors. All authors (JP, MA, NF, EB, and J-JL) provided critical feedback and helped shape the manuscript.

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REVIEW
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A Potential Link Between Oxidative Stress and Endothelial-to-Mesenchymal Transition in Systemic Sclerosis

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Systemic sclerosis (SSc), an autoimmune disease that is associated with a number of genetic and environmental risk factors, is characterized by progressive fibrosis and microvasculature damage in the skin, lungs, heart, digestive system, kidneys, muscles, joints, and nervous system. These abnormalities are associated with altered secretion of growth factor and profibrotic cytokines, such as transforming growth factor-beta (TGF-β), interleukin-4 (IL-4), platelet-derived growth factor (PDGF), and connective-tissue growth factor (CTGF). Among the cellular responses to this proinflammatory environment, the endothelial cells phenotypic conversion into activated myofibroblasts, a process known as endothelial to mesenchymal transition (EndMT), has been postulated. Reactive oxygen species (ROS) might play a key role in SSs-associated fibrosis and vascular damage by mediating and/or activating TGF-β-induced EndMT, a phenomenon that has been observed in other disease models. In this review, we identified and critically appraised published studies investigating associations ROS and EndMT and the presence of EndMT in SSc, highlighting a potential link between oxidative stress and EndMT in this condition.

Keywords: Endothelial-to-Mesenchymal Transition, oxidative stress, reactive oxygen species, scleroderma, systemic sclerosis

INTRODUCTION

Systemic sclerosis or scleroderma (SSc) is a complex multisystem autoimmune disease characterized by progressive fibrosis of the skin and visceral organs and significant vascular alterations (1). The pathogenesis of SSc remains unclear, particularly the mechanisms involved in the development of vascular lesions (2). Oxidative stress-mediated-vascular dysfunction and Endothelial-to-Mesenchymal Transition (EndMT) are likely to play a role in SSc-mediated vascular damage (3). Several studies have shown increased production of reactive oxygen species (ROS), altered redox state, and excessive extracellular matrix (ECM) deposition in organs and tissues of SSc patients (2). Although the primary cellular effector of diseases-associated fibrotic conditions remains to be identified (4) some potential sources have been proposed.

In addition to Epithelial-to-Mesenchymal Transition (EMT), which takes place *in vivo* in the lung (5–8), as well as in a variety of other fibrotic processes (9–13) including SSc (14, 15), the potential involvement of EndMT in SSc has also been suggested. EndMT accounts for the increased fibroproliferative vasculopathy and fibrosis in several diseases (16) and is considered a novel mechanism for the generation of activated myofibroblasts in SSc (17–20). On the other hand, increased ROS generation has been reported to mediate TGF-β-induced EndMT in several conditions including atherosclerosis, Fuchs endothelial corneal dystrophy, and diabetic nephropathy (21–23). TGF-β-mediated ROS generation also promotes cardiac fibroblast differentiation into myofibroblasts, which accounts for the increased production of ECM proteins such as type I and III collagen and the initiation of α-smooth muscle actin expression (α-SMA) during the EndMT process (24). Noteworthy, although not specifically in SSc, the regulation of TGF-β signaling by mitochondrial-derived ROS has also been reported in lung fibrosis (25, 26).

In this review, we summarize the most relevant research regarding the correlation between oxidative stress and EndMT, and their role in SSc-associated vascular damage and remodeling. Readers interested in a more comprehensive discussion concerning the mechanisms involved in the onset and progression of the fibrotic process can refer to other recent excellent reviews (27–30).

Oxidative Stress and SSc

The term ROS indicates oxygen-containing free radicals harboring one or more unpaired electrons in the atom or the outer molecular orbitals (31). Unpaired electrons make free radicals highly reactive. Among them, the superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH^-$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) are key oxidative molecules within the ROS family (32). In this regard, oxidative stress reflects an imbalance between the generation of ROS and the biological system's ability to counteract or detoxify their harmful effects. Therefore, when present in excessive concentrations, ROS cause oxidative stress and cellular damage, potentially leading to cell transformation and/or cell death (33).

ROS and oxidative stress are considered to play a key role in the onset and progression of SSc through several processes, such as ischemia-reperfusion injury (34–36). In addition, ligand-mediated receptor activation by cytokines and growth factors can also increase ROS generation (2, 35, 37). For example, TGF-β is a profibrotic cytokine that plays a key role in the ligand-mediated receptor process that triggers the onset and progression of SSc (2, 37–41). Other putative factors involved in the pathogenesis of SSc include the platelet-derived growth factors (PDGF), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), angiotensin II, interleukin 3, interleukin 6, tumor necrosis factor-alpha (TNF-α), nerve growth factor, and fibroblast growth factor (FGF). These factors can generate ROS in vascular smooth muscle cells, cardiac, lung and skin fibroblasts by activating signaling pathways coupled to nicotinamide adenine dinucleotide phosphate oxidase (NOX) family members (42). Cytokines can also modulate ROS generation by influencing the cellular concentrations of NOX at both RNA and protein levels, as well as by improving both stability and NOX translocation to the cell membrane by stimulating the phosphorylation of NOX complex's components (42). For instance, in rat aortic smooth muscle cells, production of ROS by IL-1β is mediated by NOX4 (43). Although IL1-β induces its expression (44), NOX1 does not appear to be involved in IL1-β-induced ROS production (43). In addition, TGF-β has been shown to induce NOX4 expression as well as ROS production in human arterial smooth muscle cells (45). IFN-γ, via the JAK/STAT pathway (46), and TNF-α, likely via the NFκB pathway (47), also induce expression of NOX1 and NOX4 in human aortic smooth muscle cells (48). Indeed, NOX inhibitors appear to attenuate the effect of TNF-α in vascular smooth muscle cells, supporting the hypothesis that this cytokine elicits its effects via NOX activity (49). The signaling pathways involved in cytokines-induced ROS generation in fibrosis and SSc are quite complex and depend on specific cytokines, NOX isoforms and target cells. For a more detailed information regarding this aspect we refer the readers to this excellent review (42) Furthermore, besides cytokines-induced ROS generation, the increased concentrations of superoxide from different cells, including fibroblasts and monocytes, can contribute *per se* to ROS elevation in SSc (2, 50–54).

About 90% of patients with SSc suffer from Raynaud's phenomenon, a condition where the cold-induced constriction of dermal arterioles is excessively augmented and results in vasospasm and skin color change. Patients with Raynaud's phenomenon secondary to underlying diseases typically present with more severe manifestations such as ulcer, scar, or gangrene (55, 56). Although the detailed molecular pathology of the Raynaud's phenomenon, and its association with SSc, is not clearly understood, both oxidative and non-oxidative pathways appear to be involved (35, 56–58). The systemic increase of ROS concentrations in SSc is likely to be an important factor for the

Abbreviations: 4-HNE, 4-hydroxynonenal, a common byproduct of lipid peroxidation during oxidative stress; ADAM17/NOTCH, Disintergrin and metalloproteinase domain-containing protein 17 involved in the activation of the Notch signaling pathway; AECA, Anti-endothelial cell antibodies; ALK5, Activin receptor-like kinase 5; BH4, Tetrahydrobiopterin; BLM, Bleomycin; c-Abl, c-Abl protein kinase; CTGF, Connective tissue growth factor; EMT, Epithelial-to-Mesenchymal Transition; EndMT, Endothelial-to-Mesenchymal Transition; ERK, Extracellular signal-regulated kinases; ET-1, Endothelial-1; FSP-1, Fibroblast specific protein-1; GSK-3β, Glycogen synthase kinase 3β; GTPase, Guanosine triphosphate intracellular signaling; HOCl; Hypochlorous acid; HPASMCs, Human pulmonary artery smooth muscle cells; I-EndMT, Induced EndMT; IL-1β, Interleukin-1β; miRNAs, MicroRNAs; MMP, Matrix metalloproteinase-1; NAC, N-Acetyl-Cysteine; NADPH oxidase (NOX), Nicotinamide adenine dinucleotide phosphate oxidase; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NO, Nitric oxide; $O_2^{\cdot -}$, Superoxide radical; OH, Hydroxyl radical; $ONOO^-$, Peroxynitrite; p38MAPK, P38 Mitogen-activated protein kinases; PAH, Pulmonary artery hypertension; PAI-1, Plasmin activator inhibitor 1; PDGF, Platelet derived growth factors; PDGFR, Platelet derived growth factor receptor; PECAM 1, Platelet endothelial cell adhesion molecule-1; PI3K, Phosphoinositide 3-kinase; PKC-δ, Protein kinase Cδ; PTU, Propylthiouracil; Ras, A small GTP-binding protein; ROS, Reactive oxygen species; SSc, Systemic sclerosis; TGF-β, Transforming growth factor-β; TIMPs, Tissue inhibitors of metalloproteinases; TNF-α, Tumor necrosis factor-alpha; VE-Cadherin, Vascular endothelial cadherin; VEGF, Vascular endothelial growth factor; vWF, Von Willebrand factor; α-SMA, α-smooth muscle actin.

worsening of the Raynaud's phenomenon. In this context, the concentrations of 8-isoprostane, a biomarker of oxidative stress, antioxidant deficiency and lipid peroxidation, have been shown to correlate with the extent of vascular lesions in Raynaud's phenomenon and the severity of fibrosis in patients with SSc (59–61). The free radical nitric oxide (NO), released by the endothelial cells, plays an essential role in the homeostatic control of vascular tone and blood pressure as well as in preventing thrombosis and cell damage. However, during the reperfusion phase in the Raynaud's phenomenon, free radicals and NO lead to peroxynitrite formation, which precedes oxidative vascular damage and endothelial apoptosis. Therefore, in this specific situation, NO further aggravates vascular damage (35, 62).

A growing number of *in vitro* and *in vivo* studies have demonstrated the direct role of ROS in the pathogenesis of SSc (61, 63, 64). Grygiel-Gorniak and Puszczewicz et al. skin and visceral fibroblasts from SSc patients spontaneously produce large amounts of ROS that initiate collagen synthesis (35). Indeed, fibroblasts from SSc patients have higher baseline NOX-inhibitable intracellular ROS concentrations (65) when compared to fibroblasts from healthy donors (65). This phenomenon appears to be triggered by the stimulation of the PDGF receptor and further maintained through ROS-ERK1/2 signals mediated by Ha-Ras (66). It is important to emphasize, however, that normal fibroblasts can also respond to stimulation by different cytokines with a NOX-dependent increase in intracellular ROS concentrations (65).

A preliminary study by Boin et al. (67) showed a significant increase in intracellular ROS concentrations in human pulmonary artery smooth muscle cells (HPASMCs) after treatment with sera from patients with SSc and pulmonary artery hypertension (PAH). NOX2ds-tat (gp91ds-tat), a specific inhibitor of NOX2, prevented the PAH-SSc sera -induced ROS generation, suggesting the mechanistic involvement of NOX2 in this phenomenon (67). Exposure of HPASMCs to SSc-PAH sera also resulted in a progressive increase of the Collagen promoter activity. Similarly, this effect was prevented by NOX2ds-tat treatment, suggesting that the collagen synthesis activation in HPAMSCs is driven by SSc-related PAH sera through NADPH oxidase-dependent ROS generation (67). Moreover, in human dermal fibroblasts, NOX inhibition caused a significant reduction in the expression of fibronectin, collagen type I and alpha-smooth muscle actin (68). Similarly, the selective NOX1/NOX4 inhibitor, GKT-137831, abolished TGF-β-induced expression of the profibrotic genes CCN2 and alpha-SMA (41). Importantly, GKT-137831 was also able to reduce collagen gel contraction as well as expression of alpha-SMA and CCN2 protein overexpression in fibroblasts isolated from dermal lesions in SSc patients (41). A NOX4-derived increase of ROS has also been reported to be involved in the vascular smooth muscle cells contractile to synthetic phenotype switch elicited by agonistic anti-PDGF receptor autoantibodies from SSc patients (69) Taken together, these findings indicate that the NOX system, in addition to ROS production, mediates the activation of collagen synthesis and profibrotic genes.

In vivo studies in BALB/c mice injected with HOCl daily for 6 weeks (a murine model of SSc) showed the induction of chronic oxidative stress and the concomitant development of cutaneous and lung fibrosis. Furthermore, HOCl-treated mice overexpressed α smooth muscle actin (α-SMA), a marker of myofibroblast activation. These processes were mediated by the ROS-activated intracellular signaling pathways ADAM17/Notch and Ras-ERK (70–72). Blockade of the Ras-ERK pathway by propylthiouracil (PTU) or simvastatin prevented both cutaneous and lung fibrosis (71, 72).

Some studies reported that, in SSc, the excessive production of ROS activates fibroblasts through the binding of stimulatory serum autoantibodies to the PDGFR (73). In this regard, it has been reported that sera from mice and patients with SSc induce fibroblast proliferation and H_2O_2 production by endothelial cells, phenomena that appear to be mediated by oxidized auto-antigens such as the oxidized DNA topoisomerase (74). Noteworthy, the same paper indicated that the nature of ROS, as well as the induction of different antibodies, appear to dictate the form of SSc in both mice and humans. BALB/c SCID mice treated with peroxynitrites developed skin fibrosis and serum anti centromere protein autoantibodies (anti-CENP-B), as reported in patients with limited cutaneous scleroderma, while mice treated with hypochlorite or hydroxyl radicals developed skin and lung fibrosis and DNA topoisomerase I autoantibodies, as reported in patients with diffuse cutaneous scleroderma (74). While pro-oxidants may cause an increase in autoantibodies, other studies failed to demonstrate a significant association between autoantibodies in endothelial cells and fibroblasts and serum-induced ROS or cell proliferation. Despite this uncertainty, there is good evidence supporting the role played by autoantibodies in SSc, particularly anti-endothelial cell antibodies (AECA), in the development of pulmonary fibrosis (35, 75). Oxidative stress may either directly activate ROS-induced differentiation of fibroblasts into myofibroblasts (70), disrupt the balance between protease and protease inhibitors, or both. TGF-β upregulates the expression of extracellular matrix proteins including collagens, but also suppresses protein degradation through enhancing the activities of protease inhibitors such as plasmin activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs) (38). This protease-antiprotease imbalance is likely to represent a critical factor in the development of SSc lung fibrosis (76). Furthermore, although not specifically demonstrated in the SSc-associated fibrotic process, a mitochondrial-generated increase of ROS has been found to induce lung fibrosis (25–27). Effect of ROS in the development of fibroproliferative vasculopathy and fibrosis in SSc (**Figure 1**).

Endothelial-to-Mesenchymal Transition and SSc

The pathogenesis of SSc involves several complex mechanisms associated with (i) microvascular fibroproliferative lesions, (ii) innate and adaptive immune system abnormalities and uncontrolled accumulation of collagen, as well as (iii) other extracellular matrix compartments produced by fibroblasts and activated myofibroblasts in the skin and other organs (17, 77). Physiologically, myofibroblasts die through apoptosis and/or the transition to a quiescent/senescent state in the

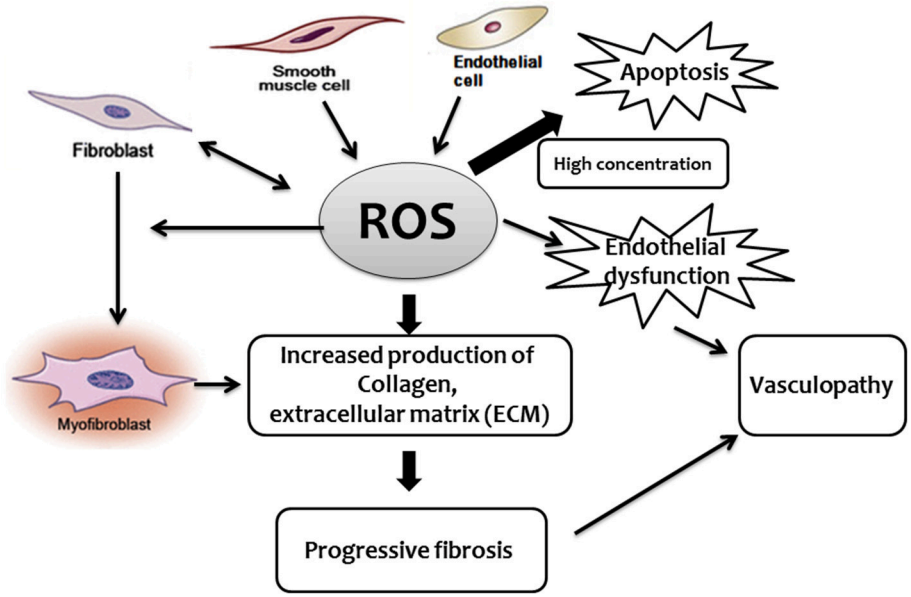

FIGURE 1 | Schematic diagram describing the central role played by ROS in the development of fibroproliferative vasculopathy and fibrosis in SSc.

late stages of wound healing. However, the persistence of activated myofibroblasts contributes to progressive fibrogenesis (78, 79) and favors the onset and progression of interstitial and perivascular fibrosis in the lungs, heart, kidneys and other organs, which accounts for the high mortality of SSc patients (80).

Myofibroblast activation in SSc has been demonstrated in pericytes and smooth muscle cells (SMCs) from vessel walls, resident fibroblasts, and bone marrow-derived fibroblasts (17, 81, 82). Although the ontogenesis of myofibroblasts in fibrotic conditions remains an area of active research (4, 83–86), an increasing number of studies indicated EMT as a potential source of activated fibroblasts by which epithelial cells transform into myofibroblasts (5–8, 14, 15). In this regard, some authors suggest that diseases-associated fibrotic processes may be the result of injury-elicited cellular stress responses such as senescence or apoptosis (28, 87–91), These processes, under yet unknown conditions, could also promote tissue repair by activating and/or recruiting resident progenitor cells (92) especially in the lungs (93–95). However, this hypothesis does not exclude the coexistence of other injury-activated processes such as the cellular transdifferentiation of endothelial cells to profibrotic activated myofibroblasts during EndMT, a phenomenon that has been reported also *in vivo* (19, 96–102). The latter might provide a plausible explanation for the excessive secretion of extracellular matrix proteins that takes place in this pathological condition (103). EndMT is considered a distinct form of EMT since vascular endothelial cells share several similar characteristics and molecular mechanisms with epithelial cells in generating fibroblasts and myofibroblasts (104, 105). EndMT contributes to the development of cardiac, pulmonary, renal, liver, and intestinal fibrosis, and idiopathic portal hypertension in SSc (7, 12, 13, 17, 18, 97, 98, 106–109). EndMT has also been reported *in vivo* in pulmonary hypertension, a process that is closely associated to SSc (101, 102, 110). It is also important to mention that, despite its role in many pathologies, EndMT may be beneficial during angiogenic sprouting, as it allows cells to lose intercellular junctions and delaminate from the parent vessel (111).

During the conversion of endothelium into mesenchyme elicited by TGF-β or Notch ligands, endothelial cells undergo morphological alterations and loss of characteristic cell-surface markers, acquiring mesenchymal, fibroblast-like properties such as spindle-shaped morphology, migratory capacity, invasiveness, and enhanced resistance to apoptosis (82, 112). During EndMT, the structure of vessel-lining is disrupted due to resident endothelial cells disaggregating from the organized cells layer in the vessel walls and invading the surrounding tissue (112–114). Cell-surface markers such as vascular endothelial cadherin (VE-Cadherin), CD31 (platelet endothelial cell adhesion molecule-1, PECAM 1), and von Willebrand factor (vWF) are gradually replaced by markers such as fibroblast-specific protein-1 (FSP-1), α-SMA, vimentin and type I and type III collagen (12, 18, 114).

Several studies have demonstrated the presence of transitional EndMT cells in the pulmonary vasculature of patients with SSc-PAH, indicating their possible contribution to vascular remodeling and fibrosis (18). For instance, the coexpression of cell surface markers specific for both endothelial and mesenchymal/fibroblastic cells, such as Willebrand factor (vWF) and α-SMA, has been reported in an experimental murine model of PAH and in pulmonary endothelium samples of SSc-PAH patients (110). Similar findings have been reported in lung tissue from patients suffering from SSc-associated interstitial lung disease (19). Cells in the intermediate stages of EndoMT have also been found in dermal vessels from

patients with SSc and in two types of SSc animal models, bleomycin-induced SSc and urokinase-type plasminogen activator receptor (uPAR)-deficient mouse model (115). When compared to normal skin microvascular endothelial cells from healthy donors, cells from SSc patients displayed a spindle-shaped figure along with co-expression of both endothelial (CD31 and VE-cadherin) and myofibroblast markers (α-SMA, S100A4, type I collagen). Moreover, exposure of healthy donor-derived microvascular endothelial cells to either SSc sera or TGFβ1 triggered the transition to a myofibroblast-like morphology, contractile phenotype, downregulation of endothelial markers and induction of mesenchymal markers (115). Similarly, exposure of pulmonary artery endothelial cells to a mixture of proinflammatory cytokines such as IL-1β, TNF-α, and TGF-β abolished their "cobblestone" structure and prompted a spindle-like appearance along with induction of mesenchymal markers (110). Induced endothelial-mesenchymal transition (I-EndMT) cells exhibit an increased secretion of proinflammatory proteins and collagen type I. In addition, the presence of I-EndMT cells in the cellular barriers leads to a significant increase in paracellular and transcellular permeability, an early sign of vascular dysfunction in SSc (76, 110).

Vascular abnormalities in SSc patients have been reported to affect the structure and function of several organs and systems, such as the kidney, lung, skin, heart, gut, penis and large vessels (17). Therefore, the mechanistic contribution of EndMT to the pathogenesis of SSc vasculopathy is postulated to involve a synergistic and complex activation of a large variety of endothelial cell types from different body districts exposed to multiple local biological mediators, particularly TGF-β, but also IL-1β, TNF-α, PDGF, VEGF, and endothelin-1 (ET-1) (17, 18, 77, 78, 112, 114, 116–119). Indeed, cytokines-mediated receptor activation induces the expression of endothelial cell adhesion molecules such as ICAM, VCAM-1, and E-selectin (120, 121), which promote both recruitment and activation of chronic inflammatory cells, such as T- and B-lymphocytes and profibrotic macrophages, in the perivascular tissue and in the parenchymal organs interstitium. Recruited chronic inflammatory cells secrete transforming TGF-β, CTGF, and other profibrotic growth factors which along with endothelial cell released mediators, such as endothelin-1, enhance the fibroproliferative vasculopathy characteristic of the disease and potentially activate EndMT (17, 122, 123). In this regard, the interaction between proinflammatory stimuli and endothelial cells has been demonstrated by a recent paper reporting the upregulation of ET-1 and TGF-β in human microvascular endothelial cells induced by IF-γ, (118). Nevertheless, the precise molecular mechanisms by which inflammatory cytokines, growth factors, and signaling pathways mediate EndMT are not completely elucidated and necessitate further investigation.

Using a transgenic mice model, a recent study demonstrated that the endothelial cell-specific activation of TGF-β1 downstream signaling pathway induces EndMT in lung vessels (20). An elegant study using co-culture of microvascular endothelial cells and fibroblasts isolated from the skin of SSc patients demonstrated the ability of SSc-fibroblasts to induce endothelial cells EndMT transition. Consistent with this finding, normal fibroblasts treated with TGF-β and ET-1 were able to promote the same effect when co-cultured with microvascular endothelial cells (124). TGF-β2 has also been reported to mediate Interferon-γ-induced EndMT in human dermal microvascular endothelial cells (118). Protein kinase C has also been reported to be involved in TG-induced EndMT of mouse pulmonary endothelial cells *in vitro* (125).

All TGF-β isoforms have been reported able to elicit EndMT although the precise role of each isoform appears to differ between species and requires further studies to be clearly elucidated. While TGF-β 1 appears to be the main isoform involved in fibrosis-associated EndMT processes, TGF-β 2 seems to be primarily involved in EndMT associated to embryonic heart development (126–128). Nevertheless, also TGF-β 2 has been recently reported to be involved in EndMT associated with some pathological process such as cardiac hypertrophy and renal fibrosis (129, 130). In this regard, Maleszewska et al. (131) showed that TGF-β can either promote EndMT *per se* or synergize with TNF-α and IL-1β to induce the transdifferentiation of endothelial cells toward the profibrotic activated myofibroblasts phenotype. The authors showed that, in the framework of an inflammatory co-stimulation, TGF-β 2 is more potent that TGF-β1 in inducing EndMT, suggesting that TGF-β 2 may be the primary EndMT trigger, while IL-1 is necessary for the efficient induction of EndMT, but is not essential for its maintenance (131). The finding that endothelial cells in proinflammatory environment respond differently to TGF1 and TGF2 suggests that, similar to what reported in the embryonic development, TGF2 might play a major role in EndMT associated to pathological inflammation. In this regard, Good et al. (110) showed that a combination of TGF-β with IL-1β and TNF-α induced EndMT in pulmonary artery endothelial cells. Notably, the withdrawal of IL-1β, TNF-α, and TGF-β after 6 days failed to revert this process, suggesting that the phenotypic change might be permanent (104, 110). This observation was in agreement with previous studies in cells exposed to activated Ras and TGF-β treatment (107). Recently, Cipriani et al. (119) reported that Macitentan, an Endothelin-1 Receptor Antagonist, blocks both Endothelin-1- and TGF-β-induced EndMT in microvascular endothelial cells isolated from healthy donors and SSc patients (119). Similar results, using both Bosentan and Macitentan, were also obtained by Corallo et al. (124) in the fibroblast and microvascular endothelial cells coculture model previously discussed (124). Other studies reported that some anti-fibrotic mediators could dedifferentiate established myofibroblasts. These findings suggest the possibility that EndMT is a reversible process, providing a new intriguing therapeutic target for fibrotic diseases (81, 132).

TGF-β plays a critical role in other signaling pathways involved in EndMT, such as the Smad-dependent and Smad-independent pathways (**Figure 2**). Smad proteins have been shown to bind directly to the Snail gene promoter and regulate its transcription (117). Snail-1 is a zinc-finger transcription factor that forms a complex with Smad3/Smad4, and by acting as transcriptional repressor, plays a crucial role in the

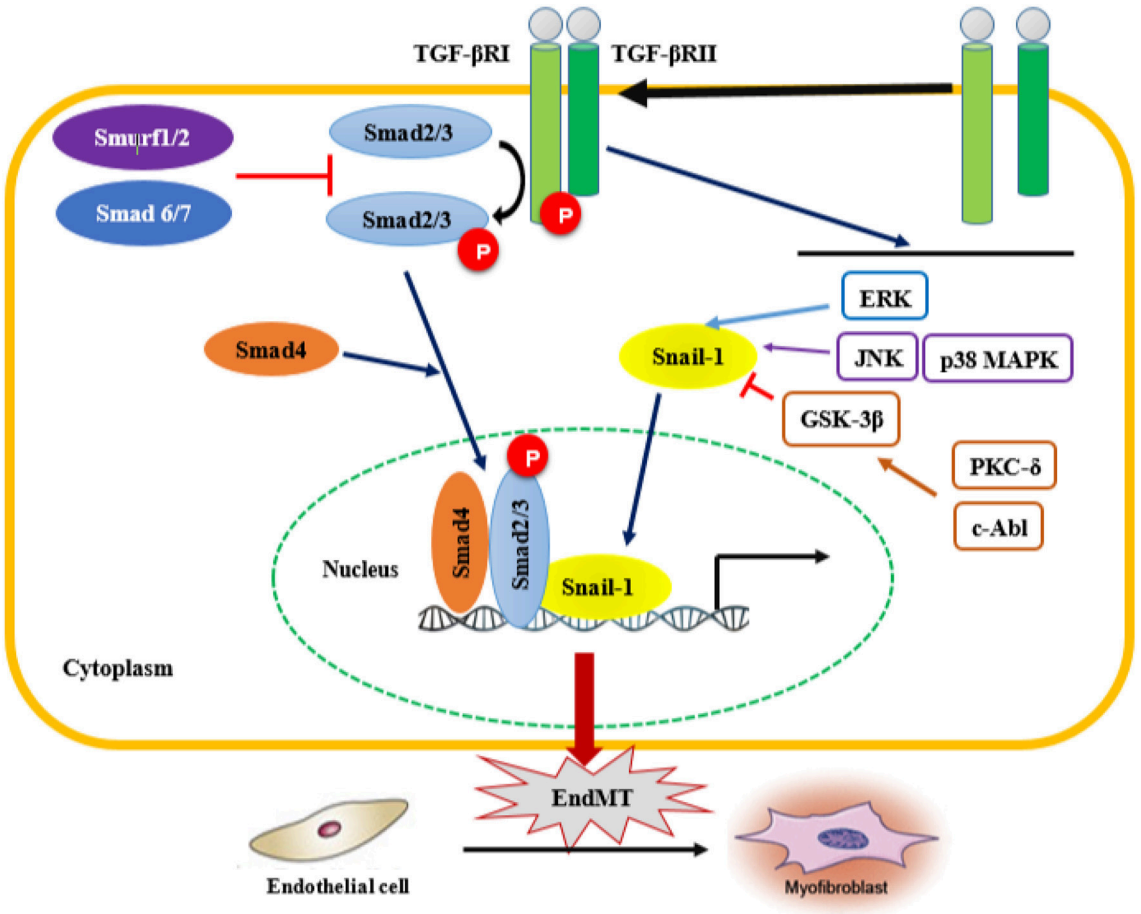

FIGURE 2 | Smad-dependent and Smad-independent pathways of TGF-β signaling associated with EndMT. Transforming growth fator-β (TGF-β) signaling activates the downstream signal transduction cascades, Smad and non-Smad pathways. TGF-β binds the TGF-β type II receptor (TGF-βRII), which recruits and activates the type I TGF-β receptor. TGF-βRI in turn phosphorylates Smad2/3, which forms a complex with Smad 4. In addition, TGF-β activates Smad-independent pathways. Activation of Smad-independent TGF-β pathway causes phosphorylation of GSK-3β mediated by PKC-δ and c-Abl. Phosphorylation of GSK-3β causes its own inhibition which then allows Snail-1 to enter the nucleus. TGF-β/Smad-dependent and Smad-independent pathways upregulate the transcription of TGF-β target genes such as α-smooth muscle actin (α-SMA), fibronectin and type I collagen, as well as the transcription factor Snail-1 involved in EndMT. EndMT leads to the transdifferentation of ECs into mesenchymal cells, which subsequently transform into myofibroblasts, therefore contributing to the progression of fibrotic diseases. ERK, extracellular signal-regulated kinase; JNK, jun N-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinases; PKC-δ, protein kinase C δ; c-Abl, c-Abl protein kinase; GSK-3β, Glycogen synthase kinase 3β.

TGF-β-induced mesenchymal transdifferentiation of embryonic stem cell-derived ECs. The active Smad3/Smad4/Snail-1 complex is a potent inhibitor of E-cadherin expression by directly integrating into specific sequences within the gene promoter and blocking its transcription. In addition to E-cadherin inhibition, Snail-1 precedes transcriptional events that lead to the expression of a mesenchymal-cell-specific phenotype (17, 18).

In the Smad-independent pathway of TGF-β signaling, there is an involvement of important kinases such as c-Abl protein kinase (c-Abl), protein kinase Cδ (PKC-δ), RhoA and glycogen synthase kinase 3β (GSK-3β). The function of RhoA in actin and microtubule cytoskeleton organization is well-established, and as such contributes to the structural/phenotypic changes observed during EndMT (133). On the other hand, activation the of Smad-independent TGF-β pathway causes GSK-3β inhibition allowing Snail-1 to enter the nucleus. Indeed, phosphorylation of GSK-3β at the specific Ser9 residue causes its own inhibition, which in turn induces Snail-1 up-regulation and promotes its subsequent translocation into the nucleus. This process increases the nuclear accumulation of Snail-1, which consequently drives the expression of mesenchymal cell-specific markers such as α-SMA and type I collagen, while reducing the expression of VE-cadherin harbored in endothelial cells. Inhibitors of c-Abl and PKC-δ, such as imatinib and rottlerin, counteract the phosphorylation of GSK-3β, which allows GSK-3β to phosphorylate Snail-1, targeting it for proteasomal degradation and ultimately abolishing the transdifferentiation of ECs into myofibroblasts. This intervention could be a therapeutic strategy to counteract the acquisition of the myofibroblastic phenotype during EndMT (125). TGF-β2-downstream signals mediated by

MEK, PI3K, and p38MAPK pathways are also essential for ECs undergoing EndMT transition (117). In addition, regulation of EndMT by Wnt (134), NOTCH (135), and Caveolin-1 signaling (136, 137) has been observed prior to the TGF-β-induced endothelial-mesenchymal transition. ET-1-mediated TGF-β1-induced EndMT has been also confirmed in skin and lungs *in vivo* in an animal model of TGF-β1-induced tissue fibrosis (138). Finally, other cytokines and growth factors such as PDGF, VEGF, ET-1, CTGF, and some MicroRNAs (miRNAs) might also be involved in the endothelial-mesenchymal transition (17, 18).

miRNAs are small non-coding RNAs containing about 22 nucleotides, which are post-transcriptional repressors of gene function (139). miRNAs have been recently reported to play important roles in SSc pathogenesis as well as in the EndMT process (140, 141). However, further studies are required to determine whether the SSc-associated profibrotic or antifibrotic effects of specific miRNAs are mediated by EndoMT. Recent studies reported the involvement of miRNAs 125b and 126 in the development of EndoMT (142–144) and the interaction between TFG β and several miRNAs in modulating EndMT. For instance, miRNA21 partially mediated TGF-β-induced EndoMT (145, 146). miR-155 is higly overexpressed during the EndoMT process and potently inhibits TGF-β induced EndoMT through a mechanism involving RhoA signaling (147). The overexpression of miR-148b increased EC migration, proliferation, and angiogenesis, whereas its inhibition promoted TGF-β-induced EndoMT (148). On the other hand, other studies indicated some miRNAs as positive modulators of TGF-β-induced EndoMT. For instance, the constitutively expression of miR-31 positively regulates TGF-β-induced EndMT in cultured endothelial cells (149). Interestingly, the overexpression of miR-130a, which is upregulated in monocrotaline-induced the Pulmonary arterial hypertension (PAH) mouse model, can elicit the expression of α-smooth muscle actin, a critical component in EndMT transition and fibrogenesis (150). Since PAH is a widely SSc-associated condition, this paper my pave the way for further experimentations in this direction. Many other miRNAs have also been reported to be involved in EndoMT modulation including let-7 (151, 152) and miRNA 29s (153). Given the pivotal regulatory effects exerted by miRNAs on the multitude of signaling pathways involved in the pathophysiology of multiple diseases, we expect that the knowledge regarding the putative contribution of miRNA to the EndMT process in conditions such as SSc will rapidly expand providing valuable information both to unravel the EndoMT mechanistic processes and to identify potential therapeutic targets for fibrotic disorders in general.

Reactive Oxygen Species and Endothelial-to-Mesenchymal Transition

ROS have been proposed as key mediators of EMT in renal tubular epithelial cells, human epithelial keratinocytes, and lung epithelial cells (32, 105, 154–156). An emerging issue is whether there is a similar tendency of endothelial cells exposed to oxidative stress to form transitional EndMT cells. Although EMT and EndMT share several similarities, in terms of signaling pathways and outcome, the two processes need to be differentiated due to the various origin, functions, and microenvironment of epithelial and endothelial cells (157, 158). Whether EndMT is a reversible biological process, similar to EMT (157), is an intriguing question that deserves further investigations.

ROS Activate/Mediate TGF-β-Dependent Signaling Pathways in EndMT

TGF-β is a multifunctional protein, including three isoforms (TGF-β1, TGF-β2, and TGF-β3), which regulates several important physiological processes such as cell proliferation, differentiation, apoptosis, adhesion, and migration (38, 159). However, a critical, potentially vicious, cycle of TGF-beta, and ROS interaction exists. For instance, TGF-β stimulates the generation of ROS in various cells while ROS can activate TGF-β and mediate its effects. Moreover, TGF-β elevates ROS production via NOX4, mitochondria, or microsomes, and ROS, in turn, can induce TGF-β gene expression and activate its signaling through oxidizing latency association protein (LAP) or activating MMPs which promotes LAP release (38). It is well known that TGF-β is synthesized as a non-active pro-form combining with LAP to create a latent complex. The ROS-oxidable redox center present in LAP can trigger a conformational change resulting in the release of TGF-β1 (160). Hence, under the stimulation of ROS, TGF-β is activated and increasingly expressed.

It is well documented that TGF-β induces EndMT in cardiac, pulmonary, renal, intestinal, and skin tissues (12, 106, 107, 161, 162). In this context, NADPH oxidases and ROS play a pivotal role in mediating TGF-β induced fibrotic responses via Smad2/Smad 3 activation (159). Of note, NOX4-dependent generation of H_2O_2 is required for TGF-β1-induced myofibroblast differentiation and ECM production (163, 164). A recent study by Montorfano et al. (32) used human umbilical vein endothelial cells (HUVECs) to investigate the role of ROS as well as the underlying mechanism in the conversion of ECs into myofibroblasts (32). The authors demonstrated that oxidative stress is a crucial factor in generating the EndMT phenotypic conversion of ECs via TGF-β1 and TGF-β2-dependent pathway and that the ALK5/Smad3/NF-κB intracellular pathway mediated the observed phenomenon. This study supports the hypothesis of an interaction between ROS, TGF-β, and EndMT. Indeed, increased ROS prompted the expression and secretion of TGF-β1/2, with consequent activation of its downstream signals, while silencing of TGF-β1/2 abolished the oxidative stress-induced conversion (32).

Another recent study by Echeverría et al. (165) provided evidence that lipopolysaccharide-induced ROS could lead to an EndMT-like process through an ALK5 activity-dependent mechanism (165). In line with this observation, Toshio et al. (109) showed that endotoxemia-derived oxidative stress promotes TGF-β-mediated EndMT in pulmonary vascular endothelial cells (109). This evidence was strongly supported by another study showing that the expression of TGF-β1 and

TGF-β2 is crucial for the development of endotoxin-induced endothelial fibrosis (166). The increase in endotoxin-induced TGF-β1 and TGF-β2 expression required the activation of NOX and the subsequent generation of ROS (166). Collectively, these data suggest that oxidative stress mediates the EndMT process induced by TGF-β.

Oxidative Stress and EndMT in SSc: Is There a Link?

Despite the fact that the relationships between ROS and SSc, and between EndMT and SSc, have been extensively investigated, relatively few studies have investigated the effects of oxidative stress on EndMT in SSc. Xu et al. (167) showed that chronic oxidative stress mediates EndMT in a murine model of SSc (167). The authors isolated microfibrils from the skin of tight skin ($Tsk^{+/-}$) mice, which showed abnormal big fibrillin-1. Culturing ECs with this abnormal extracellular matrix led to morphological and functional cellular changes, and increased the concentrations of 4-HNE, a well-known fission product of polyunsaturated fatty acid oxidation. Transdifferentiation from ECs to mesenchymal cells with the increased presence of FSP-1 and Twist (a transcription factor implicated in the endothelial cell to fibroblast transition), along with the decreased expression of VE-cadherin, were also noted (167). Furthermore, the abnormal big fibrillin expression was associated with oxidative stress (reduced nitric-oxide-to-superoxide anion ratio) suggesting changes in the intracellular redox state involved in the observed transition. Interestingly, chronic mice pretreatment with D-4F, a peptide binding with high affinity to oxidized lipids, attenuated or abolished EndMT, indicating that oxidized lipids might play a central role in other chronic conditions where oxidative stress promotes endothelial-mesenchymal transition (167). However, the authors did not dissect the role of TGF-β although, as mentioned above, TGF-β is known to be a crucial player in the initiation of EndMT in various diseases (17, 18). Of note, ECM including fibrillin functions as a reservoir for TGF-β and other growth factors to control mesenchymal differentiation (168). The possibility of an interaction between TGF-β and oxidative stress prior to EndMT is intriguing and requires further research.

It is speculated that the local availability of tetrahydrobiopterin (BH_4) contributes to endothelial physiology (169), and that its insufficiency might be involved in the endothelial dysfunction observed in SSc (170). Decreased concentration of BH_4 induces eNOS uncoupling, leading to superoxide, rather than NO, production, which in turn induces a state of oxidative stress (171). Although not able to reverse blood oxidative stress markers, the acute administration of BH_4 has been shown to improve endothelial function in patients with SSc, without affecting their blood pressure (170). However, in this paper the authors did not investigate whether endothelial, rather than systemic, oxidative stress was ameliorated. Indeed, it may be possible that the concentration of BH_4 used was sufficient to improve endothelial function but not appropriate to ameliorate the oxidative insult seen in blood samples of SSc patients. More importantly, the patients only received BH_4 for 5 h. This period might be too short to influence systemic oxidative parameters. In fact, more data support the involvement of BH_4 in SSc-associated endothelial dysfunction. For instance, circulating concentrations of BH_4 have been found to be lower in plasma and pulmonary arteries of patients with IPF, and in rats with bleomycin-induced pulmonary fibrosis, when compared to their healthy counterparts (172). Notably, TGβ1 and ET-1 were able to induce EndMT in human pulmonary artery endothelial cells by decreasing BH_4 and eNOS expression. Finally, treatment with sepiapterin, a BH_4 precursor, blunted bleomycin-induced pulmonary fibrosis, ameliorated vascular remodeling *in vivo* by increasing plasma BH_4 and vascular eNOS expression in rats and counteracted TGβ1- and ET-1-induced EndMT in human pulmonary artery endothelial cells *in vitro* (172).

In models of SSc-like bleomycin (BLM)-induced fibrosis, BLM-induced expression of collagen (I and III) synthesis mediated by ROS (173). Antioxidants such as NAC were found to attenuate BLM-induced lung fibrosis in mice and rats (174, 175) as well as collagen expression (173). The above data are consistent with earlier findings showing higher ROS production in type II alveolar epithelial cells and lung phagocytes in a rat model of BLM-induced fibrosis (176). Although the above-mentioned studies did not explore the involvement of EndoMT in the observed SSc-associated fibrotic process induced by ROS, recent evidence supports the role of ROS in promoting EndoMT in association with other pathological fibrotic conditions in the kidney (22, 177, 178). Furthermore, antioxidants have been shown to reduce EndMT of vascular endothelial cells (177). Indeed, suppressing oxidative stress has been shown to reduce *in vivo* EndMT in glomerular endothelial cells (22).

The BLM-induced fibrosis model has also been used in other studies (107, 179–181). Notably, Qi et al. (181) demonstrated that EndMT in the BLM-induced scleroderma mouse model is inhibited by geniposide, a constituent of the Chinese herbal compound Zhizi. According to Chinese herbal medicine, its "bitterness and coldness" properties are appropriate to attenuate various inflammatory conditions, including the early inflammation stage of SSc (181). The study proved the ability of Geniposide to block BLM-mediated EndMT not only *in vivo* (mouse model) but also *in vitro* (HUVECs). Further investigation of the EndMT-inhibiting effects exerted by Geniposide showed its ability to down-regulate key transcription factors involved in EndMT (Slug, Snail, Twist). Although the above-mentioned study does not report the involvement of oxidative stress, it is important to emphasize the well-known prooxidant effect of BLM which can damage surrounding cells, resulting in fibroblast activation (182). Interestingly, among all the pharmacological properties of Geniposide, its antioxidant activity was highlighted as protective in preventing cells from undergoing oxidative damage via MAP kinase pathway (183). Of note, ROS and EndMT interact through TGF-β-dependent and -independent pathways, as previously discussed. Based on the reported data, one could hypothesize that Geniposide might act as an anti-oxidant and anti-inflammatory factor, promoting an inhibitory effect on BLM-induced EndMT in the SSc mouse model. Although this hypothesis requires further investigations, a recent study demonstrated that salvianolic acid A (SSA), a natural polyphenol antioxidant, prevented EndMT both *in vitro* and

in vivo by inhibiting oxidative stress (184). In *vitro*, EndMT was induced in human pulmonary arterial endothelial cells by TFG- β treatment, while *in vivo* EndMT was studied in a rat model of monocrotaline-induced pulmonary hypertension. SSA, by reducing ROS concentrations, was able to counteract EndMT-associated cell functions, signaling, and proteins both *in vitro* and in the lung of rats with pulmonary hypertension (184).

CONCLUDING REMARKS AND FUTURE DIRECTION

SSc represents a public health and economic burden with a high rate of mortality and morbidity. ROS play a critical role in the pathogenesis of SSc. Both oxidative stress and EndMT are involved in the onset and progression of the fibroproliferative vasculopathy and fibrotic process in SSc. TGF-β is involved in the fibrotic process through EndMT, where ROS mediate and/or activate TGF-β to induce EndMT. Although numerous studies demonstrated the potential involvement of both ROS and EndMT in the development of fibrosis, only a few studies investigated the potential relationship between ROS and EndMT, and their involvement in the pathogenesis of SSc. The potential role of oxidative stress in inducing EndMT directly, or through the TGF-β-dependent pathway, is an emerging area of investigation that needs to be addressed. Elucidating the mechanism of SSc pathogenesis associated with ROS will contribute to the identification of therapeutic strategies to alleviate the costs and health burden of this disease.

AUTHOR CONTRIBUTIONS

DT and GP ideated the review. DT wrote the first draft. HZ, AE, HA-S, GN, AM, and GP contributed to the editing and review of the different versions. GP and AM performed the final editing and GP submitted the manuscript.

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REVIEW
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Regulatory T Cells in Systemic Sclerosis

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In recent years, accumulating evidence suggest that regulatory T cells (Tregs) are of paramount importance for the maintenance of immunological self-tolerance and immune homeostasis, even though they represent only about 5–10% of the peripheral $CD4^+$ T cells in humans. Their key role is indeed supported by the spontaneous development of autoimmune diseases after Tregs depletion in mice. Moreover, there is also a growing literature that investigates possible contribution of Tregs numbers and activity in various autoimmune diseases. The contribution of Tregs in autoimmune disease has opened up a new therapeutic avenue based on restoring a healthy balance between Tregs and effector T-cells, such as Treg-based cellular transfer or low-dose IL-2 modulation. These therapies hold the promise of modulating the immune system without immunosuppression, while several issues regarding efficacy and safety need to be addressed. Systemic sclerosis (SSc) is an orphan connective tissue disease characterized by extensive immune abnormalities but also microvascular injury and fibrosis. Recently, data about the presence and function of Tregs in the pathogenesis of SSc have emerged although they remain scarce so far. First, there is a general agreement in the medical literature with regard to the decreased functional ability of circulating Tregs in SSc. Second the quantification of Tregs in patients have led to contradictory results; although the majority of the studies report reduced frequencies, there are conversely some indications suggesting that in case of disease activity circulating Tregs may increase. This paradoxical situation could be the result of a compensatory, but inefficient, amplification of Tregs in the context of inflammation. Nevertheless, these results must be tempered with regards to the heterogeneity of the studies for the phenotyping of the patients and of the most importance for Tregs definition and activity markers. Therefore, taking into account the appealing developments of Tregs roles in autoimmune diseases, together with preliminary data published in SSc, there is growing interest in deciphering Tregs in SSc, both in humans and mice models, to clarify whether the promises obtained in other autoimmune diseases may also apply to SSc.

Keywords: systemic sclerosis, regulatory T cells, immune tolerance, auto-immunity, thymus

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REGULATORY T CELLS: GATEKEEPERS OF IMMUNOLOGICAL TOLERANCE

Human regulatory T cells (Tregs) expressing the transcription factor FoxP3 have a crucial role for the maintenance of immunological self-tolerance and immune homeostasis (1–3). The loss of dominant peripheral tolerance, exerted by Tregs, can lead to autoimmune diseases, immunopathology, allergy or metabolic disease (4, 5).

The pivotal role of Treg cells in the protection from autoimmunity is exemplified by spontaneous development of immunopathology in scurfy mice which are deficient for FoxP3$^+$ Tregs (4, 6). Mutations in the human ortholog result in a similar X-linked lymphoproliferative disorder characterized by immune dysregulation, polyendocrinopathy, enteropathy, defined by the acronym IPEX (7, 8). Moreover, lack of Treg-mediated control has been shown to play a role in many animal models of autoimmunity (4) but also in numerous autoimmune disorders (9–12). In this review, we will focus on CD4$^+$FoxP3$^+$ Treg cell subset.

CD4$^+$ TREGS: CELL SUBSETS AND IDENTIFICATION

Human Tregs were first characterized as CD4$^+$CD25$^+$ T cells in 2001 (13–15) based on the findings by Sakaguchi et al. that mouse Tregs constitutively express CD25, the α-chain of the IL2 receptor (16). However, CD25 is also upregulated on responder T cells upon activation. Therefore, much research has been focused on the identification of further markers to precisely distinguish the Treg population from recently activated T cells. In 2003, the transcription factor FoxP3 was shown to regulate the generation and function of Tregs in mice (17–19). Subsequently, in humans, FoxP3 was shown to be expressed predominantly by CD4$^+$CD25^{high} T cells (20). However, whereas in mice FoxP3 expression seems to be restricted to Tregs (21, 22), in humans, FoxP3 is also expressed by non-regulatory CD4$^+$CD25$^+$ T cells (23, 24), restricting the usage of FoxP3 as a specific marker for human Tregs. Moreover, FoxP3 being an intracellular protein, it cannot be used for Tregs isolation with the goal to perform functional studies. Later, it was shown that low expression of CD127, the α-chain of the IL7 receptor, acts as an additional marker for the characterization of Treg cells among CD4$^+$CD25^{high} T cells (25, 26). Indeed, FoxP3 expression and suppressive capacity are enriched in CD4$^+$ T cells that express low levels of CD127. However, CD127 expression tends to be downregulated also in activated conventional CD4$^+$ T cells (27, 28). Moreover, Klein et al. demonstrated that a high percentage of CD127$^+$ cells expressed FoxP3 and, reciprocally, that there was a high percentage of CD127$^{low/-}$ cells that did not express FoxP3. These results suggest that these markers did not represent the same population of Tregs (29). A number of additional cell markers for the identification of CD4$^+$ Tregs have been proposed (30–32) but many of these are also induced upon activation of non-regulatory CD4$^+$ T cells.

In this context, Miyara and coworkers further delineated the Treg cell compartment into three subpopulations using the combination of FoxP3 and CD45RA expression: (i) CD45RA$^+$FoxP3^{low} resting Tregs (rTregs); (ii) CD45RA$^-$FoxP3^{high} activated Tregs (aTregs), both of which are strongly suppressive *in vitro*; and (iii) non-suppressive cytokine-secreting CD45RA$^-$FoxP3^{low} non-Tregs (33). *In vitro*, CD45RA$^-$FoxP3^{high} aTregs were activated, highly suppressive and died by apoptosis after exertion of suppression, whereas rTregs were in a quiescent state, proliferated upon activation and converted into aTreg cells *in vitro* and *in vivo*. A major stake of this combination is based on the identification of a non-regulatory FoxP3$^+$ T cell population, enabling to overcome the contamination by this cell population when studying Treg cells.

CD49d (α-chain of the integrin VLA-4) was also described as a marker that could discriminate contaminating effector cells from immune-suppressive Foxp3$^+$ Treg cells. This marker is present on the majority of proinflammatory effector cells but absent from Foxp3$^+$ Treg cells. Therefore, depletion with α-CD49d removes proinflammatory effector cells from CD25^{high}CD4$^+$ cells and, in combination with α-CD127, it provides access to hugely pure populations of Foxp3$^+$ cells (34).

MECHANISMS OF ACTION AND FUNCTIONAL CHARACTERIZATION OF CD4$^+$ TREG

The best common way to analyze Treg function is based on their capacity to suppress target cell proliferation, and consist of *in vitro* suppression assays. This method relies on isolation of effector and regulatory cell populations immunomagnetically or by fluorescence activated cell sorting (FACS). Effector cells are then activated in the presence or absence of the regulatory population. After a defined period of time, their proliferation, and/or cytokine production are examined. However, FoxP3 being an intracellular protein, live human Tregs cannot be isolated using FoxP3 as a marker, and the lack of specific Treg cell surface markers precludes the isolation of a pure Treg population to test in these *in vitro* suppression assays.

Numerous mechanisms have been described as to how Tregs exert their suppressive function, including cell-cell contact dependent suppression, inhibitory cytokine release (IL-10, TGFβ, IL-35, Granzymes A et B), IL-2 deprivation, modulation of antigen-presenting cell function via CTLA-4, cytolysis and metabolic disruption of the target cell. These mechanisms have been extensively reviewed (35–38) and will not be further discussed in this article.

Defects in the number and/or function of Treg cells could each lead to a suboptimal T cell regulation, and subsequently to the development of autoimmunity.

SYSTEMIC SCLEROSIS

Systemic sclerosis (SSc) is an orphan connective tissue disease characterized by extensive immune abnormalities, microvascular injury and fibrosis of skin and internal organs (39). It is the most severe connective tissue disease, associated with a high mortality risk (40). Patients with SSc are classified according to skin involvement extent: limited cutaneous SSc (LcSSc), with skin involvement restricted to the hands, arms, and face; and diffuse cutaneous SSc (DcSSc), with more extensive skin thickening (truncal and proximal) and more frequent visceral involvement (41).

Although the pathogenesis of SSc is complex and remains incompletely understood (42), research in the area has

confirmed that immune dysfunction is one of the most important component of the pathogenesis. Innate and adaptive immune abnormalities can be observed, and culminate in auto-antibodies production and activation of cell-mediated autoimmunity. Moreover, immune cells may trigger the complex biochemical and molecular changes that promote vasculopathy and fibrosis. Indeed, there is increasing evidence that places immune activation as a cause and not a consequence of the vasculopathy and fibrosis. First, histological studies indicate that an inflammatory infiltrate is present in the very early stages, preceding the onset of fibrosis (43). This cellular infiltrates consist mostly of T cells which are predominantly $CD4^+$ cells (44). Second, fibroblasts with increased expression of type I and III procollagen mRNA can often be identified in areas adjacent to the infiltrating mononuclear cells (45, 46). Third, T cells in the skin and in the peripheral blood of SSc patients express an oligoclonal T cell receptor (TCR) repertoire, strongly suggestive of a proliferation and clonal expansion of these cells in response to a specific Ag(s) (47, 48). Furthermore, several studies have demonstrated an association of particular HLA alleles with SSc (49–52), which supports the concept of an Ag-driven T cell response in SSc. It should be noted that the genotype varies particularly strongly according to the presence of different types of autoantibodies associated with SSc: anti-centromere antibodies was associated with DRB1*01:01, DRB1*01:04, DRB1*01:08, DQB1*05:01, DPB1*04:02 and anti-topoisomerase I with DRB1*11-*15:02, DPB1*13:01 and DPB1**09:01 (51, 52). In a large study of HLA class II genes carried out in 1,300 SSc cases and 1,000 controls, the *DRB1*11:04*, *DQA1*05:01* and *DQB1*03:01* haplotypes and the *DQB1* allele were the strongest associations identified (49). The association of *DRB1*11:04*, *DQA1*05:01* and *DQB1*03:01* haplotype with SSc was confirmed in a similar study (944 Caucasian SSc patients and 1,320 unaffected controls) (50). Although not specific to SSc, these HLAs were not found in many other autoimmune diseases.

Therefore, among this aberrant immune response, T lymphocytes seem to be of particular importance in the pathogenesis of SSc. These cells are predominantly $CD4^+$ cells, display markers of activation, with a predominant Th2 cytokine profile characterized by high levels of IL-4, IL-5, and IL-13 in skin, lung and peripheral blood (53–56). This key role of T cell proliferation and cytokine secretion in SSc suggests that this condition could be associated with a defective control of T cell activation.

CIRCULATING TREG IN SSc

Evidence for numerical and functional changes of Treg population in SSc has been obtained in several studies (**Table 1**). The majority of the studies reported decreased frequencies and/or impaired function of circulating Tregs in SSc patients compared to controls (12, 57–65, 74). Banica et al. investigated Treg cells in peripheral blood of patients with different connective tissue diseases, as compared with blood from healthy controls. They found a reduced percentage of $CD4^+CD25^{hi}$ T cells in SSc compared to controls but also to other connective tissue diseases (12). Antiga et al. also reported fewer $CD4^+CD25^{bright}FoxP3^+$ cells in SSc patients naïve to any systemic treatment compared with healthy controls and with patients having other common immune-mediate dermatoses (psoriasis, atopic dermatitis) (57). This decrease was associated with reduced total TGFβ1 and IL-10 serum levels. The authors concluded that this reduced frequency of Tregs, together with that of total TGFβ1 and IL-10, may be responsible for the loss of tolerance observed in SSc. Papp and coworkers observed decreased $CD4^+CD25^+FoxP3^+$ T cells percentages in peripheral blood of patients with SSc associated with increased Th17 cell percentages and decreased circulating IL-10 levels (59). In addition, both $CD4^+$ and $CD8^+$ central memory T-cell percentages were increased representing an immunologically active state. Similarly, the results of Fenoglio et al. supported an imbalanced ratio between Th17 and Treg cell subsets in SSc patients, with increased proportion of circulating Th17 cells, and decreased proportion of both $CD4^+CD25^+CD127^-$ and $CD8^+CD28^-$ Treg cells (60). Lower frequencies of $CD4^+CD25^+FoxP3^+$ T cells in SSc patients were reproducibly reported by other groups (62–64). Kataoka et al. found reduced frequencies of $CD4^+CD25^+FoxP3^+$ T cells in treatment-free SSc patients compared to healthy controls, particularly in patients with late-stage disease (65).

Among other reports, some studies have reported decreased frequency of circulating Tregs but not reaching statistical significance (66, 67, 75).

Some studies have reported an increase in circulating Tregs (68–73), particularly in early phase and active disease (68–70). Radstake et al. reported an increase in the frequency of circulating $CD25^+FoxP3^+CD127^-$ T cells in SSc patients, especially in early phase of the disease. However, despite this increase, Tregs from SSc patients harbored a defective suppressive capacity correlated with a dramatic reduction in CD62L and CD69 expression. Interestingly, co-incubation of Treg cells from healthy donors with plasma from SSc patients abrogated suppressive activity, suggesting the presence of specific soluble factors inhibiting Treg function in SSc patients (68). Two studies reported higher number of $CD25^+FoxP3^+$ T cells in SSc patients, correlated with disease activity and severity (69, 70). Three more studies reported a significantly higher frequency of circulating Tregs in SSc compared to controls, although no functional studies were performed (71–73).

These discrepancies reflect the challenge of the phenotypic characterization of Treg cells and possible contamination by activated $CD25^+$ T cells. In this context, two studies have used the combination of markers described by Miyara et al. (33) discriminating $CD4^+FoxP3^{low}CD45RA^+$ resting Treg (rTreg), $CD4^+FoxP3^{high}CD45RA^-$ activated Treg (aTreg) from non-regulatory $Foxp3^+$ cells. Mathian and coworkers found that the percentages and absolute counts for both aTreg and rTreg were decreased in SSc compared to controls, but not those for non-regulatory $FoxP3^+$ $CD4^+$ T cells (61). Interestingly, aTreg were decreased at any disease stage while rTreg frequency declined in late phases of SSc. Moreover, the quantitative Treg defect was less pronounced in diffuse cutaneous and/or active disease. Similarly, Liu et al. reported lower proportions

TABLE 1 | Circulating regulatory T cells in systemic sclerosis.

Study (year) (Ref.)	Phenotype used to quantify	Study population (*n*)	Frequencies	Functionality and immune association	Clinical association
REDUCED					
Banica et al. (12)	CD4+CD25hi	SSc: 13 Active: 7 Inactive: 6 SS: 8 SLE: 20 PM/DM: 11 HC: 18	Mean: SSc: 1.0% SS: 1.3% SLE: 1.5% PM/DM: 1.3% HC: 3.3% $p = 0.0001$ (SSc compared to HC)	NA	Treatment with glucocorticoids and immunosuppressive therapy in association was associated with reduced Tregs frequency.
Antiga et al. (57)	$CD4^+CD25^{bright}FoxP3^+$	SSc*: 15 LcSSc: 10 DcSSc: 5 Morphea: 15 Psoriasis: 10 Atopic dermatitis: 10 HC: 10	Median (range): SSc: 1.5% (1.0–2.1) Morphea: 1.8% (1.1–2.6) Psoriasis: 2.4% (1.9–3.3) Atopic dermatitis: 2.5% (1.6–3.5) HC: 3.5 (3.1–3.9) $p < 0.05$ (SSc compared to HC)	Decreased of TGFβ1 and IL-10 serum levels	NA
Liu et al. (58)	$CD4^+FoxP3^{high}CD45RA^-$ activated Treg $CD4^+FoxP3^{low}CD45RA^+$ resting Treg	SSc: 31 LcSSc: 15 DcSSc: 16 HC: 33	Mean ± SD: Activated Treg SSc: 0.25 ± 0.16% HC: 0.66 ± 0.41% $p < 0.001$ Resting Treg SSc: 1.87 ± 0.94% HC: 1.63 ± 0.97% $p = 0.320$	Impaired suppressive capacity of $CD4^+CD25^+FoxP3^+$ cells Immune imbalance Treg/Th17 with elevated Th17 cells	NA
Papp et al. (59)	$CD4^+CD25^{bright}FoxP3^+$	SSc: 21 (only DcSSc) HC: 15	Mean ± SD: SSc: 5.03 ± 2.3% HC: 6.21 ± 0.1% $p = 0.031$	Suppression capability of $CD4^+CD25^+$ T cells reduced in SSc patients. Increased $CD4^+$ and $CD8^+$ central memory T-cell percentages. Immune imbalance Treg/Th17 with elevated Th17 cells. Decreased peripheral Il-10 level.	NA
Fenoglio et al. (60)	$CD4^+CD25^{hi}CD127^{low}$	SSc: 36 LcSSc: 24 DcSSc: 12 HC:10	Decreased $p < 0.05$	Impaired suppressive capacity of $CD4^+CD25^{hi}CD127^{low}$ cells in patients with diffuse and active disease. Immune imbalance Treg/Th17 with elevated Th17 cells	NA
Mathian et al. (61)	$CD4^+CD45RA^-$ $FoxP3^{bright}CD25^{bright}$ activated Treg $CD4^+CD45RA^+$ $FoxP3^+CD25^+$ resting Treg	SSc: 53 LcSSc: 18 DcSSc: 35 HC: 24	Median (range): Activated Treg SSc: 0.66 (0.17–2.0) HC: 1.51 (0.79–3.03) $p < 0.0001$ Resting Treg SSc: 0.72 (0.1–5.3) HC: 1.63 (0.57–4.94) $p < 0.0001$	Suppressive capacity of aTregs ($CD4^+CD45RA^-CD25^{bright}$) and rTregs ($CD4^+CD45RA^+CD25^+$): no significant difference with HC	Percentage of aTregs correlated with the EScSG activity index and the MRSS LcSSc patients had significantly less aTregs than DcSSc patients rTregs in SSc negatively correlated with disease duration Late SSc patients had significantly less circulating rTregs than early SSc patients

(Continued)

TABLE 1 | Continued

Study (year) (Ref.)	Phenotype used to quantify	Study population (*n*)	Frequencies	Functionality and immune association	Clinical association
Cordiali-Fei et al. (62)	$CD4^+CD25^+FoxP3^+$	SSc*: 25 LcSSc: 14 DcSSc: 11 HC: 15	Median (range): LcSSc: 1.67% (1.2–3.3) DcSSc: 1.85% (1.2–3.2) HC: 2.1 (1.1–3.2) $p < 0.05$	NA	Significant correlation between disease duration and reduced Treg cell percentages in LcSSc patients
Wang et al. (63)	$CD4^+CD25^+FoxP3^+$	SSc: 18 LcSSc: 6 DcSSc: 12 HC: 17	Mean ± SD: SSc: 6.2 ± 1.8% HC: 11.1 ± 2% $p = 0.024$	Decreased FoxP3 expression in CD4+ T cells and increased methylation levels of FoxP3 gene. Inhibition of DNA methylation enhanced FoxP3 expression.	Promoter methylation status and expression level of FoxP3 significantly associated with disease activity.
Baraut et al. (64)	$CD4^+CD25^{hi}FoxP3^+$	SSc*: 7 (only DcSSc) HC: 7	Mean ± SD: SSc: 2 ± 0.5% HC: 4.2 ± 1.1% $p < 0.01$	Impaired suppressive capacity of $CD4^+CD25^{hi}CD127^{low}$ cells	NA
Kataoka et al. (65)	$CD4^+CD25^+FoxP3^+$	SSc*: 23 LcSSc: 8 DcSSc: 15 HC: 22	Mean ± SD: SSc: 2.7 ± 1.5% HC: 4.8 ± 1.5% $p < 0.0001$	Decreased Runx1 mRNA expression in purified Treg (especially in early disease)	Reduced frequency more pronounced in late disease
SIMILAR					
Klein et al. (66)	$CD4^+CD25^{++}FoxP3^+$	SSc: 20 LcSSc: 14 DcSSc: 6 HC: 29	Median (range): SSc: 1.1% (0.7–3.5) HC: 1.3 (0.6–2.8) $p = 0.953$	Suppressive capacity of $CD4^+CD25^+FoxP3^+$ cells similar to that of HC	NA
Yang et al. (68)	$CD4^+CD25^+CD127^-$	SSc: 45 LcSSc: 20 DcSSc: 25 HC: 24	Mean ± SD: Active SSc: 6.2 ± 1.2% Stable SSc: 6.5 ± 1.5% HC: 7.1 ± 1.6% $p > 0.05$	Increased Th17 cell percentages	NA
Krasimirova et al. (67)	$CD4^+CD25^+FoxP3^+$	SSc: 24 LcSSc: 11 DcSSc: 13 HC: 16	Mean ± SD: SSc: 4.02 ± 0.52% HC: 4.16 ± 0.53%	Increased Th17 cell percentages Raised levels of IL-6, TGFβ1, IL-10, IL-17A	No association with disease activity, disease duration or visceral involvement.
INCREASED					
Radstake et al. (68)	$CD3^+CD25^+FoxP3^+CD127^-$	SSc: 68 LcSSc: 20 DcSSc: 48 HC: 26	Mean ± SEM: SSc: 17.3 ± 1.9% HC: 2.9 ± 0.5% $p < 0.0001$	Expression of CD62L and CD69 markedly lower in SSc ($CD4^+$ $CD25^{hi}$ cells) Impaired suppressive capacity of $CD4^+CD25^+FoxP3^+CD127^-$ cells	Significantly higher number of $CD25^+FoxP3^+CD127^-$ cells in early DcSSc (<2 years) compared to late DcSSc
Slobodin et al. (69)	$CD4^+CD25^{bright}$ $FoxP3^+$	SSc: 10 LcSSc: 6 DcSSc: 4 HC: 10	Increased	Production of TGFβ1 and IL-10 by activated CD4+ cells similar in patients and controls	Correlation with the EScSG disease activity index and the Medsger disease severity index
Giovannetti et al. (70)	$CD4^+CD25^{high}FoxP3^+$	SSc*: 35 LcSSc: 20 DcSSc: 15 HC: 39	Mean ± SD: SSc: 6.7 ± 2.2% HC: 4.5 ± 1.5% $p < 0.0001$	NA	Correlation with the EScSG disease activity index and DLCO

(Continued)

TABLE 1 | Continued

Study (year) (Ref.)	Phenotype used to quantify	Study population (*n*)	Frequencies	Functionality and immune association	Clinical association
Rodriguez-Reyna et al. (71)	$CD4^+CD25^+FoxP3^+$	SSc: 135 LcSSc: 78 DcSSc: 57 HC: 16	SSc: 6.0% HC: 3.3%	Increased Th17 cell percentages	NA
Jiang et al. (72)	$CD4^+CD25^+FoxP3^+$	SSc: 53 HC: 27	SSc: 3.04% HC: 2.24% $p = 0.018$	Increased Th17 cell percentages	Association with high ILD score on CT and with low DLCO
Ugor et al. (73)	$CD3^+CD25^+FoxP3^+CD127^-$	SSc: 26 LcSSc: 7 DcSSc: 19 HC: 10	Increased $p < 0.05$	Increased $CD62L^+$ Tregs, decreased $IL\text{-}10^+$ Tregs	NA

*Patients not receiving systemic treatment.

CT, computed tomography; DLCO, diffusing capacity of the lung for carbon monoxide; DcSSc, diffuse cutaneous systemic sclerosis; FC, flow cytometry; HC, healthy controls; ILD, interstitial lung disease; LcSSc, limited cutaneous systemic sclerosis; NA, not available; PM/DM, poly- and dermatomyosite; SD, standard deviation; SLE, systemic lupus erythematosus; SS, Sjögren syndrome; SSc, systemic sclerosis.

of aTreg and higher proportions of non-regulatory $Foxp3^+$ cells in SSc patients compared to healthy controls (58). In the latter study, the frequency of $CD4^+CD25^+FoxP3^+$ Treg cells was significantly increased in patients with SSc, suggesting that the increase in this cell population was mainly due to elevated $CD4^+CD25^+FoxP3^{low}CD45RA^-$ non-Treg cells. These results within the same patient population support the notion that the complexity of the phenotypic characterization of this cell population explains conflicting results in the literature.

However, the discrepancies among these studies cannot be solely explained by the use of different Treg markers. Indeed, it should be emphasized that flow cytometry gating is rather subjective and depends partly on researcher selection. Therefore, studies using same Treg markers are not necessarily comparable. Moreover, the patients' characteristics, such as disease duration, disease severity and activity, concomitant treatments, might also contribute to the discrepancy among different studies as previously stated.

As regards clinical association, most of the studies reporting increased frequency of circulating Tregs have demonstrated a correlation with disease activity (69, 70) and severity (69, 72), and with early disease (68), whereas reduced frequency of Tregs seemed to be associated with late disease (61, 62, 65). Elevated Tregs were also reported in patients with a high interstitial lung disease (ILD) score on computed tomography (72) and with low DLCO (70, 72). No other clinical association was found, in particular, no difference was observed between the two subsets of the disease.

Effect of medications on Tregs frequency have been raised by some authors. It is of note that most of the patients in studies reporting increased or similar Tregs frequency received systemic treatment which may have bias the results, since some authors reported that immunosuppressive therapy is able to increase the pool of circulating Treg (76, 77). On the other hand, immunosuppressive therapy and bosentan showed no significant effect on the frequency of Tregs in some reports (66, 73). In contrast, treatment with glucocorticoids and immunosuppressive therapy in association was associated with reduced Tregs frequency in the study of Banica et al. (12). Thus, more investigations are needed to evaluate the impact of therapies on Treg cells.

Regarding the functional capacity of circulating Tregs, almost all studies agree that Tregs fail to produce inhibitory cytokines or suppress the effector T cells in SSc (58–60, 64, 68).

Pulmonary arterial hypertension (PAH) is one of the most severe complication of SSc. Several studies have investigated the role of Tregs in PAH. Two studies reported elevated $CD4^+CD25^+FoxP3^+$ T cells in the peripheral blood of idiopathic PAH patients (78, 79), but not in the lungs (78). Huertas et al. investigated the functional status of $CD4^+CD25^+FoxP3^+$ Treg function by measuring Treg STAT3 phosphorylation in patients with idiopathic PAH, heritable PAH or SSc-PAH, compared to controls (80). Although Treg cell numbers were similar between patients and controls, they found that Tregs were dysfunctional in all these PAH subgroups, including SSc-PAH, with reduced proportion of Treg-$pSTAT3^+$ cells compared to controls.

MECHANISMS OF TREGS DYSFUNCTION IN SSc

Mechanisms of Tregs dysfunction in SSc have been investigated by several studies. Aberrant epigenetic modifications, such as microRNA, DNA methylation, histone modifications, affecting FoxP3 and other key genes in Tregs have been shown to contribute to disease activity and tissue inflammation in autoimmune diseases (81). In SSc, Wang et al. reported elevated methylation levels of the FoxP3 promoter, inversely correlated with FoxP3 mRNA expression, and accompanied by reduced proportion of $CD4^+CD25^+FoxP3^+$ Tregs (63). Furthermore, treatment of SSc $CD4^+$ T cells with 5-azacytidine, a DNA methylation inhibitor, reduced the mean methylation levels, increased FoxP3 expression and induced Treg generation. Interestingly, the promoter methylation status and expression level of Foxp3 were significantly associated with disease activity. D'Amico et al. provide evidence of the association between rs2294020 FoxP3 polymorphism and disease progression in a female Italian population (82). Otherwise, it has been reported a significantly higher frequency of skewed X chromosomal inactivation in patients with SSc compared with controls, correlated with lower FoxP3 expression in $CD4^+CD25^+$ cells and less efficient suppressive activity (83). Kataoka and al. reported reduced expression of the transcription factor Runx1 mRNA correlated with decreased proportion of Tregs even in early stages of the disease (65). Semaphorin 3A serum levels along with cell expression on Tregs were reported to be low in SSc patients (84).

In addition, recent evidence indicate that Tregs could contribute to SSc pathogenesis by conversion into pathogenic effector T cells in the presence of appropriate environment. Thus, because accumulating evidence suggest that Th17 cells could be responsible for prominent features of SSc (53, 71, 85), it has been hypothesized that a Treg/Th17 imbalance could be a pivotal component of SSc pathogenesis. Indeed, Fenoglio et al. found a significant correlation between increased circulating Th17 cells and alteration of the Treg compartment (60). One could argue that the observed decrease in Treg cells could be the result of conversion to Th17. Several groups have reported the conversion of Tregs to Th17 cells in both mouse and human (86–88), supporting this hypothesis. Moreover, IL-6 and IL-1β, that are highly expressed in inflammatory conditions, have been shown to convert Tregs to Th17 cells (89, 90). Liu et al. found that $CD4^+CD25^+FoxP3^{low}CD45RA^-$ non-regulatory T cells produced high levels of IL-17 (58). They hypothesized that this population of $FoxP3^+$ non-regulatory T cells expressing IL-17 could represent a transitional phase in the conversion process from Treg to Th17 cells. Consistent with this result, T cells that co-express IL-17 and FoxP3 have been identified by other groups (91, 92). In contrast, although they reported decreased Tregs proportions, no IL-17 amplification was observed in blood and skin of SSc patients in the study of Mathian et al. (61).

More recently, MacDonald et al., using flow cytometry, analyzed FoxP3 and cytokine expression among skin-resident T cells isolated from cultured explants (93). They found that Tregs from SSc-skin produced significant amount of Th2 cell-associated cytokines IL-4 and IL-13 compared to controls. On the other hand, circulating Tregs of SSc patients did not produce Th2 cytokines, but they contained a significantly higher proportion of skin-homing cells expressing Th2 cell-associated chemokine receptors. The authors also found evidence that IL-33 might be an important stimulator of tissue-localized loss of normal Tregs function and polarization into Th2-like cells. Altogether, these results further support the hypothesis that the skin of SSc patients provides the appropriate environment for transdifferentiation of Tregs toward a Th2-like phenotype, that might contribute to fibrosis in patients with SSc.

TREGS AT THE SITE OF INFLAMMATION IN SSc

When studying Treg cells in such diseases, one needs to consider potential differences between Treg cells derived from the peripheral blood vs. the inflamed organs (skin, lungs) in terms of function and frequency. In contrast to the lung, skin-resident Tregs are being actively investigated, probably due to relative ease of access for tissue samples. Therefore, data from mice and human subjects have revealed the importance of correct Treg cell positioning in the skin for the maintenance of immune homeostasis and prevention of spontaneous autoimmune and inflammatory disease (94). Contradictory results have been reported by the few studies investigating the presence of Tregs in the skin of patients with SSc (**Table 2**). Earlier studies have found fewer $FoxP3^+$ cells by immunohistochemistry as compared to healthy controls or control diseases (psoriasis and atopic dermatitis) (57, 66). Interestingly, no significant difference was revealed when comparing lesional and non-lesional skin of SSc patients (66). This decrease was associated with reduced TGFβ and IL-10, which are regulatory cytokines involved in Treg suppressive function, both in skin and blood of SSc patients in the study of Antiga and coworkers (57). By contrast, in the study of Yang et al. $FoxP3^+$ Treg cells was reported to be enriched in both the dermis and epidermis of patients with early SSc compared with patients with late SSc and healthy controls (75). The authors hypothesized that this expansion of $FoxP3^+$ cells in early SSc skin may reflect a regulatory feedback mechanism to restore cellular tolerance and ameliorate harmful autoimmune responses. It should be noted that disease duration was not reported in the two studies that have found fewer $FoxP3^+$ cells. More recently, MacDonald et al., found that $FoxP3^+$ cells with high IL-4 and IL-13 production could be detected more frequently in the skin of SSc patients compared to normal controls (93). This study provides the first evidence for the differentiation of human Treg cells into Th2 cytokine-producing cells that might contribute to fibrosis in patients with SSc.

TREGS IN SSc MOUSE MODELS

Numerous inducible and genetic mouse models of SSc have been developed and characterized in the last years (95). Conversely to other autoimmune diseases, very scarce data about Tregs in SSc mouse models have been produced. In the topoisomerase mouse model, treatment with topoisomerase I and Freund's

TABLE 2 | Regulatory T cells in systemic sclerosis skin.

Study (year) (Ref.)	Phenotyped by	Phenotype used to quantify	Study population	Frequencies	Functionality and immune association
INCREASED					
Yang et al. (75)	IHC	FoxP3$^+$ cells	SSc: 13 LcSSc: 1 DcSSc: 12 HC: 4	Mean ± SD: Early SSc: Superficial dermis: 10.5 ± 1.6%; deep dermis: 6.9 ± 1.7% Late SSc: Superficial dermis: 2.2 ± 1.3%; deep dermis: 1.2 ± 10.8% HC: Superficial dermis: 0.8 ± 0.4%; deep dermis: 0.8 ± 0.4% $p < 0.01$	Higher in early disease
MacDonald et al. (93)	FC	FoxP3$^+$ cells	SSc: 19 HC: 13	Mean ± SD: SSc: 30.3 ± 2.8% HC: 23.7 ± 4.5% $p > 0.05$	Production of high amounts of Th2 cell-associated cytokines IL-4 and IL-13 by Tregs from skin
DECREASED					
Antiga et al. (57)	IHC	FoxP3$^+$ cells	SSc*: 15 LcSSc: 10 DcSSc: 5 HC: 10	Median (range): SSc: 2% (1–4.5) HC: 9% (4.2–10)	Reduced TGFβ$^+$ and Il-10$^+$ cells
Klein et al. (66)	IHC	FoxP3$^+$ cells among CD4$^+$ cells	SSc: 12 Psoriasis: 10 Lichen planus: 10 Atopic dermatitis: 10	Median (range): SSc: 17.2% (9.1–21.7) Psoriasis: 45.4% (14.9–57.7) Lichen planus: 51.3 (16.3–78.7) Atopic dermatitis: 33% (17–55.1) $p < 0.005$ (compared with control diseases) No significant difference when comparing lesional and non-lesional skin biopsies of SSc patients	NA

**Patients not receiving systemic treatment.*
DcSSc, diffuse cutaneous systemic sclerosis; FC, flow cytometry; HC, healthy controls; IHC, immunohistochemistry; LcSSc, limited cutaneous systemic sclerosis.

complete adjuvant (CFA) induces SSc-like skin, lung fibrosis and autoimmune abnormalities with anti-topoisomerase I autoantibody production (96). This was associated with increased IL-6, TGFβ1, and IL-17 production and decreased IL-10 production. In this model, mice treated with topoisomerase I and CFA exhibited significantly increased frequencies of Th1 cells, Th2 cells, Th17 cells and Treg cells in bronchoalveolar lavage fluid compared with mice treated with saline or with topoisomerase I. Functional characteristics of Tregs was not assessed in this study.

In the mouse model of bleomycin-induced pulmonary fibrosis, contradictory results have been produced. Birjandi et al. found that treatment by IL-2 complex, used to expand CD4$^+$CD25^{high}FoxP3$^+$ cells in the lung, leads to immune deviation that is dominated by type 2 immune response within the lung, and associated with exacerbate lung fibrosis (97). Moreover, they showed that bleomycin had a modifying and profibrotic effect on the CD4$^+$CD25^{high}Foxp3$^+$ cells. This was corroborated by adoptive transfer experiments in Rag$^{-/-}$ mice. The authors concluded that a therapeutic strategy of expanding CD4$^+$CD25^{high}FoxP3$^+$ in humans may be harmful via the augmentation of Th2 immune responses in patients with idiopathic pulmonary fibrosis and other fibroproliferative lung diseases. On the other hand, adoptive transfer of Tregs on day 14 after a bleomycin challenge significantly reduced pulmonary fibrosis in another report (98). Moreover, although splenocytes significantly improved bleomycin-induced pulmonary fibrosis when they were administered on day 14, this effect was abolished by depleting Tregs with an anti-CD25 monoclonal antibody. Finally, another group found that early depletion of Tregs with an anti-CD25 antibody led to favorable outcomes whereas late depletion of Tregs led to increased fibrosis, suggesting that Tregs play a detrimental role in early stages but protective role in late stages of pulmonary fibrosis in mice (99).

Although these results have to be taken into account in future therapeutic strategy using Treg cells in such diseases, it should be noted that extrapolation of these data from a mouse model to human is challenging since bleomycin may not accurately recapitulate human SSc ILD or other human fibrotic lung diseases. Moreover, there remains much controversy in the field about the actual role of Tregs.

THERAPEUTICS APPLICATION

Taking into account that Tregs are immunodominant suppressors, there is a huge interest in the therapeutic potential of Tregs in several immune-mediated diseases. Indeed, adoptive cellular therapies may offer fewer risks and better efficacy than

traditional pharmacological strategies. So far, clinical research has involved mostly hematopoietic stem cell transplantations, solid organ transplantations, and autoimmunity. Mechanisms of actions are incompletely understood but Tregs protect from auto-aggression and damage to tissues; the effect is executed mainly via cell-to-cell contacts, and also "control by starvation/theft" of IL-2. In general, what is critical is the balance between Tregs and effector T cells (Teff). Therefore, changing the balance between Tregs and Teff is a promising avenue to restore immune homeostasis and to treat autoimmune diseases. Moreover, since the recognition of antigen is a central part in Treg function and their therapeutic use, the modulation of T cell receptor specificity may be offer very stimulating perspectives (100).

Early reports demonstrated that adoptive transfer of Treg cells associated with hematopoietic stem cell transplantation (HSCT) in mice promoted the graft vs. leukemia effect (GvL) and protected from graft vs. host disease (GvHD). Unfortunately, this simple strategy cannot be translated to humans, such as the clinical efficacy requires the administration of a high number of cells whereas Treg cells represent a very low percentage of leukocytes in the blood. Thus, manufacturing procedures to expand Tregs *in vitro* before administration was developed. Multiple sclerosis (MS) is a well-defined autoimmune disease with solid evidence of Treg involvement. Remarkably, remission or prevention of experimental autoimmune encephalomyelitis (EAE), was associated with the induction of $CD4^+CD25^+$ Tregs. The adoptive transfer of Tregs further confirmed this hypothesis: transfer before EAE induction prevented EAE, and transfer to mice that already had EAE alleviated symptoms (101).

In humans, infusion of Tregs that is the direct approach to increase Tregs have been used in several phase 1 clinical trials for the prevention of GvHD or type 1 diabetes. Currently, several trials are registered in clinicaltrials.gov website for various conditions, such as liver transplantation (NCT01624077), autoimmune hepatitis (NCT02704338), chronic graft vs. host disease (NCT02385019 and NCT01937468), kidney transplantation (NCT02088931), type 1 diabetes (NCT01210664), systemic lupus erythematosus (NCT02428309). Although it is manageable to produce large numbers of alloantigen-reactive Tregs through selective stimulation by allogeneic antigen presenting cells, producing a high number of tissue antigen-specific Tregs for autoimmune diseases is far more challenging. Indeed, the low frequency of Treg precursor and the tendency of Tregs to destabilize after repeated *in vitro* stimulation impair easy large quantity production (102, 103).

Manipulating Tregs is another avenue that may even be complementary to adoptive cell transfer. In order to restore a safe equilibrium between Treg and Teff, anti-CD3 strategies mainly using antibodies was first developed (104, 105). However, efficacy was restricted to some patients and only at early stages. Co-stimulation may also be used and low-dose CTLA4-Ig therapy can enhance Treg and prevent immune activation (105). Finally, the most stimulating findings have been reported with IL2 and relate to the harboring by Tregs of high affinity IL-2 receptor promoting preferential expansion in conditions of low amounts of IL-2. Very stimulating data have been reported in humans in type 1 diabetes (106), GvHD (107), and regrowth of the scalp and/or body hair could be seen in alopecia areata (108). Patients with hepatitis C virus-induced vasculitis have a set of symptoms including fatigue, skin purpura, arthralgia, neuropathy and kidney involvement. In eight out of ten patients treated with low-dose IL-2, these symptoms progressively disappeared. It must be pointed out that in most cases, clinical improvements started to be observed after the second or third course of IL-2 therapy (109). Systemic lupus erythematosus (SLE) shares with SSc several immune disturbances. A dysbalance between Treg and Teff was recently confirmed in SLE with correlations between these findings and disease activity. *In vitro* experiments showed that lack of IL-2 production by $CD4^+$ T cells accounted for the loss of CD25 expression in SLE Treg (110). Preliminary data in few patients receiving low-dose IL2 showed effectiveness to expand Treg (111). Clinical effects are now under investigations. Thus, IL-2 therapy is a promising avenue for expanding Treg cells and improving clinical outcomes for patients with autoimmune disease and trials are ongoing in connective tissues diseases including systemic sclerosis (TRANSREG study NCT01988506).

Similarly, anti-CD25 therapy results in prevention of activation and proliferation of T cells and inhibition of T cell responses. It is indicated for the prevention of acute organ rejection in adult and pediatric renal transplant recipients in combination with other immunosuppressive agents and has been studied in some immune diseases. Basiliximab is a chimeric (human/murine) anti-CD25 monoclonal antibody. It was administered to 10 SSc patients with severe skin involvement in addition to concomitant immunosuppressive and vasoactive treatments (112). Outcomes showed a reduction in skin fibrosis at week 68 and improvement in lung function at week 44. Treatment with basiliximab was well-tolerated. Although erythema, transient nausea, fatigue and weakness were common, severe reactions with significant dyspnea occurred in only one case. No patient had a documented severe infection and only one patient needed antibacterial therapy because of suspected respiratory infection. However, the application of this therapy in auto-immune diseases could be questionable. Indeed, since CD25 is also expressed on regulatory T cells, elimination or inhibition of the functional capacity of this subset using CD25 antibody might be counterproductive.

Use of autologous hematopoietic stem cell transplantation SCT (aHSCT) has recently gained interest in systemic sclerosis (64, 113–115) although the right regimen and best patient profile remain a matter of debate. It must be pointed out that sustained regression of skin and lung fibrosis has been reported for some patients. After aHSCT, reappearance of functional B cells, T-cell development, reconstitution of effector cells and efficient antigen presentation to reconstitute the pre-transplantation immune repertoire has been described. In SSc, there is scarce data about Treg restoration after aHSCT and contradictory results have been reported. A preliminary report about 7 patients focused on Treg showing a decrease number of Treg at baseline and altered suppressive capacity contrasting with restoration of Treg numbers and suppressive activity 24 months after aHSCT although a high variability was observed (64). On the other hand, in another report about 11 patients, both $CD4^+$ activated effector T cells and Tregs did not reconstitute well

after aHSCT with high dose cyclophosphamide conditioning, although the patients displayed resolution of clinical SSc (115). Therefore, the authors concluded that a complete reconstitution of the immune system, including Tregs, is not necessary for a treatment effect. Besides reconstitution of Treg numbers and suppressive capacity, increase in diversity of the TCR repertoire of Treg could be a crucial mechanism for the re-establishment of immune tolerance after aHSCT (116). Thus, future research into the effect of aHSCT on Treg cell compartment in SSc is required to clarify the underlying mechanisms of Treg cell pool renewal and the potential link with clinical outcome.

CONCLUSION

Evidence for numerical and functional changes of Tregs in SSc has been obtained in several studies. While the majority of the studies reported reduced frequencies of circulating Tregs in SSc patients compared to controls, it seems that some patients, especially in early phase and active disease have increased number of circulating Tregs. This paradoxical situation could be the result of a compensatory, but inefficient, amplification of Tregs in the context of active inflammation. In addition to diminished suppressive capacity, recent evidence indicate that Tregs could contribute to SSc pathogenesis by conversion into pathogenic effector T cells in the presence of appropriate environment, such as Th17 cells and Th2 cytokine-producing cells.

Nevertheless, these results should be tempered with regards to the heterogeneity of the studies in terms of patient's phenotype, and of the most importance regarding Tregs definition and activity markers. Moreover, although most previous studies analyzed peripheral blood of patients, the studies on Treg cells investigating phenotype and function in the site of inflammation are still sparse. Furthermore, conversely to other auto-immune diseases, very scarce data about Tregs in SSc mouse models have been produced. Thus, since the available data points toward a central role of Treg cells in SSc, future research is definitively needed to clarify the role of this cell population in SSc pathogenesis.

Finally, since the effect of existing treatment modalities on Tregs in SSc has not been elaborated sufficiently, gaining a better understanding of the natural history of Treg function and the affected mechanisms in SSc will certainly lead to new avenues in therapy, and will help to clarify whether the promises obtained in other autoimmune diseases may also apply to SSc.

AUTHOR CONTRIBUTIONS

CF wrote the first draft of the manuscript; YA wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Autologous Hematopoietic Stem Cell Transplantation for Treatment of Systemic Sclerosis

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Systemic Sclerosis (SSc) is a complex autoimmune disease, characterized by high mortality and morbidity. The heterogeneity in terms of extent, severity, and rate of progression of skin and internal organ involvement gives rise to many difficulties in finding the optimal therapeutic interventions for SSc and, to date, no disease-modifying agents are available. In this scenario, it is not surprising that SSc was one of the first autoimmune diseases challenged with high-dose immunosuppressive treatment followed by autologous hematopoietic stem cell transplantation (AHSCT). In the last decades, AHSCT has emerged as a treatment option for refractory SSc through a reduction of the aberrant immune cells, followed by re-constitution of a new, self-tolerant immune system. After several case series and pilot studies, more recently three randomized controlled trials have shown a benefit in skin involvement, organ functions and quality of life measures in AHSCT compared to monthly cyclophosphamide. In addition, although AHSCT presents a certain risk of mortality, it has been shown that the overall survival is better, compared to the cyclophosphamide group. Current evidence suggests that SSc patients who are most likely to benefit from AHSCT are early, active, with rapidly progressing diffuse skin disease, and mild involvement of internal organs. As the studies have progressed, it has become evident the need for a more rigorous patient selection, the optimization of transplant and post-transplant procedures, and the intervention of multidisciplinary teams of specialists to increase the safety and efficacy of AHSCT in SSc.

Keywords: systemic sclerosis (Scleroderma), stem cells, therapy, transplantation, haematopoietic

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INTRODUCTION

Hematopoietic stem cell transplantation, commonly used to treat hematological malignancies, has evolved in the last 20 years as a specific therapy for severe and therapy-refractory autoimmune diseases. Since the first case series, and following prospective and retrospective studies [recently reviewed by Eyraud et al. (1)] reporting the feasibility and efficacy of AHSCT in SSc on skin thickening and stabilization of internal organ function, randomized controlled trials have recently provided evidence that AHSCT is a real disease-modifying treatment for diffuse SSc inducing better long-term survival in comparison with intravenous cyclophosphamide (2–4). These data have provided convincing proof of the superiority of AHSCT over conventional therapies even considering the intrinsic risk of the procedure. Really, the risk of transplant-related

mortality (TRM) has decreased over the years (5) thanks to the careful selection of patients, growing experience in organ pre-transplant screening and ongoing refining of transplant procedures. Although AHSCT has been proved to be effective in patients with SSc, more in-depth knowledge about the mechanisms of action related to AHSCT-induced remission is required. AHSCT is considered a sort of intense immune-suppressive therapy able to ablate the aberrant auto-reactive immune cells to allow for a subsequent resetting of the host immune system. Indeed, the effects of AHSCT are now believed to be more complex, due in part to the intense immune ablative conditioning regimen, the modulation of mediators of innate immunity and adaptive immune cells, and the regenerative effects of the infusion of hematopoietic progenitors on damaged tissues (6).

In this review, we will examine the outcome of conventional therapy of SSc, provide a detailed description of the procedure, the clinical results and risks of AHSCT for SSc. We will present a comparison of the design and outcomes of published randomized trials regarding AHSCT in SSc. We will further discuss the options and recommendations for a better pre-transplant selection and evaluation of SSc patients to refer for AHSCT. We will also provide an analysis of the actual unmet needs in AHSCT and the issues that determine why AHSCT is still seen as a rescue therapy rather than an effective therapy for long-term suppression of SSc.

CURRENT IMMUNOSUPPRESSIVE MANAGEMENT OF SSC

SSc is an immune-mediated disorder characterized by inflammatory, vascular, and fibrotic features resulting in skin fibrosis and multiple organ manifestations (7). Despite the fact that in the last 20 years there has been improved understanding in the early diagnosis of the disease, and in identifying early internal organ involvement, therapy is still an unsolved problem (8, 9). While some patients display an indolent course, others exhibit a rapid and severe progression of the fibrotic processes with early manifestations of vital organ dysfunction. The lack of validated biomarkers that could be used for diagnosis, disease classification, identification of probable organ involvement and evaluation of therapeutic response, increases the difficulties in the management of scleroderma patients (10). The ability to identify different clinical phenotypes with a heterogenous course, to recognize the existence, like other autoimmune diseases, of flare and remission phases, and to estimate the prognosis of the disease would also be of remarkable importance from different points of view. In this perspective, the therapeutic strategies to treat SSc patients should not be addressed to a simple "organ-based" treatment, but to a more complex evaluation of the patient aimed at the prompt detection of active phases of the disease, assessment of possible organ damage and patient prognosis.

Current treatment options for SSc have targeted different pathogenic processes, including inflammation, immune dysregulation and fibrosis, showing only limited efficacy, and, to date, SSc still continues to carry a very high morbidity and mortality rate, mainly in the rapidly progressive form of the disease (11, 12). For a long time, cyclophosphamide has been considered a first line therapy for SSc, namely in patients with skin disease and concomitant interstitial lung disease (ILD) (13). More recently, two randomized, placebo-controlled trials involving patients with SSc showed that mycophenolate and cyclophosphamide were effective against ILD associated with SSc and, in particular, mycophenolate was useful in terms of tolerability, improvement of lung function and dyspnea, thickening of the skin, and health-related quality of life. However, all the effects, except for a sustained impact on dyspnea, disappeared approximately 1 year after stopping oral administration of cyclophosphamide (14–19). Furthermore, two meta-analyses of prospective studies using oral or IV cyclophosphamide in SSc-related ILD did not report any improvement in lung function (20, 21).

With regard to other immunosuppressive agents, a wide variety of treatments for SSc have been explored including azathioprine, methotrexate and, more recently, targeted therapies with monoclonal antibodies, including tocilizumab (anti-interleukin-6 receptor antibody), rituximab (an anti-CD20 antibody), and fresolimumab (an anti-TGFß antibody) (22). However, although showing a possible improvement of skin and lung involvement, they have been used in small series, pilot or short-term studies without yielding any definite evidence that they may be effective in changing the natural history of scleroderma disease (9, 19). Based on this uncertainty and frustration, AHSCT has been seen as a hopeful opportunity to treat SSc.

AHSCT Procedure

The AHSCT procedure is based on three main steps: the first one consists of the mobilization of stem cells from bone marrow to the peripheral blood by priming regimens followed by the collection of the mobilized stem cells; the second step is the administration of conditioning regimens with an immunoablative or myeloablative effect and, finally, the infusion of autologous (CD34+) stem cells (so called "transplantation").

The mobilization protocols include the administration of cyclophosphamide (2–4 gr/m^2) in combination with granulocyte-colony stimulating factor (G-CSF). Mobilized stem cells are then harvested by leukapheresis with or without CD34 selection before cryopreservation. Before transplantation, the patient undergoes the conditioning phase that can be either non-myeloablative or myeloablative. The goal of non-myeloablative regimens is to maximally suppress the immune system without destroying the bone marrow stem cell compartment. The bone marrow is not completely wiped out, which makes the treatment less dangerous and allows the patient to recover faster. Myeloablative AHSCT is the more stringent type of treatment. The term myeloablation refers to the administration of total body irradiation (TBI) and/or alkylating agents, at doses which will not allow autologous hematologic recovery. This is designed to almost completely wipe out both the autoreactive lymphocytes, as well as the bone marrow. Both forms are equal in that they both ablate the lymphocytes in the body that are

self-intolerant, and are ultimately responsible for the underlying autoimmune disease. However, current evidence supports the concept that non-myeloablative AHSCT has an improved level of safety and tolerability with lower mortality when compared to myeloablative regimens (23). In this regard, it is well known that in hematological diseases TBI is associated with the occurrence of secondary myelodysplasia and acute myeloid leukemia and solid tumors, even many years after the treatment (24, 25).

The available data do not allow making conclusions as to which autologous transplant regimen is best since head-to head studies have not been performed. To date, a few studies analyze the features and outcomes of mobilization in patients with autoimmune diseases and particularly with SSc. The use of G-CSF alone has been frequently associated with flare of disease activity in different autoimmune diseases, so the combination with cyclophosphamide is considered advantageous not only in protecting against disease flares but reducing the number of autoreactive T cells in the graft (26–30). With regard to cyclophosphamide dosage, doses used for mobilization in patients with malignancies could not be the best regimen for patients with autoimmune diseases. In patients with SSc and visceral involvement, mobilization-related complications, particularly cardiotoxicity, hemorrhage as well as infections, are largely dependent from cyclophosphamide doses (31–33). Besides the mobilization regimen, stem cell yields could vary according to the underlying disease and prior medication history. There are limited reports investigating the efficacy of stem cell mobilization and harvesting in patients with autoimmune diseases treated by AHSCT. In general, patients with SSc have not shown any relevant difference in terms of progenitor mobilization in comparison with patients suffering from other autoimmune disease and no correlation has been observed with respect to disease duration and previous exposure to methotrexate, cyclophosphamide or prednisone (26, 34, 35). With regard to the selection of cells for the graft, a recent multicenter retrospective study has demonstrated that the use of selected CD34+ cells for AHSCT in patients with SSc did not add any benefit to the outcome with respect to the use of un-manipulated cells (36).

IMMUNOLOGIC MECHANISMS OF AHSCT

SSc is considered a pleiomorphic disease deriving from the complex interaction of endothelial damage, autoimmune inflammatory reaction and excessive fibrosis, and is related to Th1/Th2 dysregulation, with prevalence of Th2 cells and development of autoreactive T and B cells targeting self-antigens causing organ damage (7). Considering this scenario, hematopoietic stem cell transplantation aims to reconstitute the hematopoietic niche after chemotherapy treatment or irradiation to obliterate autoreactive cells.

The result of this procedure is an "immune resetting," that is the eradication of the preexisting immune system, which is replaced by a new immune repertoire, with re-instatement of an appropriate immune regulation. These three well-defined mechanisms of immune resetting may be synergistic and their relative contribution to disease control depends on the transplantation regimen and on the underlying disease (37).

The combination of lymphotoxic chemotherapy, such as cyclophosphamide and anti-thymocyte globulin, leads to a profound and long-lasting lymphopenia and persistently reduced levels of putative pathogenic autoantibodies. Apart from this non-specific immunosuppression, there is growing evidence that autologous AHSCT can also re-establish immunological tolerance through different mechanisms. Firstly, AHSCT leads to an increased number of regulatory, FoxP3+ T cells, which are important in the preservation of tolerance (38). Secondly, the reactivation of thymic function after autologous AHSCT potentially leads to a tolerant, "juvenile" immune system. This has been illustrated by the recurrence of recent thymic emigrating cells, characterized by T-cell receptor excision circles (TREC) and CD31 expression, reestablishing T-cell receptor diversity in the years after AHSCT in patients with SLE (39). Moreover, anti-thymocyte globulin directly targets long-living, autoantibody-producing plasma cells by complement mediated lysis and apoptosis (40).

The immune reconstitution post-therapy results in profound changes of circulating immune cell populations, which involve a functional reactivation and volumetric enlargement of the thymus, defined as thymic rebound. However, the presence of pre-transplant B cell clonal expansion and faster T cells recovery after transplantation represent specific immunologic characteristics of long-term non-responder/relapsing patients.

T Cells and T Cell Receptor Repertoire

The cytopenia following the conditioning phase of AHSCT affects the lymphocyte subsets differently, and the kinetics of reconstitution depends on different timing of recovery for each cell type. The levels of B cells, natural killer cells (NKs), and CD8+ T cells begin raising rapidly and achieve a complete reconstitution to pre-transplantation levels after 2–3 years. The recovery of CD4+ T cells has consistently been observed to be slower and is often incomplete. Particularly, delayed CD4+ T cells recovery appears more pronounced in SSc patients who present good response after HSCT in comparison to poor responders (41). When compared with pre-transplant levels, absolute regulatory T cells (Treg) numbers increased significantly at 12 months post-transplantation, concurrent with thymic rebound (42). It is noteworthy that good responders to transplant present higher CD4+CD25^{high}FoxP3+ Treg percentages than non-responders (42). This data suggests that these regulatory molecules probably play an important role in the renewal of the immune system. Furthermore, increase of PD-1+ (programmed death-1 positive) T-cell numbers has been described in a group of SSc patients with better outcomes after AHSCT (42). PD-1 expression is critical to induce self-tolerance in newly generated T-cells under lymphopenic conditions, and its absence is associated with development of a systemic multi-organ inflammatory disease (43). In other words, PD-1 positivity in responders suggest an additional mechanism of negative regulatory control on autoreactive pathogenic T-cells (44, 45).

The influx of newly generated thymic-derived naïve T-cells results in changes in the T-cell receptor (TCR) repertoire

following transplantation. In the early period post graft, TCR repertoire has been demonstrated to be disturbed with a higher number of families presenting a skewed and oligoclonally expanded profile. Later, thymic rebound results in elevated TCR repertoire. Estimation of TCR diversity showed that, for responder patients, overall specificities increased following thymic rebound at 1 and 2 years, whereas non-responder patients failed to achieve higher TCR diversity (46).

Although AHSCT includes high dose immunosuppression, potentially pathogenic T cells are not completely depleted, because specific T-cell clones can still be detected post graft (47). There are two sources to consider for persistent autoreactive clones after autologous HSCT: residual clones in the host resisting after the immunoablative therapy, and cells reinfused with the graft. The intensity of the immunosuppressive regimen (myeloablative, high-dose or non-myeloablative, reduced intensity conditioning) and the manipulation of the graft (enriched in stem cells or unmanipulated), are the main factors that determine the number of residual T cells (37).

The new immunological arrangement after grafting will depend on the different antigenic stimuli, on the affinity for the ligand and the function of the responding cells (effector or regulatory), which are reprogrammed during the immune system reset.

B Cells

Naïve B-cell counts progressively begin increasing from the sixth month after AHSCT. Particularly, SSc patients with good response to transplantation presented a sustained B cell reconstitution compared to non-responders. Percentage and absolute numbers of $CD24^{high}CD38^{high}$ Bregs increase significantly in the following months post-AHSCT and responders are shown to present significantly higher frequencies of Bregs than non-responders and Breg levels correlate with a favorable outcome in SSc patients (42).

It should be mentioned that thymic rebound, as well as increased bone marrow output of newly generated naïve B cells, are exclusive of the post-transplant setting and are not observed in SSc patients receiving conventional treatment (42).

Autoantibodies

To date, literature data about the presence and the modification of anti-Scl70 antibodies after AHSCT are inconclusive. Some studies suggest a correlation between anti-Scl70 titer and clinical response, whereas others show uncertain association (48–50). Henes and colleagues demonstrated the anti-Scl70 reactivity significantly decreased after transplantation but remained positive in 10 of the 11 patients followed for up to 24 months. This decrease did not correlate with the clinical outcome after grafting (49). Farge et al. reported long-term anti-Scl70 negativity after transplantation in responder patients, although this decrease was not associated with a reduced B cells counts (46). Recently Glaeser et al. recognized a specific epitope recognized by anti-topo-1-antibodies in SSc sera. Interestingly, SSc patients with a good response to AHSCT had lower reactivity towards this peptide (p39, aa647-671) in comparison to non-responders (50).

Cytokines

The immune reconstitution process following the AHSCT may involve also the serum levels of the inflammatory cytokines profile and pro-fibrotic molecules concentration.

Detection of circulating and tissue cytokine levels has provided evidence for a balance between Th1/Th2 cytokines in the course of SSc, supporting a predominant Th2 immune response (7, 51, 52). A correlation between cytokine levels and SSc severity, in terms of extent of skin and organ fibrosis, has been widely reported (52), suggesting cytokines as a target of new therapeutic strategies, including AHSCT.

Currently, three studies examined the evolution of serum cytokine profile in SSc patients underwent AHSCT. The first study analyzed the serum levels of inflammatory cytokines (IL-2, IL-6, IL-8, and IFN-γ), pro-fibrotic molecules (TGF-β, IL-4, and PDGF), pro-angiogenic factors (VEGF and PDGF), endothelial markers (E-selectin and P-selectin) and MCP-1 chemokine before and up to 4 years after HSCT in 20 SSc patients. Even though a decrease in IL-2 and IL-8 levels, along with a slight but significant decrease in TGF-ß levels after 6 months has been demonstrated, these fluctuations did not reflect the skin score improvement after AHSCT (53).

In a cohort study of 11 patients, the concentrations of tumor necrosis factor alpha (TNF-α), soluble Interleukin 2 receptor (sIL-2r) and IL-6 levels were detected 12 months after AHSCT. Levels of TNF-α, sIL-2r, and IL-6 were significantly decreased, although reached normal values after 3 and 6 months post-AHSCT. TGF-β1 titers were not statistically significant decreased. Serum levels of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) did not decrease (48).

Assassi et al. analyzed gene expression patterns in the peripheral blood from patients participating in the SCOT Trial (4), and showed that the interferon signature was decreased by AHSCT. SSc patients presented a significant up regulation of genes that are induced by IFN. After 26-months of follow up, the IFN transcript score decreased significantly in patients receiving grafting, whereas it remained stable in the patients treated with monthly cyclophosphamide. Although this may support the "resetting" hypothesis, it remains to be investigated whether the IFN signature remains durably suppressed in the long term (54).

Recent studies have tried to understand if there is an immunological signature characterizing and predicting the clinical response to AHSCT (**Table 1**), but the findings are not univocal and sometimes confusing. In this subset, further immune reconstitution analysis will guide the clinicians for establishing new targeted therapeutic protocols.

CLINICAL USE OF AHSCT IN SSC

Efficacy

Several case reports and different phase I-II studies formed the basis for randomized controlled studies (RCTs) with AHSCT in SSc (1–4). All the RCTs have similar eligibility criteria and control treatment but exact comparability of cohorts, procedures and outcomes is questionable (see **Tables 2**, **3**).

TABLE 1 | Immunological profile in patients responders to AHSCT.

Before and after AHSCT	After AHSCT
- High number of T-reg and B-reg - Broad T-cell receptor diversity	- High number of PD-1+ T cells - Low reactivity to a specific epitope recognized by anti-topo-1-antibodies - Delayed CD4+ T cells recovery

AHSCT, Autologous haematopoietic stem cell transplant; PD-1, programme death-1.

The ASSIST is a single-center randomized phase II study, whereas the others (ASTIS and SCOT) are multicenter randomized phase III studies (2–4). In addition, the ASSIST is a treatment failure study and allowed a cross over to transplant due to unsatisfactory response to cyclophosphamide in eight out of nine control patients (2). The ASTIS and SCOT are survival studies and cross over from the control arm to transplant because of disease progression was not allowed in SCOT, except in the case of 2 years of cyclophosphamide therapy in the ASTIS trial (3, 4). Two trials are non-myeloablastive (ASSIST and ASTIS) using cyclophosphamide at 200 mg/kg in the conditioning phase, whereas the SCOT has used a TBI-based myeloablative regimen with cyclophosphamide 120 mg/kg. Two RCTs used the CD34+ selection of the graft, while the ASSIST did not. The primary end-point of the ASSIST trial was improvement in the modified Rodnan skin score (mRSS) or in pulmonary forced vital capacity (FVC) (2). By contrast, the ASTIS trial had event-free survival (EFS) as the primary end point and the treatment-related mortality (TRM) and toxicity, and progression-free survival as major secondary end points (3). The ASTIS and SCOT trials were initially matched in terms of entry criteria, control arms and end points (3, 4). In 2010, the primary end point of the SCOT trial was changed to a non-clinical outcome, the global rank composite score (GRCS). The GRCS ranked subjects based on a hierarchy of several important outcomes, including mortality, EFS (without organ failure), lung function, scleroderma health assessment questionnaire and the mRSS (4). Composite endpoints are increasingly used as primary efficacy measures in several clinical trials to capture a comprehensive picture of the treatment effect and to improve trial efficiency by increasing the event rate and reducing the sample size required. However, there are also some limitations to use this methodology. First, a true survival curve (e.g., Kaplan-Meier estimate) cannot be obtained for the composite outcome score since all the events that occur throughout the trial are shown. Furthermore, the overall global rank comparison may be statistically significant, though the individual components used for the primary analysis may not be significantly different between the two arms. In addition, confirmation of their validity is needed before they can achieve widespread acceptance and, in the case of SSc trials, they have never been used and validated.

Despite all these differences, the outcome data from these RCTs definitely support the greater benefit of AHSCT in comparison to cyclophosphamide for severe SSc (2–4). All the patients in the AHSCT arm experienced a significant improvement in the mRSS and functional capacity (HAQ, Disability Index). These results are consistent with previous observations from observational, pilot studies and registries that showed a marked impact of AHSCT on skin thickness (1). At the moment, no other studies on therapeutic intervention in diffuse scleroderma have shown to be so effective in stopping and reversing scleroderma skin involvement. The impressive efficacy of AHSCT on skin thickening has to be regarded as an important issue since high mRSS values have been recognized to be a predictor of poor prognosis and high mortality and, on the contrary, improvement in skin thickness is associated with better survival (55, 56).

Although showing some statistically significant improvement in FVC at 1 year and 2 year, AHSCT has yet to demonstrate a clear clinical significant improvement in DLCO and total lung capacity in all three RCTs, confirming the data from observational and retrospective studies (33, 57–59). With regard to DLCO, in a retrospective case-control study Del Papa et al. reported that the cumulative probability that DLCO values may fall under 50% is lower in the AHSCT group compared to the control group (60). In addition, in a cohort of 89 patients transplanted according to the ASSIST protocol, Burt et al. observed that DLCO was not improved significantly after AHSCT, but when the patients were stratified according to the pre-AHSCT echocardiogram and electrocardiogram characteristics, the DLCO was improved in a subgroup of patients with normal cardiac features, raising the question of the relationship between DLCO and cardiac function (33). Using high resolution computed tomography (HRCT), Launay et al. assessed lung involvement after AHSCT in a small group of SSc patients. The extent of SSc lung fibrotic involvement on HRCT rapidly but transiently regressed 6 months after AHSCT. However, longer-term follow-up showed that the impressive early treatment effects of AHSCT on the extent of pulmonary fibrosis decreased over time and were transient in some patients, returning to the pre-transplant extent two years after AHSCT. Moreover, pulmonary fibrosis appeared to be rather stable up to 60 months of follow-up (61). These data are not surprising since they reflect the known effects of cyclophosphamide on SSc skin and lung fibrosis. In the Scleroderma Lung Study (SLS I), the maximum improvement in lung function was observed at 18 months with 12 months of cyclophosphamide use, but at the end of one additional year off therapy, the beneficial effects on FVC and TLC were lost, whereas the skin score stabilized (16). SLS II showed that immunosuppression with either 2 years of mycophenolate or 1 year of oral cyclophosphamide led to slight improvement in lung function (17, 18). Some additional observational studies showed that a strategy combining IV cyclophosphamide followed by oral maintenance azatioprine or mycophenolate for worsening SSc-ILD was associated with stabilization or improvement of pulmonary function tests in approximatively 50% of patients after 12 months of mycophenolate and 24 months of azatioprine respectively (15, 19). Based on these observations, we could speculate that in the direct comparison between the efficacy of AHSCT and that of cyclophosphamide therapy, the lack of maintenance immunosuppression in the control group might be responsible for better outcomes in the transplanted arm. However, unmet

TABLE 2 | Overview of study characteristics, inclusion and exclusion criteria of ASSIST, ASTIS and SCOT trials.

	ASSIST (2)	**ASTIS (3)**	**SCOT (4)**
Trial design	Randomized Phase II	Randomized Phase III	Randomized Phase III
Patients number	19	156	75
Recruitment period	2006–2009	2001–2009	2006–2011
Mobilization	CYC 2 g/m^2, G-CSF	CYC 4 g/m^2, G-CSF	G-CSF only
Conditioning	CYC (200 mg/kg), rabbit ATG	CYC (200 mg/kg), rabbit ATG	CYC (120 mg/kg), equine ATG
Total body irradiation	No	No	Yes (800 cGy, lung and kidney shielding)
Stem cell manipulation	None	CD34+ selection	CD34+ selection
Comparator arm	CYC 6 monthly IV courses (1,000 mg/m^2)	CYC 12 monthly IV courses (750 mg/m^2)	CYC 12 monthly IV courses (750 mg/m^2)
Inclusion criteria	<60 years	18–65 years	18–69 years
	Diffuse SSc	Diffuse SSc	Diffuse SSc
	Disease duration ≤ 4 years	Disease duration ≤ 4 years	Disease duration ≤ 4 years
	mRSS ≥ 15	mRSS ≥ 15	mRSS ≥ 16
	Internal organ involvement	Internal organ involvement	Internal organ involvement
Exclusion criteria	Mean PAP > 25 mmHg or PAP sys > 40 mmHg	Mean PAP > 50 mmHg	Mean PAP >30 mmHg
	LVEF < 40%	LVEF < 45%	LVEF < 50%
	–	–	FVC<45% predicted DLCO<40% predicted
	Creatinine > 177 umol/L	Creatinine Clearance < 40 mL/min	Creatinine Clearance < 40 mL/min
	CYC >6 g IV	CYC cumulative IV dose >5 g or >3 g months oral	CYC cumulative IV dose >3 g/m^2 or >4 months oral or >6 months IV
	–	–	Active GAVE

ASSIST, American Scleroderma Stem cell versus Immune Suppression Trial; ASTIS, Autologous Stem cell Transplantation International Scleroderma Trial; SCOT, The Scleroderma Cyclophosphamide Or Transplantation; SSc, systemic sclerosis; CYC, cyclophosphamide; ATG, antithymocyte globulin; mRSS, modified Rodnan skin score; LVEF, left ventricular ejection fraction; PAP, pulmonary arterial pressure; IV, intravenous; GAVE, gastric antral vascular ectasia.

TABLE 3 | Comparison of outcomes among ASSIST, ASTIS, and SCOT.

	ASSIST (2)	**ASTIS (3)**	**SCOT (4)**
Primary outcomes measures	>25% decrease in mRSS, or >10%% increase in FVC at 12 months	Survival without new onset of heart, lung or kidney failure	Global Rank Composite Score at month 54
Follow up	2.6 years (mean)	5.8 years (median)	Up to 4.5 years
12-months treatment related mortality in comparator arm	0 (0%)	0 (0%)	0 (0%)
12-months transplant-related mortality	0 (0%)	8 (10.1%)	1 (3%)
Overall mortality	0 (%)	19 (24%)	3 (9%)
P/EFS	80% (2.6 years)	81% (4 years)	79% (4.5 years)

ASSIST, American Scleroderma Stem cell vs. Immune Suppression Trial; ASTIS, Autologous Stem cell Transplantation International Scleroderma Trial; SCOT, The Scleroderma Cyclophosphamide Or Transplantation; mRSS, modified Rodnan skin score; FVC, forced vital capacity; P/EFS, progression/event free survival without mortality, relapse or progression of the disease.

needs exist for post-transplant immunosuppressive treatment and future research should address the question whether an additional post-transplantation management is therefore useful to improve AHSCT outcomes.

Suppression or control of disease activity by AHSCT can be regarded as an additional optimal goal of the AHSCT procedure. A study published in 2017 demonstrated that lowering disease activity can be achieved with AHSCT in a population of SSc patients with high disease activity scores evaluated by a validated scoring system, namely the ESSG by Valentini et al. (60, 62). Furthermore, in the myeloablative trial, only 9% of transplant recipients showed scleroderma relapses by 24 months without any significant changes at 54 months (4). This rate was lower than that observed in the non-myeloablative RCT (3). These results further strengthen the profound effects of AHSCT on the course of the disease characterized by poor prognosis. This is a key issue, since patients presenting a rapidly evolving and active disease could be the best candidates to undergo such an extremely

aggressive treatment, and have the best possible results. Indeed, similarly to other rheumatic diseases, the opportunity to early "switch off" the inflammatory and active phases of the disease may offer an opportunity to stop and prevent disease organ damage, finally changing the natural history of an aggressive disease. In this regard, both the long-term RCT studies of AHSCT in SSc showed that EFS and overall survival rates are better for patients in the AHSCT arm than for patients in the control arm (82% at 5 years in the ASTIS and 86% at 54 months in the SCOT) (3, 4). These findings confirmed previous long-term follow-up data from a phase I/II study showing that death from disease progression occurred in 8% of severe SSc patients treated with AHSCT (59). This rate is considerably lower compared to the 5-year mortality rate estimated at 40% in such severe SSc patients (63). Similarly, Del Papa et al. observed a significant reduced disease-related mortality in patients with severe SSc treated with AHSCT in comparison with a historical cohort of age-and sex-matched SSc patients with analogous clinical features treated with conventional immunosuppressive agents (60).

Safety

Supported by preclinical studies and case reports, and more recently by 3 RCTs providing proof of efficacy of AHSCT over conventional therapies, the uptake of AHSCT in SSc has increased over the last decades and evolved as a specific treatment of patients with the severe rapidly progressive form of the disease (5, 13). This is indeed remarkable when we consider that, among the different severe autoimmune diseases treated with AHSCT, SSc has shown the higher risk of mortality (5). This feature can be explained by the fact that, compared with other autoimmune diseases, SSc patients have an involvement of vital organ function resulting in poor tolerance of AHSCT. However, the risks and adverse effects of AHSCT in SSc have changed over the last 20 years.

Adverse Effects

Expected complications related to the intense AHSCT-related immunosuppression, are opportunistic infections, urinary infections, neutropaenic fever and viral reactivation (5, 64). They represent the leading cause of mortality after AHSCT for autoimmune disease (64, 65) and cluster within the first month after AHSCT. In the ASTIS trial, viral infections were detected in 27.8% of patients in the AHSTC group *versus* 1.3% in the control arm (3). Conversely, overall infection rates were similar in the two arms of the SCOT trial with the exception of varicella zoster infection that developed in 12 out of 33 transplanted patients (36%) (4).

AHSCT can also induce other off-target adverse effects including transient alopecia and amenorrhea, and permanent infertility is a real risk (66). Recently, a multicenter retrospective analysis of pregnancy and childbirth in patients who underwent AHSCT for different autoimmune diseases, including SSc, reported 15 pregnancies (68%) with healthy life births and no congenital, developmental or any other disease in the children. There were no reports with regard to maternal mortality associated with pregnancy or postpartum (67).

AHSCT-related late adverse events include malignancies (64). SSc patients who undergo myeloablative regimens receive TBI with lung and kidney shielding. TBI guarantees a stronger immune suppression in comparison to the non-myeloablative regimen. However, it is well known that in patients who have been given a TBI-based regimen there is a higher risk of secondary malignancies, particularly myelodysplasia/acute myeloid leukemia and later (only in trials with >10 years of follow-up) solid tumors (24, 25, 68). The relative long-term follow-up of transplanted SSc patients suggests that TBI is related to an increased risk of malignancies as shown by their onset in 9% of patients (two cases of myelodysplastic syndrome and one of medullary thyroid cancer) in the SCOT trial vs. 2 instances of EBV-positive lymphoproliferative disorder in the ASTIS trial (2.5%) (3, 4). It is interesting to note that, for unclear reasons, recent meta-analysis studies confirmed an increased incidence of cancer in SSc patients compared with the general population. The tumor types included lung cancer, non-Hodgkin's lymphoma and hematopoietic cancers (69–71). Thus, we can speculate that the intense immunosuppression related to transplantation might represent an additional risk factor for malignancies in SSc.

As observed in patients receiving HSCT for different indications (72), AHSCT, as do adverse events, can induce the onset of secondary autoimmune diseases. The AHSCT in autoimmune diseases is aimed at the immune system, inducing an intense immune-depletion and the consequent re-establishment of tolerance. However, during the immune reconstitution new autoreactive clones may arise and induce *de novo* autoimmunity (64, 73). In a retrospective EBMT registry analysis published in 2011, the incidence of secondary autoimmune disease was 9.8%. The most frequent secondary autoimmune diseases were organ-specific, including autoimmune thyroiditis, hemolytic anemia, autoimmune thrombocytopenia, and myasthenia gravis (64).

Treatment Related Mortality

Despite the proven efficacy of AHSCT in the treatment of SSc, transplant-related mortality (TRM) still represents a thorny issue and makes it difficult to view AHSCT as a standard therapy rather than a salvage option for the early and rapidly progressive forms of SSc.

AHSCT was performed safely in the first published RCT comparing transplantation with cyclophosphamide (ASSIST) (2). This trial enrolled a small number of patients (19), 10 of whom were allocated to receive AHSCT, with a follow-up of at least 2 years. The results were extremely positive in terms of efficacy and eight control patients, who progressed or did not improve by 1 year, were allowed to crossover at the AHSCT arm. Notably, no death or serious adverse events were registered during the study (2). Other studies failed to demonstrate such a high level of safety (74, 75). A retrospective analysis of a large cohort of SSc patients treated with the same non-myeloablative ASSIST regimen, reported a TRM of 6% (33) and this was mainly ascribable to cardiovascular complications. In the ASTIS trial, the TRM was 10% during the first year, and again cardiac events were suggested as the main cause of death (3). In the SCOT trial, TRM was lower than that previously reported (3% at 54

months and 6% at 72 months) and no deaths occurred during the first year (4). These differences in TRM in different AHSCT-RCTs may have multiple explanations and all of them essentially emphasize the key role of cardiac function in the safety of AHSCT in SSc. As a matter of fact, high dose cyclophosphamide is the agent most frequently associated with cardiac toxicity and preserved cardiac function is generally required for enrollment in clinical trials of high-dose chemotherapy (76). High-dose regimen of cyclophosphamide (4 gr/m^2 in the mobilization phase and 200 mg/kg in the conditioning phase) used in the non-myeloablative AHSCT might be too toxic for those severe SSc patients, possibly with heart involvement. On the contrary, the SCOT trial, based on a low-dosage of cyclophosphamide, was not characterized by important cardiac events. Secondly, the low TRM reported in the studies by Burt et al. (2, 33) may be related to a more accurate cardiac evaluation. Similarly, none of the patients included in the SCOT trial had heart involvement or pulmonary hypertension (SCOT). Basal evaluation for the inclusion in the ASTIS trial consisted of echocardiogram for the detection of pulmonary hypertension and, only in this case, right heart catheterization was performed. However, it has been agreed that echocardiography cannot be reliable alone in making a diagnosis of pulmonary arterial hypertension. Right heart catheterization (RHC) is currently considered the gold standard for the evaluation of arterial pulmonary hypertension, further providing direct and accurate measurements of hemodynamics of the cardiovascular system (77). Furthermore, fluid challenge during RHC can give additional information to understand the cardiopulmonary response to increased volume load (as happens during AHSCT) and to identify patients with subclinical signs of SSc-related involvement of heart or pulmonary vasculature, neither of them being detectable at rest (33). Important complementary information may be provided by cardiac magnetic resonance imaging (CMR). CMR has emerged as the reference standard for assessment of left ventricular and right ventricular morphology, volumes and function (78) and it is considered a useful tool for the early assessment of cardiac involvement in SSc (79). The experience gained from a better knowledge of cardiac involvement in SSc and AHSCT studies is reflected in the current recommendations by the EBMT-Autoimmune Disease Working Party for a correct and extensive cardiopulmonary pre-transplant evaluation combining lung function tests, echocardiography, CMR imaging and invasive hemodynamic tests (80). Based on these recommendations, only patients without any evidence for PAH, even in the presence of fluid challenge during RHC, and good cardiopulmonary function can be considered for AHSCT.

Finally, both in the ASTIS and SCOT Trials, smoking status raised as an important element in compromising the AHSCT outcomes (3, 4). Compared to never smokers, previous and current smoking patients had a poorer overall survival. This finding is not new since it is common to other transplant setting (81), however the explanation of this correlation is largely speculative. Smoking is associated with impaired NK cytotoxic activity, unbalanced production of pro- and anti-inflammatory cytokines (81, 82) that may increase the risk of both respiratory and systemic infections in patients who experience compromised immunity. Furthermore, different studies provided strong evidence that tobacco use is detrimental to lung function in SSc patients further boosting abnormalities related to scleroderma lung disease (83–85).

In conclusion, in SSc patients, the risks of transplant-related complications and TRM are relatively high and depend on careful patient selection and evaluation, and the intensity of the transplantation regimen (agent and doses). These two features are strictly related to each other. Toxicity of the conditioning regimen largely depends on organ involvement and disease stage. In this perspective, the center experience and the close interspeciality networking provide an additional advantage (5).

Recommendations

All the available studies prove the general concept of AHSCT as an effective, safe and feasible therapy in severe SSc. The potential of AHSCT to suppress, or at least ameliorate SSc features might be even more encouraging, if in the coming years the profile of the ideal SSc candidate for AHSCT will be better defined.

The criteria for the patient's selection and the timing of providing stem cell transplantation in patients with SSc are greatly needed. The key point is to identify patients with the highest possibility to have an improvement by the transplant procedure and the lowest risk of developing post-transplant life-threatening complications. The mortality risk of the disease being treated by AHSCT might justify the risk related to the transplant procedure. Several papers have concluded that many variables detected at the first evaluation of SSc patients are associated with reduced survival during long-term follow-up (55, 86, 87). These variables include male sex, older age, diffuse skin thickening, involvement of the heart, lung and kidney, and total skin thickness score. Domsic et al. (55) found that the rapid skin thickness progression rate (STPR) is an independent predictor of early mortality and the development of SRC. Observational studies have demonstrated that in diffuse scleroderma, most of the pathological processes in the internal organs or systems (gastrointestinal, lung, heart and kidney) occur within the first 3 years of the disease onset. Keeping these considerations in mind, patients with early and rapidly progressive diffuse SSc with evidence of at least mild involvement of heart, lung or kidney are the best candidates for AHSCT (**Table 4**). Thus, a more accurate cardiac assessment before directing a patient to AHSCT is certainly needed. Right catheterization with fluid challenge and cardiac magnetic resonance evaluation are the diagnostic tools proposed for this purpose (80). The lower rate of treatment-related mortality reported in most of the studies recently published, can probably be explained by the exclusion of patients with severe organ involvement. Certainly, one can consider that most trials have chosen arbitrary cut-offs for respiratory and cardiac function, and data are not so convincing for these measures. Anyway, it would be considered prudent to avoid patients with endstage lung disease to minimize infectious complications related to the different immunosuppressant drugs used during the mobilization and conditioning prior to AHSCT. Likewise, the presence of advanced heart disease may increase the risk of poor outcome in situations requesting higher heart

TABLE 4 | Main indications and contraindications to transplantation.

Indications to AHSCT	Contraindications to AHSCT
Acute onset	Long standing disease
Rapidly progressive disease refractory to conventional therapy	Indolent course of the disease
Mild organ damage	Irreversible organ involvement

performance as fever, infections and liquid overload infusion related to the transplant procedure.

A large body of evidence suggests the concept that, as for other rheumatic diseases, SSc has flogistic and active flares too, and therapeutic options, including AHSCT, should be tailored by considering the phase of the disease. Measuring disease activity in SSc has been particularly difficult in comparison with other autoimmune diseases in which it is possible to easily differentiate flares from quiescent phases. In 2001, the European Scleroderma Study Group (EScSG) developed a preliminary activity index that was subsequently validated and endorsed by the European Scleroderma Trials and Research group (EUSTAR) (62, 88). Recently, Del Papa et al. showed that AHSCT in patients with rapidly evolving dcSSc is effective in lowering both disease activity and severity of skin involvement (60). The same study showed that the patients selected in the control group (treated with conventional therapies) with comparable levels of disease activity had a 5-year probability of survival of around 40% in comparison to the higher percentage observed in the AHSCT group (80%) (60). This figure certainly demonstrated that the decrease in the disease activity is effective in prolonging survival and preserving organ damage related to persistent active disease.

A recent retrospective analysis of the EBMT autoimmune disease working party recognizes the great importance of center experience in the AHSCT outcome in autoimmune diseases, and in particular in SSc (5). Given the low prevalence of SSc patients with a severe form of the disease, the difficulty in identifying patients with a poor prognosis in the early phases, the experience needed for a correct evaluation of organ involvement, the complexity of AHSCT in these patients, a real benefit can certainly be achieved by creating close interaction between hematologists, rheumatologists, cardiologists and pulmonologists.

CONCLUSIONS

Evidences from trials suggest that AHSCT is more effective than conventional immunosuppressive therapies at inducing a better long-term survival, ameliorating skin thickening and stabilizing internal organ function in severe SSc. The patients who can likely benefit from AHSCT are those with a rapid progressive and diffuse skin involvement, persistent high levels of disease activity, and mild initial organ damage. Center experience and specialist expertise are further important factors for improving outcomes of AHSCT strategies. Positive results from the published trials for AHSCT in SSc raise questions and new prospects of transplant activities. These challenges include (1) the definition of an optimal regimen intensity and in order to decrease TRM; (2) the availability of biomarkers or gene profiles able to select patients most likely to benefit from AHSCT; (3) a longer follow-up to identify late-onset adverse events; (4) the opportunity of a post-transplant immunosuppression to reduce the risks of disease relapse post-transplantation.

AUTHOR CONTRIBUTIONS

ND designed the plan of the review, revised the literature and wrote the paper. FP revised the recent literature and the final paper. EZ, WM, and AM revised the literature and the final version of the paper.

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Pharmacological Inhibition of Toll-Like Receptor-4 Signaling by TAK242 Prevents and Induces Regression of Experimental Organ Fibrosis

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Systemic sclerosis (SSc) is a poorly understood heterogeneous condition with progressive multi-organ fibrosis. Recent genetic and genomic evidence suggest a pathogenic role for dysregulated innate immunity and toll-like receptor (TLR) activity in SSc. Levels of both TLR4, as well as certain endogenous TLR ligands, are elevated in skin and lung tissues from patients with SSc and correlate with clinical disease parameters. Conversely, genetic targeting of TLR4 or its endogenous "damage-associated" ligands ameliorates progressive tissue fibrosis. Targeting TLR4 signaling therefore represents a pharmacological strategy to prevent intractable fibrosis. We examined the effect of TAK242, a small molecule TLR4 inhibitor, in preclinical fibrosis models and in SSc fibroblasts. TAK242 treatment prevented, promoted regression of, bleomycin-induced dermal and pulmonary fibrosis, and reduced the expression of several pro-fibrotic mediators. Furthermore, TAK242 ameliorated peritoneal fibrosis and reduced spontaneous hypodermal thickness in TSK/+ mice. Importantly, TAK242 abrogated collagen synthesis and myofibroblasts differentiation in explanted constitutively active SSc fibroblast. Altogether, these findings identify TAK242 as an anti-fibrotic agent in preclinical models of organ fibrosis. TAK242 might potentially represent a novel strategy for the treatment of SSc and other fibrotic diseases.

Keywords: systemic sclerosis, SSc, fibrosis, IL-6, toll-like receptor, TLR, TAK242, resatorvid

INTRODUCTION

A unique feature of SSc is synchonous fibrosis in multiple organs. Despite recent advances in understanding cellular and molecular basis of fibrosis, SSc still carries significant mortality. Most SSc patients develop interstitial lung disease (ILD), which currently accounts for >30% of all SSc deaths (1). There are no therapies effective in halting or resolving fibrosis (2). While multiple intracellular signaling pathways are implicated in SSc fibrosis, the nature of their persistent deregulation in pathological inflammation and fibrosis remains poorly understood (3).

We recently showed that toll-like receptor 4 (TLR4) and its endogenous ligands damage associated molecular patterns (DAMPs) such as fibronection-EDA and tenascin C are markedly elevated in SSc skin and lungs (4–6). Conversely, genetic targeting of TLR4 or its endogenous DAMPs, was shown to ameliorate tissue fibrosis in multiple preclinical models of SSc (4–6). Stimulation of fibroblasts TLR4 was associated with the induction of extracellular matrix (ECM) remodeling and tissue repair programs, as well as synergistic enhancement of TGF-beta-mediated fibrotic responses (6). Thus, in a fibrogenic milieu enriched with both TGF-beta and endogenous TLR4 ligands, fibroblasts expressing elevated TLR4 are likely to engage in uncontrolled ECM production and myofibroblast differentiation, contributing to progression of fibrosis. Disrupting persistent TLR4 signaling with TLR4 inhibitors therefore might represent a potential strategy for breaking the vicious fibrosis cycle in SSc or other fibrotic diseases (7, 8).

TAK242 is a small molecule inhibitor of TLR4 signaling that blocks the production of lipopolysaccharide-induced inflammatory mediators (9). TAK242 binds to Cys747 in the intracellular domain of TLR4, and disrupts the interaction of TLR4 with adaptor molecules, thereby inhibiting TLR4 signaling (9, 10). While TAK-242 was shown to suppress the increase in serum TNF-a, IL-1, and IL-6 in murine and porcine models of sepsis, it had no effect on survival (10, 11). In published clinical trials of sepsis, TAK-242 demonstrated non-significant reduction in patient mortality, but failed to demonstrate a beneficial effect on inflammatory cytokine levels. Moreover, treatment was associated with methemoglobinemia in some patients (12). Therefore, TAK-242 does not appear to be an optimal therapy for the treatment of sepsis, whereas its potential efficacy and safety in other diseases linked to excessive TLR4 signaling remain to be investigated. The strategy of converting the indications of existing drugs from one therapeutic area to include the treatment of other diseases, known as "drug repurposing" curtails the time required for clinical application. In view of our recent findings implicating the DAMP-TLR4 axis in driving sustained fibroblasts activation underlying fibrosis progression in SSc, we sought to investigate the effect of TAK242 in preclinical models of organ fibrosis, and in SSc fibroblasts. Our results demonstrate that selective pharmacological blockade of TLR4 signaling with TAK242 prevented and reversed organ fibrosis in multiple distinct mouse models. Moreover, TAK242 reduced constitutive collagen synthesis and myofibroblasts transdifferentiation in explanted SSc fibroblasts. These results provide a rationale for further exploring selective targeting of TLR4 signaling as a potential therapeutic strategy in patients with SSc.

MATERIALS AND METHODS

Cell Culture and Reagents

Primary cultures of human fibroblasts were established by explantation from patients with SSc, healthy, or mouse skin (6). Biopsies were performed with written informed consent and in accordance with protocols approved by the Institutional Review Board for Human Studies at Northwestern University. Mouse fibroblast cultures were established from skin biopsy specimens of 8-week-old female TLR4-mutant C3H/HeJ mice and WT control C3H/HeOuJ mice (Jackson Laboratories, Sacramento, CA) and studied in parallel. Low-passage fibroblasts were grown in monolayers in plastic dishes, and studied at early confluence. Cultures were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 1% vitamin solutions, and 2 mM L-glutamine. All other tissue culture reagents were from Lonza (Basel, Switzerland). TAK242 was synthesized as previously reported by TAKEDA (Osaka, Japan) (10). For experiments using SSc fibroblasts, cultures were placed in low serum media containing 0.1% FBS with or without the TLR4 inhibitor TAK242. For other experiments, cultures were placed in serum-free media containing 0.1% bovine serum albumin (BSA) with or without indicated concentrations of TAK242. The inhibitor was added 60 min prior to endotoxin-free Fn^{EDA} (1 μg/ml) isolated and purified from embryonic IMR90 fibroblasts (4).

Cytotoxicity Assays

Cell death or cytotoxicity was evaluated by quantification of plasma membrane damage using LDH Cytotoxicity Assay Kit II (Colorimetric Assay Kits, Biovision, Milpitas, CA).

Experimental Models of Fibrosis

Animal experiments were performed according to institutionally approved protocols and in compliance with guidelines of the Northwestern University Animal Care and Use Committee. A series of complementary fibrosis models were employed to evaluate pharmacological TLR4 blockade *in vivo*. First, 8-week-old female C57BL/6J (The Jackson Laboratory) mice received subcutaneous (s.c.) injections of bleomycin (10 mg/kg/day) or PBS daily for 10 days (5 days/week), along with TAK242 (10 or 30 mg/kg) by daily intraperitoneal (i.p.) injections starting concurrently with bleomycin, and sacrificed on day 7 or 22. Another group of mice received TAK242 injections started at day 15, and continued until sacrifice at day 28. A third group of mice received PBS, and a fourth received bleomycin alone. In a complementary non-inflammatory fibrosis model, 6 week-old Tsk1/+ mice (C57BL/6 background, The Jackson Laboratory) received TAK242 (30 mg/kg) injections i.p. daily till sacrifice at 12 weeks.

Tissue collagen content was determined by hydroxyproline assays using Colorimetric Assay Kits (Biovision, Milpitas, CA) (5). Lung fibrosis was quantitated in histological lung sections using the modified Ashcroft score determined from 5 h.p.f. per mice (Hubner score) (13).

Peritoneal fibrosis was induced by i.p. injections of 0.1% chlorhexidine gluconate (CG) (Wako Pure Chemical Industries, Osaka, Japan) dissolved in 15% ethanol/PBS and injected every other day. C57BL/6 male mice (∼25 g) received CG alone, or together with TAK242 (PO, BID; 30 or 100 mg/kg) or vehicle starting concurrently with CG injection for up to 21 day. At day 22, mice were sacrificed, and peritoneal tissues were carefully dissected. To avoid damage to the peritoneum, injections were made at the caudal part of the peritoneum, while the rostral portion of the parietal membrane was taken for analysis.

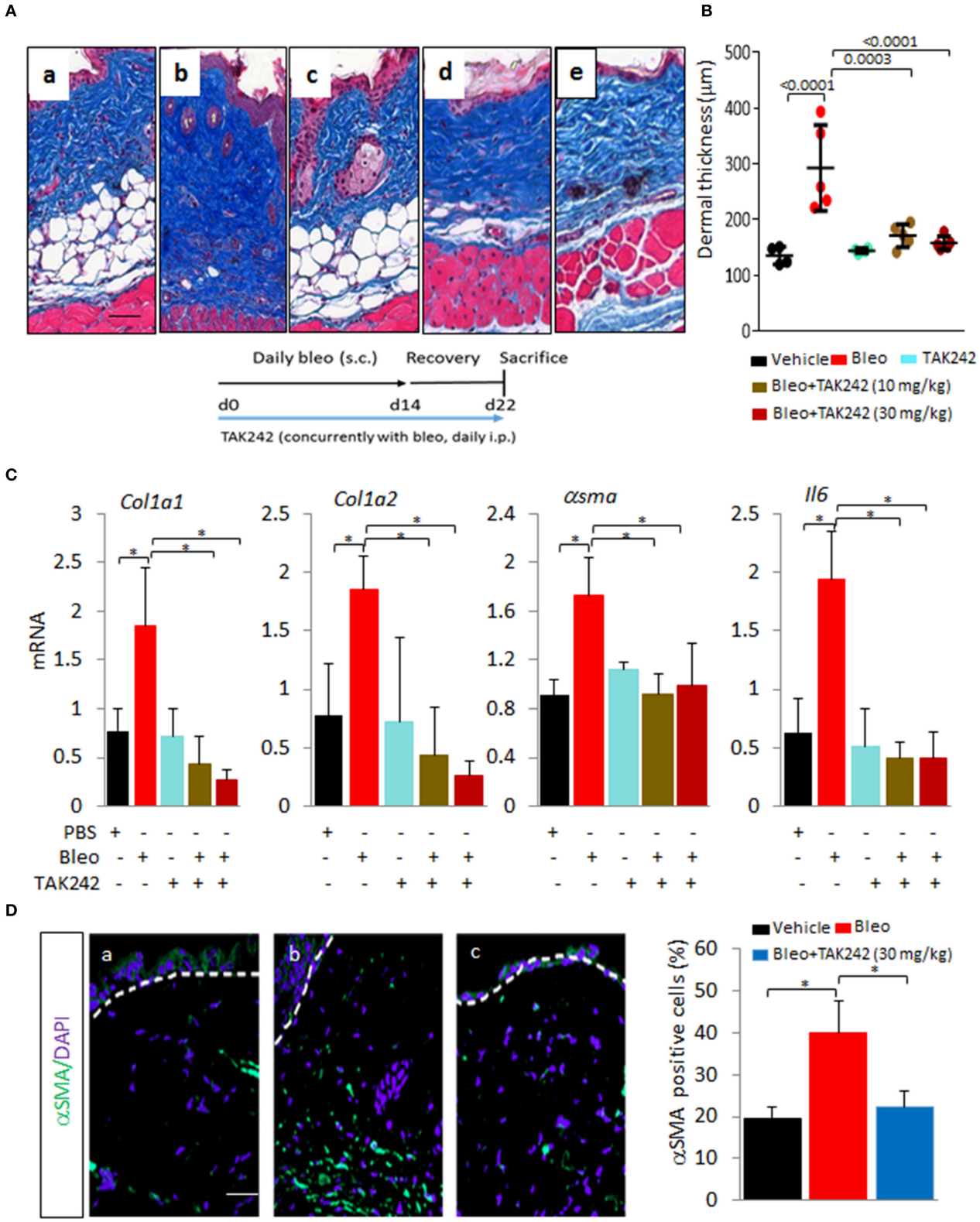

FIGURE 1 | Inhibition of TLR4 signaling by TAK242 prevents skin fibrosis. C57/BL6 mice received daily s.c. injections of PBS or bleomycin alone, or together with TAK242 (10 or 30 mg/kg) or vehicle. Mice were sacrificed at day 22 and skin was harvested for analysis. **(A)** Masson's Trichome stain. (a) vehicle, (b) bleomycin for 2

(Continued)

FIGURE 1 | weeks (5 days a week), (c) TAK242, and (d) low dose TAK242 (10 mg/kg) and bleomycin, and (e) high dose of TAK242 and bleomycin (30 mg/kg) injected concurrently for 2 weeks (5 day/week)**.** Representative images. Bar = 25 μm. **(B)** Dermal thickness (means ± s.d. of five determinations/hpf from five mice/group). One-way analysis of variance followed by Sidak's multiple comparison test. *p <0.05. **(C)** Real-time quantitative PCR. Results, normalized with GAPDH, are means ± s.d. of triplicate determinations from four mice/group; One-way analysis of variance followed by Sidak's multiple comparison test. *p < 0.05. **(D)** Immunofluorescence using antibodies to α-smooth muscle actin (αSMA, green) and DAPI (blue). Treatment groups as indicated inset. bleomycin alone for 2 weeks, (c) bleomycin plus TAK242 started concurrently. Representative images. Bar = 50 μm. Relative fluorescence intensities (means from four randomly selected from thee mice/group).

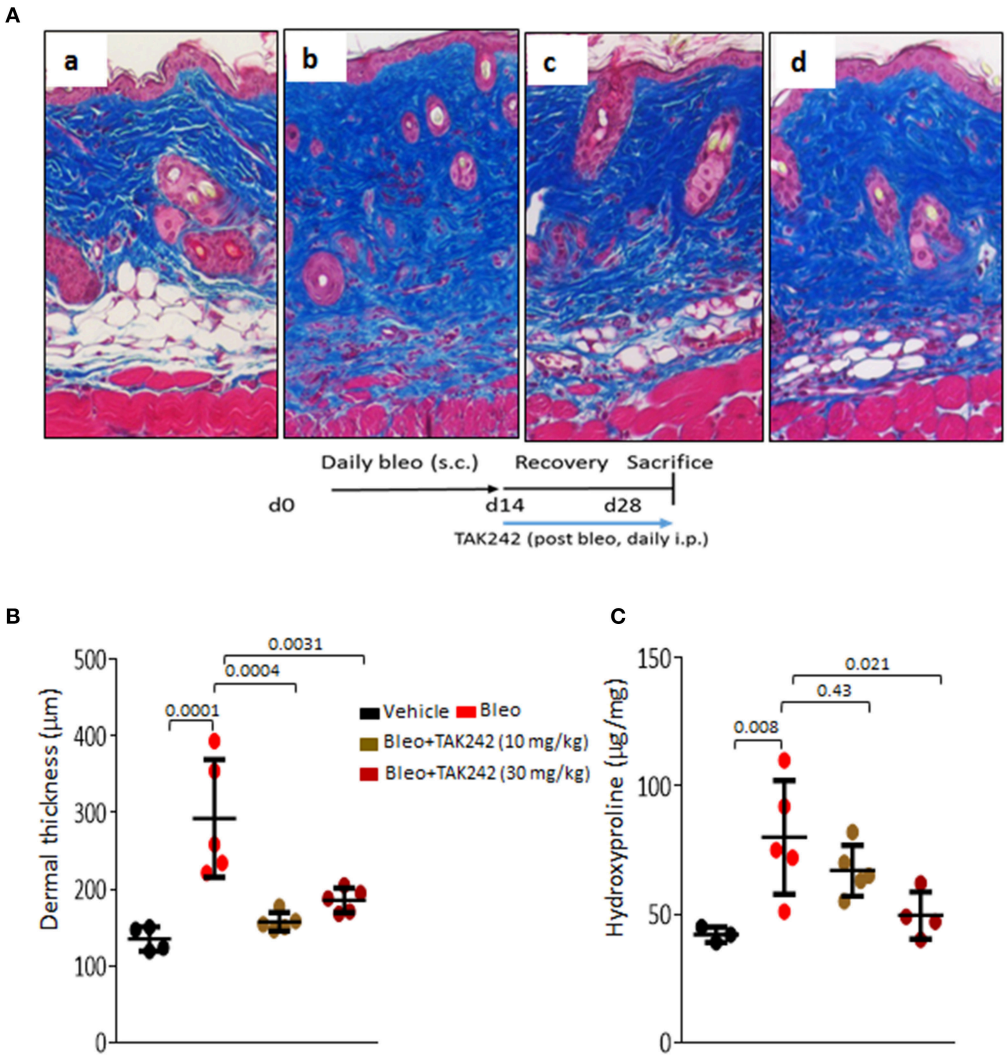

FIGURE 2 | Inhibition of TLR4 signaling by TAK242 induces regression of established skin fibrosis. C57/BL6 mice received daily s.c. injections of PBS or bleomycin alone, or TAK242 (10 or 30 mg/kg) or vehicle started after 2 weeks of bleomycin injections. Mice were sacrificed at day 28 and skin was harvested for analysis. **(A)** Masson's Trichome stain. (a) vehicle, (b) bleomycin for 2 weeks (5 days a week), (c) bleomycin followed by low dose (10 mg/kg), and (d) high dose (30 mg/kg) of TAK242 post-treatment until harvest at 28 days. Representative images. Bar = 25 μm. **(B)** Dermal thickness (means ± s.d. of five determinations/hpf from five mice/group). **(C)** Skin hydroxyproline content. One-way analysis of variance followed by Sidak's multiple comparison test.

Isolation and Analysis of RNA

At the end of the experiments, total RNA was isolated and reverse-transcribed to cDNA using Supermix (cDNA Synthesis Supermix; Quanta Biosciences, Gaithersburg, MD) as described (6). Amplification products (50 ng) were amplified using SYBR Green PCR Master Mix (Applied Biosytems, Foster City, CA) on an Applied Biosystems 7500 Prism Sequence Detection System. Data were normalized to GAPDH RNA, and -fold change in samples was calculated (5).

Transient Transfection Assays

The reporter constructs 772COL1A2-luc, harboring the 2772/58-bp fragment of the human proα1(I) collagen was used in transient transfection assays. Subconfluent cultures of wt and

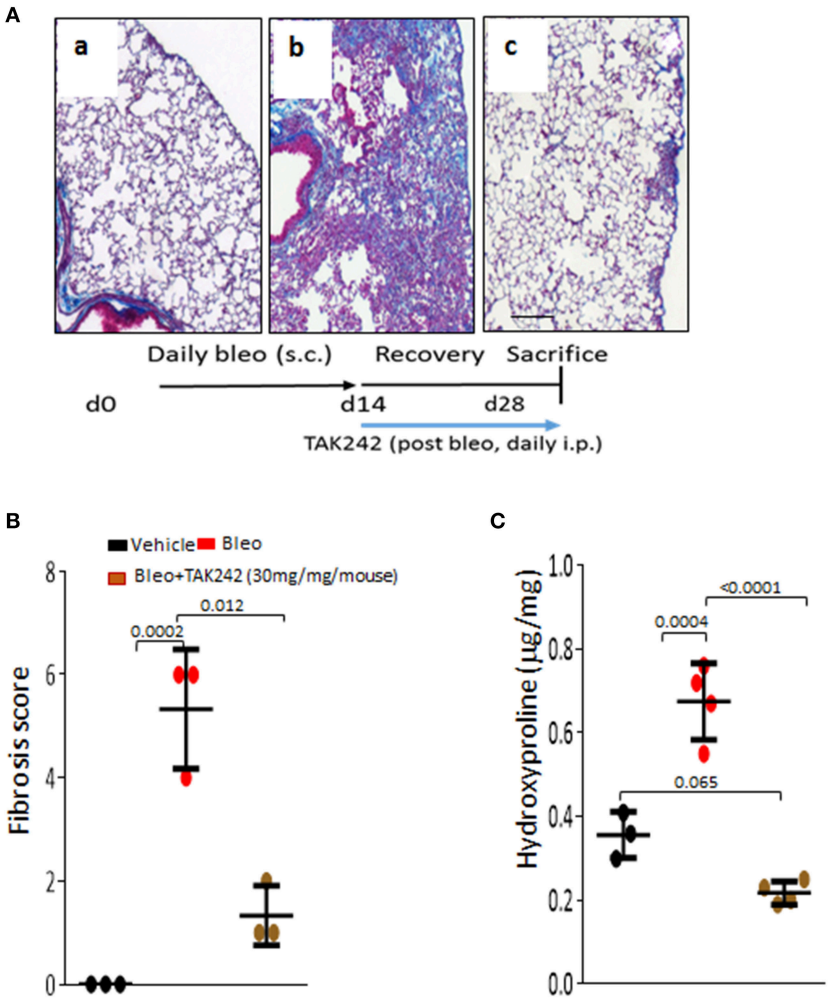

FIGURE 3 | Inhibition of TLR4 signaling by TAK242 induces regression of established lung fibrosis. C57/BL6 mice received daily s.c. injections of PBS or bleomycin alone, or TAK242 (10 or 30 mg/kg) started after 2 weeks of bleomycin injections or vehicle. Mice were sacrificed at day 28 and lung was harvested. **(A)** Masson's Trichome stain. (a) vehicle, (b) bleomycin for 2 weeks (5 days a week), (c) bleomycin followed by high dose (30 mg/kg) of TAK242 post-treatment until harvest at 28 days. Representative images. Bar = 25 μm. **(B)** Fibrosis score (Hubner) (13) determined in lungs from 5 hpf per mice. Results are means±s.d. from three mice per group. One-way analysis of variance followed by Sidak's multiple comparison test. **(C)** Lung hydroxyproline content. One-way analysis of variance followed by Sidak's multiple comparison test.

TLR4 mutant mouse skin fibroblasts in serum-free media were transfected using Superfect reagent (Qiagen, Valencia, CA). After 72 h of incubation with Fn^{EDA} (10 ug/mL) in absence or presence of TAK242 (3 uM), cultures were harvested and whole cell lysates were assayed for their luciferase activities. In each experiment, fibroblasts were cotransfected with Renilla luciferase pRL-TK plasmids (Promega, Madison, WI) as a control for transfection efficiency.

Immunofluorescence Confocal Microscopy

To assess TAK242 modulation of fibroblast responses in SSc or normal skin fibroblasts were treated with TAK242 for 24 h. Cells were then fixed, permeabilized, and incubated with antibodies to type I collagen (Southern Biotech) and α-smooth muscle actin (αSMA, 1:500) (Sigma, St. Louis, MO) at 1:500 dilution, followed by Alexa-fluor-labeled secondary antibodies (Invitrogen). Nuclei were identified using 4,6-diamidino-2-phenylindone (DAPI). Subcellular distribution of immunofluorescence was evaluated under an immunofluorescence microscope or Zeiss UV Meta 510 confocal microscope (Carl Zeiss Inc, Jena, Germany) and quantitated using ImageJ (National Institutes of Health) (6). For tissue immunofluorescence, paraffin-embedded sections were incubated with primary rabbit antibodies against α-SMA (Sigma, 1:500), followed by incubation with Alexa Fluor–conjugated IgG secondary antibodies (Invitrogen). Mean fluorescence intensities were quantified by using ImageJ (means from four randomly selected hpf/subject). Sections were imaged using Zeiss UV Meta 510 confocal microscope (Carl Zeiss Inc, Jena, Germany) and quantitated using ImageJ (National Institutes of Health) (5).

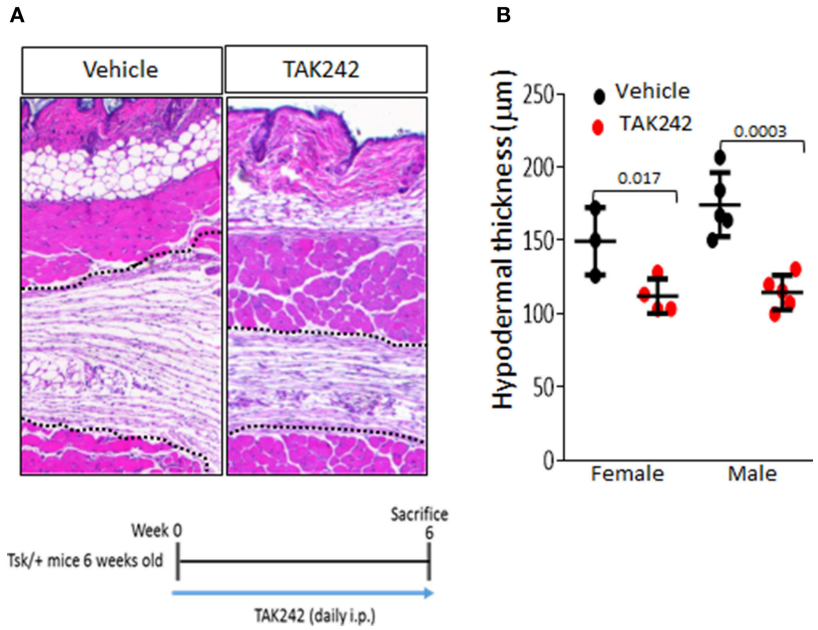

FIGURE 4 | Inhibition of TLR4 signaling by TAK242 exerts antifibrotic effects in Tsk1/+ mice. Six week-old Tsk1/+ mice received TAK242 (i.p.; 30 mg/kg) daily, sacrificed at 12 weeks of age, and dorsal skin was harvested for analysis. **(A)** H and E stain (dotted line indicate hypodermis). **(B)** Hypodermal thickness (means ± s.d. of five determinations/hpf from four or five mice/group). Bar = 200 μm. Student's t-test.

Statistical Analysis

Data are presented as means ± S.D. Two-tailed Student's *t*-test or Mann Whitney test were used for comparisons between two groups. Differences among groups were examined for statistical significance using analysis of variance (ANOVA) followed by Sidak's correction. A *p*-value less than 0.05 denoted the presence of statistically significant difference. Data were analyzed using Graph Pad prism (Graph Pad Software version 5, Graph Pad Software Inc., CA).

RESULTS

In light of the profibrotic activities associated with TLR4 (6, 7), we sought to evaluate the anti-fibrotic potential of TAK242 using mouse models of skin fibrosis. To induce fibrosis, we injected C57BL/6J mice with bleomycin by daily s.c. injections for 2 weeks (5 days/week). Mice were given concurrent TAK242 (10 or 30 mg/kg) or vehicle by daily i.p. injections (5 days/week) in parallel. Mice were sacrificed at day 22, and lesional skin was harvested and analyzed. Injection of TAK242 for up to 2 weeks was well tolerated by mice, and no significant weight loss or other signs of toxicity. The thickness of the dermis was markedly increased in mice injected with bleomycin compared to PBS (**Figures 1A,B**). Both excessive collagen deposition (left panel) and increased dermal thickness (right panel) were markedly ameliorated when TAK242 was administered concomitantly with bleomycin. Moreover, the dramatic loss of the intradermal adipose layer accompanying dermal fibrosis was substantially attenuated in mice treated with TAK242. Furthermore, up-regulated expression of multiple fibrotic marker genes (*Col1a1*,*Col1a2*, *asma,* and *Il6)* in lesional skin was attenuated in TAK242-treated mice (**Figure 1C**). Moreover, α-SMA expression in the lesional dermis notably attenuated in in TAK242-treated mice (**Figure 1D**).

Next, to determine if TLR4 inhibition might promote fibrosis regression, treatment was initiated when dermal fibrosis is already established by bleomycin treatment. The results showed that TAK242 treatment reduced the increased dermal thickness ($p < 0.01$) and hydroxyproline content, even when treatment was initiated after fibrosis has developed (**Figure 2**). Subsequent experiments sought to explore the role of TLR4 in lung fibrosis, a major fibrotic complication of SSc. Chonic s.c. injection of bleomycin elicited prominent lung changes, with an influx of inflammatory cells, and emergence of fibrotic foci those were primarily subpleural, along with sparse perivascular and interstitial fibrosis. Lung fibrosis was associated with substantial collagen accumulation and increase in pathological fibrosis score (**Figures 3A,B**). Trichome stain and hydroxyproline assays confirmed marked pulmonary accumulation of collagen (**Figure 3C**). Each of these parameters of bleomycin-induced pulmonary fibrosis showed substantial attenuation in TAK242-treated mice.

In order to evaluate the effect of TLR4 inhibition in an inflammation-independent model of fibrosis, we used Tsk1/+ mice that spontaneously develop hypodermal fibrosis in the absence of inflammation (5). We therefore treated male and female TSK1/+ mice with TAK242 or vehicle in parallel. Treatment was initiated at 6 weeks of age, when skin fibrosis is already detectable, and continued for 6 weeks. When

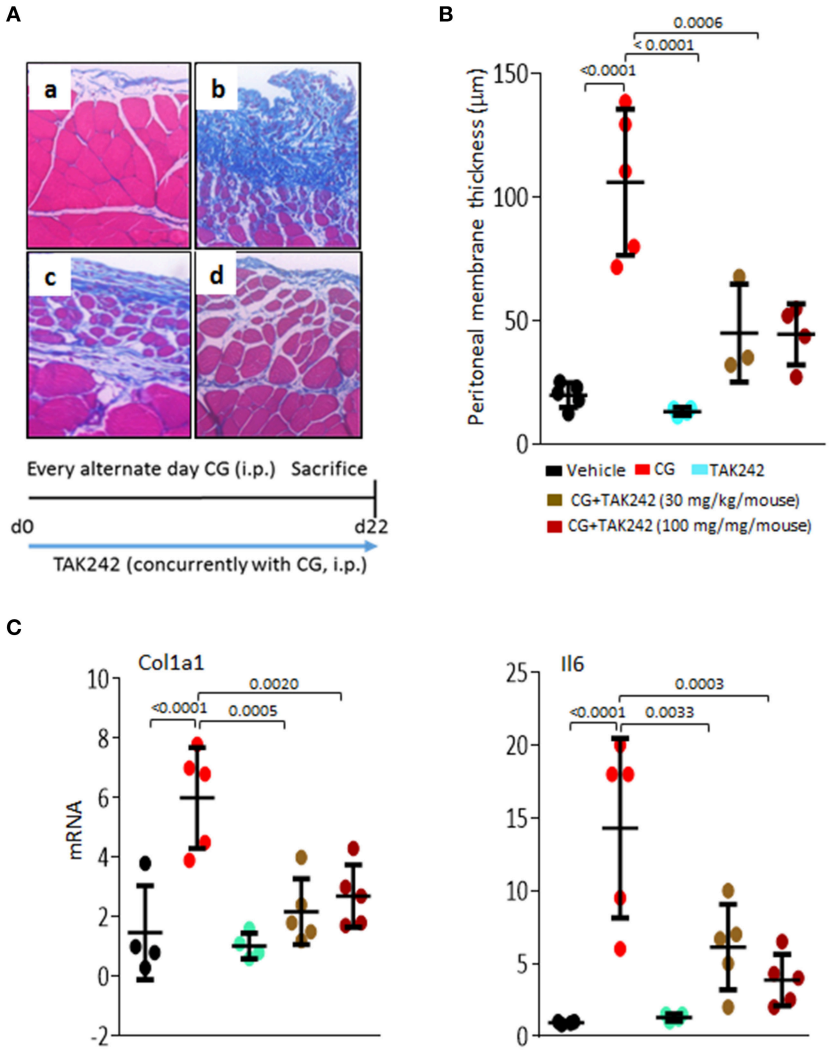

FIGURE 5 | Inhibition of TLR4 signaling by TAK242 ameliorates peritoneal fibrosis. C57/BL6 mice received i.p. injections of vehicle or chlorhexidine gluconate (CG) alone (i.p.; every other day), or together with TAK242 (daily PO, BID; 10 or 30 mg/kg) started concurrently with CG injections. Mice were sacrificed at day 22, and parietal peritoneal membranes were harvested for analysis. **(A)** Left panel, Masson's trichome stain. (a) vehicle, (b) CG treatment, (c) CG plus concurrent treatment of TAK242 (low dose, 30 mg/kg; PO, BID), or (d) TAK242 (high dose, 100 mg/kg; PO, BID). Representative images. Bar = 25 µm. **(B)** Submesothelial compact zone thickness. Results are means ± s.d. of five determinations/hpf from five mice/group. One-way analysis of variance followed by Sidak's multiple comparison test. **(C)** Real-time qPCR. Results, normalized with GAPDH, represent the means ± s.d. of triplicate determinations from at least four mice/group; One-way analysis of variance followed by Sidak's multiple comparison test.

sacrificed at 12 weeks of age, TSK1/+ mice displayed a substantial increase in hypodermal thickness (5). In contrast, both male and female TSK1/+ mice that received TAK242 for 6 weeks showed significantly attenuated hypodermal thickening compared to vehicle-treated TSK1/+ mice (**Figure 4**). Chonic i.p. administration of TAK242 was not associated with adverse effects in either mouse strain.

TAK242 Protects Mice From Peritoneal Fibrosis

Long-term peritoneal dialysis is frequently complicated by peritoneal fibrosis, a process that can be phenocopied in mice injected with CG (14). To explore the impact of TLR4 inhibition in peritoneal fibrosis, male C57BL/6 mice were administered CG on alternate days, and treatment with daily TAK242 (PO) was initiated concurrently with CG. Mice were sacrificed at day 22 and the parietal peritoneal membranes were harvested for analysis. The results showed a nearly 5-fold increase in thickness ($p < 0.0001$) and marked accumulation of collagen, in CG-treated mice (**Figure 5A**). Treatment with TAK242 was highly effective in preventing fibrosis. We found >60% reduction in membrane thickness, and attenuated expression of fibrotic and inflammatory genes (*Col1a1*and *Il6*) within the fibrotic peritoneal lining (**Figures 5B,C**).

TAK242 Treatment Mitigates the TLR4-Specific Profibrotic Fibroblast Phenotype

In preliminary experiments with normal fibroblasts, TAK242 showed no cytotoxicity in concentrations up to 10 μM (**Supplementary Figure 1A**). To examine the TLR4 specific role of TAK242, confluent cultures of fibroblast cultures were incubated with Fn^{EDA} for 72 h in presence or absence of TAK242 to block TLR4-mediated signaling. In response to TAK242, fibroblasts showed reduced collagen and αSMA levels as shown by real-time qPCR and immunofluorescence (**Figures 6A,B**). Importantly, TLR4 mutant mouse (nonfunctional TLR4) skin fibroblasts showed no inhibition of Fn^{EDA}-induced COL1A1-luc promoter activity with TAK242 treatment compared to wt control (**Supplementary Figure 1B**).

TAK242 Treatment Mitigates the Activated SSc Fibroblast Phenotype

To examine the cell-autonomous role of TLR4 in the persistently activated SSc fibroblast phenotype, we established confluent cultures of fibroblast monolayers from four different SSc donors (**Supplementary Table 1**). Cultures were incubated with TAK242 for 24 h to block TLR4-mediated signaling. In response to TAK242, each SSc cell line showed reduced collagen gene expression (mean = 50%, $p < 0.05$) and αSMA levels (**Figures 7A,B**). In contrast, healthy donor showed no significant inhibition of collagen and αSMA levels (**Supplementary Figure 2**).

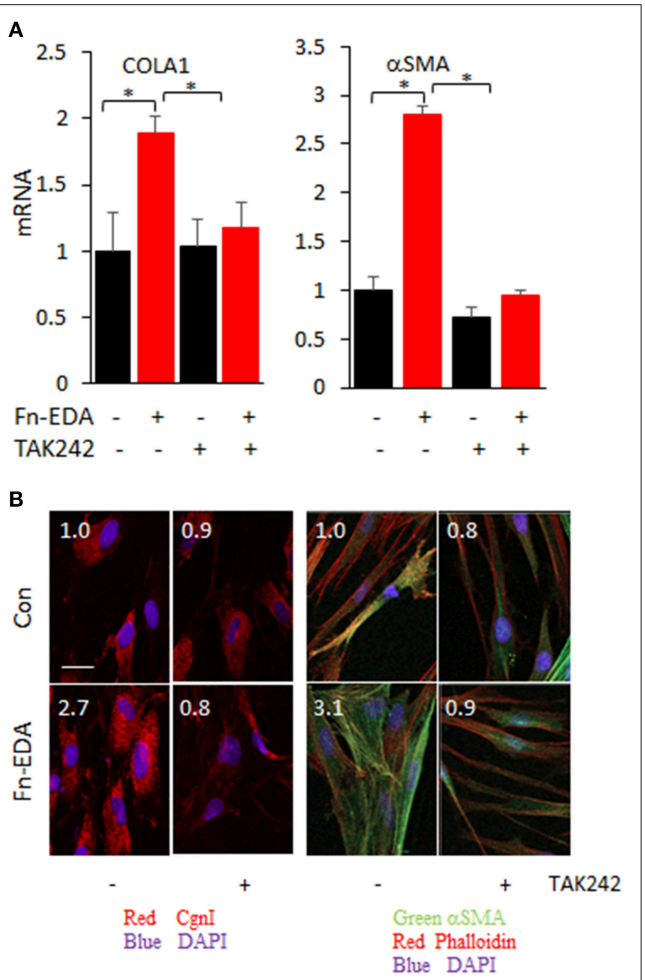

FIGURE 6 | Inhibition of TLR4 signaling by TAK242 abrogated activated profibrotic fibroblast phenotype. Normal skin fibroblasts were incubated in medium with Fn^{EDA} (10 μg/ml) for 72 h in absence or presence of TAK242 (3 uM). **(A)** mRNA levels were determined by real-time qPCR. Results, normalized with GAPDH, are the means ± SD of triplicate determinations from two independent experiments ($P < 0.01$, one-way ANOVA followed by Sidak's multiple comparison test). **(B)** Immunofluorescence using antibodies to type I collagen (red), αSMA (green), and Phalloidin (red). Representative images. Scale bar, 50 μm. Relative fluorescence intensities (inset, means from four randomly selected field/well). *$p < 0.05$.

DISCUSSION

In contrast to more common organ-based fibrotic diseases, fibrosis in SSc synchronously affects the skin and internal organs such as lungs and heart (15, 16). Currently, there are limited therapeutic options for patients with SSc (17, 18). Immunomodulatory treatments that are highly effective in rheumatoid arthitis and other inflammatory diseases generally show only modest and variable efficacy in SSc, and at best slow disease progression (1). As dysregulated TLR4 signaling underlies the pathology of diverse chonic conditions, substantial effort has been devoted to the design and development of selective TLR4 inhibitors. TAK-242 was developed as a small-molecule selective inhibitor of TLR4 signaling that suppresses the increase in serum cytokine levels TNF-a, IL-1, and IL-6 in murine and porcine models of sepsis, as well as in LPS challenged healthy volunteers (9–11).

Disease heterogeneity is a hallmark of SSc and contributes to the lack of effective therapies to date (1). Our previous studies implicated both TLR4 and its certain endogenous "damage-associated" ligands in the pathogenesis of fibrosis in SSc (5–7). Using an experimentally-derived "fibroblast TLR4-responsive gene signature" to interrogate SSc skin biopsies, we found that biopsies displaying a strong TLR4 gene signature largely mapped to the previously defined inflammatory intrinsic gene subset of patients (7, 8, 19). Fibroblast TLR4 signatures might therefore represent biomarkers of on-going TLR4 activity; and might have potential utilities to identify SSc patients potentially responsive to TLR4 inhibition. Drug repurposing using TAK242 thus appears to be an attractive anti-fibrotic strategy for treating fibrosis in patients in SSc subset demonstrating inflammatory intrinsic gene signatures. We therefore sought to investigate the anti-fibrotic effect of TAK242 in preclinical models of organ fibrosis. Chonic subcutaneous bleomycin injection induces a local inflammatory response in mice that is followed by fibrosis in the skin and lungs. Treatment with TAK242 exerted potent antifibrotic effects in both bleomycin-induced skin fibrosis that recapitulate the inflammatory stage of SSc in fibrotic skin (20) and in the Tsk1/+ mice that resembles non-inflammatory SSc (5),

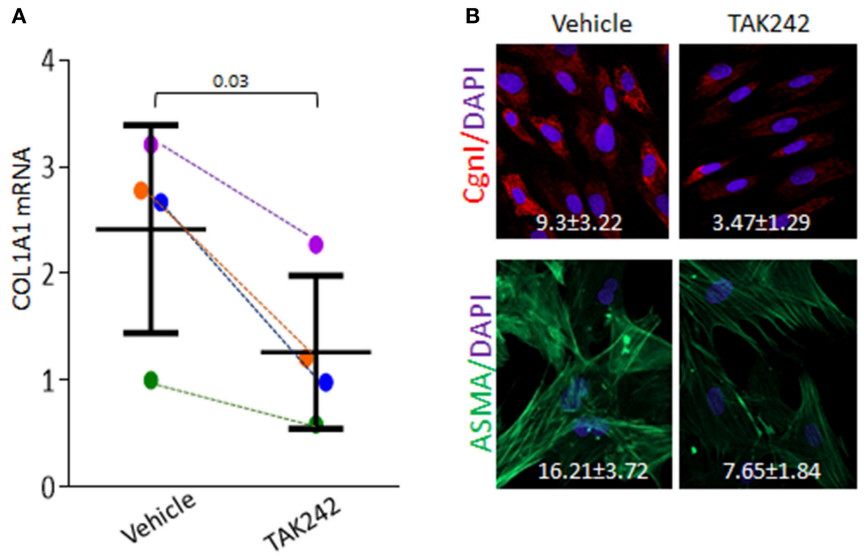

FIGURE 7 | TAK242 attenuates the activated SSc fibroblast phenotype. Confluent cultures of early-passage SSc skin fibroblasts were incubated in media with TAK242 (10 μM) or vehicle for 24 h. **(A)** Real-time qPCR. Results, normalized with GAPDH, are means ± s.d. compared to vehicle-treated controls. **(B)** Immunofluorescence using antibodies to type I collagen (red) and αSMA (green). Representative images. Bar = 50 μm. Inset, quantification of immunofluorescence. Quantification of fluorescence intensities using ImageJ (means from four randomly selected hpf from four SSc fibroblasts). Paired *t*-test. Each dot and color represent an individual.

and attenuated the constitutively-activated phenotype of SSc fibroblasts in culture. Moreover, the antifibrotic effects of TAK242 were not restricted to preventive application, but also when treatment was initiated after fibrosis had already been established. Most importantly, TAK242 exhibited marked efficacy in bleomycin-induced lung fibrosis, accounting for ∼30% of all SSc deaths. Anti-fibrotic effects of TAK242 have been reported in experimental models of hepatic, kidney and myocardial fibrosis in preclinical treatment regimen (21–23). Our presented data demonstrate efficacy of TAK242 in complimentary inflammation-dependent and -independent preclinical models of organ fibrosis in preventive as well as in therapeutic settings.

Together, our results indicate that a novel small molecule selectively targeting TLR4 signaling prevented as well as reversed organ fibrosis in a variety of preclinical disease models, and abrogated fibrotic responses in SSc fibroblasts *in vitro*. Transcriptome-based selection of SSc patients demonstrating on-going TLR4 activity, therefore, might provide entirely new opportunities for safe and effective targeted therapy of SSc and other chonic fibrosing conditions.

ETHICS STATEMENT

Animal experiments were performed according to institutionally approved protocols and in compliance with guidelines of the Northwestern University Animal Care and Use Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the IRB.

AUTHOR CONTRIBUTIONS

SB and JV conceived the project, designed experiments and interpreted. SB and JV wrote the manuscript. WW, ZT, and BS performed the major experiments, data acquisition and analysis. YT and MY contributed to reagents materials analysis tools and critical reading of the manuscript. AY performed lung histological and pathological analyses and interpretation of the results. SB and ZT performed statistical analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02434/full#supplementary-material

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Prevalence and Clinical Associations of Antiphospholipid Antibodies in Systemic Sclerosis: New Data From a French Cross-Sectional Study, Systematic Review, and Meta-Analysis

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Objectives: Antiphospholipid antibodies (aPL) can be present in the sera of systemic sclerosis (SSc) patients. This study aimed to determine the prevalence of aPL in a cross-sectional study of SSc patients, to assess their clinical associations, to perform a systematic review of published reports and a meta-analysis to estimate the worldwide prevalence of aPL in SSc.

Methods: Two-hundred and forty-nine SSc patients were consecutively tested once for lupus anticoagulant (LA), anticardiolipin (aCL), and anti-β2glycoprotein I (anti-β2GpI) antibodies. Clinical associations with aPL positivity were studied using a logistic regression model. A systematic review of the literature was carried out in PubMed and Embase. Meta-analysis was performed using number of aPL positive (at least one of the three antibodies positive) and negative patients. Meta-regression was used to study potential factors explaining the heterogeneity between studies.

Results: In our cross-sectional study, aPL positivity was found in 16 patients (prevalence 6.4%; 95%CI [3.8–10.4]). In multivariate analysis, there was a significant association between aPL positivity and venous thrombosis (VT) (OR 6.25 [1.18–33.00]; $p = 0.028$) and miscarriage (OR 5.43; 95%CI [1.31–22.13]; $p = 0.017$). Twenty-four studies were included in the meta-analysis, representing a total population of 3036 SSc patients. The overall pooled prevalence of aPL in SSc was 14% (9–20) with a high degree of heterogeneity among studies.

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Conclusion: This study found a prevalence of aPL positivity in our SSc population of 6.4% (3.8–10.4) and an overall worldwide pooled prevalence of 14% (9–20). In our SSc population, aPL positivity was associated with VT and miscarriage. These data provide additional insights into the role of aPL in the vasculopathy observed in SSc.

Keywords: systemic sclerosis, antiphospholipid antibodies, pulmonary hypertension, venous thrombosis, miscarriage

INTRODUCTION

Systemic sclerosis (SSc) is a severe and chronic connective tissue disorder with skin and internal organ involvement. Immune activation, vasculopathy, and excessive synthesis of extracellular matrix with collagen deposition are known to play a role in the pathophysiology of this disease (1). In SSc, vasculopathy can manifest by Raynaud's phenomenon, digital ulcers (DU), pulmonary arterial hypertension (PAH) as well as venous thrombosis (VT) (1, 2). Many autoantibodies can be detected in patients' sera. The most common are antinuclear auto-antibodies as anti-centromere (ACA), anti-topoisomerase I (anti-topo I), and anti-RNA polymerase III (anti-RNA pol III) antibodies (3). There are some evidences that certain SSc specific autoantibodies, but also newly discovered endothelium-related antibodies, are associated to vasculopathy (4). For example, an association between levels of antibodies and vascular manifestations has been described for antibodies against angiotensin II type 1 receptor and endothelin-1 type A receptor (5). Among antibodies with a possible association with vasculopathy in SSc, antiphospholipid antibodies (aPL) are a heterogeneous group.

The aPL, namely lupus anticoagulant (LA), anticardiolipin antibody (aCL), or anti-β2 glycoprotein-I antibody (anti-β2GpI) are usually found in the primary antiphospholipid syndrome (APS), but can be associated with other connective tissue diseases (mainly systemic lupus erythematosus), infections, drugs, and malignancies. In connective tissue diseases, the significance of aPL in patients who have never suffered from a thrombotic event remains unclear, but could reflect the endothelial activation (6).

In the literature, there are important variations (from 0 to 57%) in the prevalence of aPL in SSc. Moreover, associations of these antibodies with thrombotic events, miscarriage, or SSc clinical manifestations are still debated (7). Some studies reported an association between aPL positivity in SSc and PAH (8–10), digital ulceration (DU) (10, 11), interstitial lung disease (ILD) (10), while others did not (12, 13). Most of these studies have tested a relatively small number of patients, which could be responsible for a lower statistical power. These heterogeneous results preclude any firm conclusion on a link between aPL positivity and clinical manifestation in SSc.

The aims of this study were: (i) to determine the prevalence of aPL in a new cross-sectional study of well-phenotyped SSc patients (ii) to assess their clinical associations with a focus on vasculopathy (iii) to perform a systematic review and a meta-analysis of published reports to estimate the worldwide prevalence of aPL in SSc and to assess the factors associated with the observed heterogeneity.

PATIENTS AND METHODS

Patients Included in This Study

Population

Two hundred and forty-nine unselected patients with SSc were consecutively included and studied in the Internal Medicine Department of University Hospital of Lille, France, between October 2014 and January 2016. Patients fulfilled the following criteria for inclusion: age>18 years, and a diagnosis of SSc according to ACR/EULAR criteria (14). Disease subtype was classified based on LeRoy and Medsger criteria: diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc) (15).

Data Collection

All variables were entered into a standardized questionnaire fulfilled by the clinician at the time of the inclusion. In all patients, at the time of inclusion, the complete medical history of the patients was retrospectively reviewed. Physical examination variables, laboratory and imaging exams were prospectively collected for all patients. Systemic hypertension was defined as blood pressure ≥140/90 mmHg after 10 min of rest. Interstitial lung disease (ILD) was defined as subpleural pure ground-glass opacities and/or interstitial reticular pattern with or without traction bronchiectasis and/or honeycomb cysts, on high resolution computed tomography (HRCT). PAH was diagnosed based on right-heart catheterization if mean pulmonary arterial pressure was ≥25 mmHg and pulmonary capillary wedge pressure ≤15 mmHg in a patient with either no ILD or ILD with forced vital capacity % predicted ≥70% and extent of ILD on HRCT ≤20% (16). Scleroderma renal crisis (SRC) was defined as the abrupt onset of severe hypertension and/or decline in renal function, with proteinuria without an alternate etiology. The 2001 European and Scleroderma Trials And Research (EUSTAR) disease activity score was calculated for each patient as described in (17).

Biological Parameters

All patients were tested for LA, aCL (IgG isotype) and anti-β2GpI (IgG isotype). LA was detected in plasma by a dilute Russell's viper venom time (Siemens), and partial thromboplastin time test (HemosIL Silica Clotting Time Werfen) as screening and confirmation tests with calculating a normalized ratio. aCL and anti-β2GpI were measured using commercial ELISA assays (Orgentec, Trappes, France), positive titer was defined as ≥10 UGPL/mL (aCL) or ≥10 UA /mL (anti-β2GpI). Identification of antinuclear antibody specificities using both specific immunofluorescence patterns on HEp-2 cells and the Luminex approach (Bio-Plex 2200; Bio-Rad) for anti–topo I,

ACA, anti–U1 RNP, anti-SSA/Ro, and anti-SSB/La antibodies was performed as part of routine clinical care. Anti–RNA pol III antibodies were identified by immunodot (Euroline Systemic Sclerosis [Nucleoli] Profile [IgG]; Euroimmun). Other laboratories tests performed at the inclusion were: creatinine, CRP, platelet count, uric acid, serum protein electrophoresis, immunoglobulin G, M, and A plasma levels, LDL-cholesterol, triglycerides and glycated hemoglobin. Diabetes mellitus was defined as a glycated hemoglobin $\geq$6.5% and/or anti-diabetic medication intake. Dyslipidemia was defined as a LDL-cholesterol $\geq$1.6 g/L and/or triglycerides $\geq$1.5 g/L and/or lipid-lowering medication intake.

Ethics Approval and Consent to Participate

This study was authorized by the French Competent Authority dealing with Research on Human Biological Samples namely the French Ministry of Research. The Authorization number is DC 2008 642. To issue such authorization, the Ministry of Research has sought the advice of an independent ethics committee, namely the "Comité de Protection des Personnes," which voted positively. French legislation on non-interventional studies requires collecting the non-opposition of patients but does not require written consent. As such, non-opposition was obtained from each patient included in the study for the use of their de-identified medical record data.

Systematic Review and Meta-Analysis

The statement on Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) was used as a guide to conduct the review and analysis (18).

Search Strategy

Two of the authors (VS and AL) performed a search of published studies between May 1975 and November 2015, in PubMed and Embase databases. We used combinations of the terms "systemic sclerosis," "scleroderma," "antibodies, antiphospholipid," "antibody syndrome, antiphospholipid," "lupus anticoagulant," "antibodies, anticardiolipin," "antibodies, anti-β2GP1," "thrombosis," "pulmonary embolism," "digital ulceration," "pulmonary hypertension," "deep vein thrombosis," "cavernous sinus thrombosis," "stroke," "myocardial infarction," "acute limb ischemia," "pregnancy," "miscarriage." We adapted the search strategy to meet the specificities of each database. The reference lists of the retrieved reports were searched to identify additional relevant publications.

Study Selection

Inclusion criteria were: French or English-language publication, patients >18 years old, and diagnosed as having SSc, and at least 30 patients with SSc were tested in each study for LA, or/and aCL or/and anti-β2GpI. Reports that failed to provide sufficient information for the data analysis were excluded. Two of the authors (VS and AL) independently screened the titles and abstracts of the articles that were retrieved and applied the selection criteria to identify relevant material to be read in full. The reviewers' selections were compared and, in cases of disagreement, a third author (DL) was involved and decisions were made by consensus. The reviewers independently read the complete articles and applied the selection criteria to determine whether the studies would be included in the meta-analysis. The selections were again compared, and in cases of disagreement, a third author (DL) was again involved and decisions were made by consensus. Since the studies that were initially selected included some overlapping cohorts for a given center assessed during the same period, we chose to include 1 study per center (whichever study included the highest number of patients). Studies of 2 or more cohorts were included if extraction of data for each cohort was feasible. In this case, each cohort was analyzed as an independent cohort. Multicenter studies were excluded if participating centers had published single-cohort reports that were already included. Therefore, for each center, only 1 source of information was analyzed in order to avoid duplicate data (19).

Quality

Two authors (VS and AL) independently assessed the quality of the studies (risk of bias) using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool (16). In accordance with the QUADAS-2 user guidelines (16), items were modified for this study. In domain 1 (Patient selection), the item "Was a case–control design avoided?" was omitted. In domain 2 (Index test), the items "Were the index test results interpreted without knowledge of the results of the reference standard?" and "If a threshold was used, was it pre-specified?" were substituted with the item "Was the method of antibody determination described?" In domain 3 (Reference standard), the items "Is the reference standard likely to correctly classify the target condition?" and "Were the reference standard results interpreted without knowledge of the results of the index test?" were omitted. In domain 4 (Flow and timing), the item "Was there an appropriate interval between index test and reference standard?" was omitted, and the item "Did all patients receive the same reference standard?" was substituted with the item "Were all patients tested for aPL?" In accordance with the QUADAS-2 guidelines, articles were assessed for each item according to the following rating scale: high risk of bias, low risk of bias, or unclear (**Supplementary Table 8**).

Data Extraction

Following information were extracted from each selected study: continent, country, center, disease duration, disease subtype (percentage of diffuse form), age of patients, sex ratio, percentage of patients with ILD, DU, SRC, PAH, ACA, and anti-topo I, number of patients tested for aPL, number of patients positive for aPL and which type of aPL (LA, aCL, or anti-β2GpI) and isotype of aCL and anti-β2GpI (IgG and/or IgM). Thrombosis and miscarriage events were not collected because of missing data or high variability in definitions. Authors were contacted in case of missing data for the calculation of aPL prevalence.

Statistical Analysis

Characteristics of patients were described using mean and standard deviation (SD) for continuous variables and count and percentage for categorical variables. Characteristics of patients as a function of aPL status (aPL+/aPL–) were compared using

Student's test for continuous variables and Fisher's exact test for categorical variables. The associations between aPL status and complications (arterial or venous thrombosis, miscarriage, PAH, and DU) were studied using binomial logistic regressions. Adjustments were done (i) a priori for gender, age at aPL testing, SSc type (dc/lcSSc) and disease duration, and (ii) for the characteristics that differed significantly between aPL+ and aPL– patients ($p < 0.20$). Regression diagnostics were performed.

Similar analyzes were performed considering the titers of aCL and anti-β2GpI rather than the aPL status. Because these variables had a majority of zero values, they were categorized (visual analysis of their distribution as shown in **Figure 1** and **Supplementary Figure 1**), as follows: (i) 0, ≥1 and <5, ≥5 and ≤20 UGPL/mL for aCL, and (ii) 0, ≥1 and <5, ≥5 and <10, ≥10 and ≤100 UA/mL for anti-β2GpI.

As a sensitivity analysis, we then focused on patients who were tested twice for LA, aCL, and anti-β2GpI ("repeat testing"). Patients with "persistent aPL" were defined as having the same positive test for at least one aPL at two different times. We performed similar comparisons and associations studies than with the "single testing."

For the meta-analysis, we calculated weighted pooled summary estimates of aPL prevalence. For each meta-analysis, the DerSimonian-Laird method was used. Accordingly, studies were considered to be a random sample from a population of studies. Heterogeneity was quantified using a chi-square heterogeneity statistic and by means of an I^2 statistic for each analysis. A random-effects model was used to combine data. The overall effect was estimated using a weighted average of the individual effects, with weights inversely proportional to variance in observed effects. Freeman-Tukey transformation was used. Meta-regression was performed to assess the impact of continent, country, center, disease duration, disease subtype (percentage of diffuse form), age of patients, sex ratio, percentage of patients with ILD, DU, SRC, PAH, ACA, and anti-topo I, risk of bias using QUADAS-2 tool. For aCL and anti-β2GpI prevalence, the isotype of aCL and anti-β2GpI tested (IgG and/or IgM) were also included, respectively.

All statistical analyses were performed using R software, version 3.1.2 (20); the threshold for statistical significance was set to $p < 0.05$. For meta-analysis, R metafor package was used.

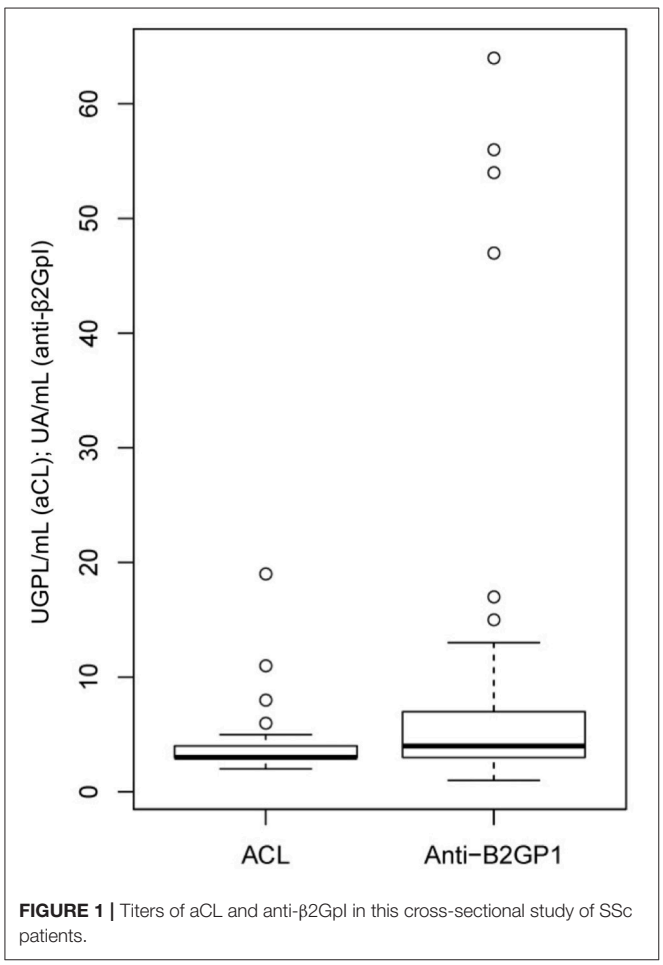

FIGURE 1 | Titers of aCL and anti-β2GpI in this cross-sectional study of SSc patients.

RESULTS

Lille Cross-Sectional Study

Patients and Disease Characteristics

The 249 patients included in our study were predominantly female (82%), with lcSSc (82%). Mean age of patients at the time of the study was 59.5 ± 13.3 years and mean disease duration was 10.7 ± 8.9 years (**Table 1**). The prevalence of ILD was 45%, DU 33% and PAH 6%. Forty-five (18%) patients had a history of arterial ($n = 22$) or venous ($n = 29$) thrombosis, and 40 (21%) patients had a history of miscarriage (characteristics of the individual thrombotic events and miscarriage are described in **Supplementary Table 1**).

Frequency of aPL

One or more aPL were found in 16 (6.4%, 95%CI [3.8–10.4]) patients. One patient was positive for both LA and anti-β2GpI, and one was positive for aCL and anti-β2GpI. The prevalence of positive LA was 1.6% (0.4–4.1). The prevalence of aCL was 1.2% (0.3–3.5) with a mean value of 14 UGPL/mL for positive patients. The prevalence of anti-β2GpI was 4.4% (2.3–8.0) with a mean value of 28 UA/mL for positive patients (**Figure 1**).

Among patients with aPL, LA was found in 25.0%, aCL in 18.8%, and anti-β2GpI in 68.8% (**Table 2**). Double positivity was seen in 12.5%. At the time of the study, 2 patients had been previously diagnosed with an APS based on clinical and biological criteria. Among the 29 patients with a history of VT, five had aPL positivity (LA and/or anti-β2GpI, but no aCL), 2 were diagnosed as having APS (they were both positive for LA, none of them were positive for anti-DNA antibodies or fulfilled systemic lupus erythematosus criteria).

Associations With Clinical Manifestations

The associations of aPL positivity with disease manifestations are presented in **Table 1** and **Supplementary Table 2**. Mean age of aPL positive patients was higher than aPL negative patient (65.9 ± 7.4 vs. 59.1 ± 13.5 yrs, $p = 0.047$). A higher BMI (29.2 ± 8.3 vs. 24.8 ± 5.4, $p = 0.003$) was found in aPL positive patients group,

TABLE 1 | Characteristics of the population included in the study, and comparison between aPL positive or negative patients (single testing).

	N (N aPL+)	Whole population ($n = 249$)	aPL+ ($n = 16$)	aPL– ($n = 233$)	*p*
Sex, *n* (%) female	249 (16)	205 (82)	14 (88)	191 (82)	0.745
Age, mean ± SD years	249 (16)	59.5 ± 13.3	65.9 ± 7.4	59.1 ± 13.5	**0.047**
Age at onset of disease, mean ± SD years	204 (11)	47.7 ± 13.7	48.2 ± 11.5	47.6 ± 13.9	0.897
Disease duration, mean ± SD years	204 (11)	10.7 ± 8.9	15.3 ± 10.9	10.5 ± 8.8	0.082
BMI mean ± SD	232 (16)	25.1 ± 5.7	29.2 ± 8.3	24.8 ± 5.4	**0.003**
Tobacco use, n (%)	248 (16)	99 (40)	2 (13)	97 (42)	**0.032**
Systemic hypertension, n (%)	249 (16)	125 (50)	11 (69)	114 (49)	0.195
Diabetes mellitus, n (%)	249 (16)	12 (5)	1 (6)	11 (5)	0.558
Dyslipidemia, n (%)	249 (16)	112 (45)	11 (69)	101 (43)	0.068
Disease subtype n (%)	249 (16)				
Limited		203 (82)	15 (94)	188 (81)	0.318
Diffuse		46 (18)	1 (6)	45 (19)	
mRSS, mean ± SD	247 (16)	5.1 ± 5.8	4.3 ± 4.5	5.1 ± 5.9	0.581
Pulmonary arterial hypertension, *n* (%)	233 (16)	15 (6)	1 (6)	14 (6)	1.000
Interstitial lung disease, *n* (%)	230 (16)	104 (45)	6 (38)	98 (46)	0.608
Digital ulceration, *n* (%)	236 (14)	79 (33)	2 (14)	77 (35)	0.150
Renal crisis, *n* (%)	231 (16)	1 (0)	0	1 (1)	1.000
2001 EUSTAR SSc activity score	234 (16)	1.2 ± 1.2	1.2 ± 1.1	1.2 ± 1.2	0.943
Arterial or venous thrombosis, *n* (%)	246 (16)	45 (18)	6 (38)	39 (17)	0.086
Arterial thrombosis, *n* (%)	247 (16)	22 (9)	3 (19)	19 (8)	0.160
Stroke/transient ischemic attack		11 (4)	2 (13)	9 (4)	0.154
Acute limb ischemia		3 (1)	0	3 (1)	1.000
Myocardial infarction		5 (2)	1 (6)	4 (2)	0.287
Venous thrombosis, n (%)	248 (16)	29 (12)	5 (31)	24 (10)	**0.027**
DVT		22 (9)	4 (25)	18 (8)	**0.041**
PE		9 (4)	2 (13)	7 (3)	0.108
Miscarriage, *n* (%)	187 (12)	40 (21)	5 (42)	35 (20)	0.136
ANA specificity, *n* (%)	238 (16)				
ACA		139 (58)	12 (75)	127 (57)	0.196
Anti–topo I		50 (21)	3 (19)	47 (21)	1.000
Anti-RNA pol III		7 (3)	0	7 (3)	1.000
Anti–U1RNP		9 (4)	0	9 (4)	1.000
Patients ANA negative, *n* (%)		2 (1)	0	2 (1)	1.000
CRP > 10 mg/L, *n* (%)	248 (15)	21 (8)	1 (7)	20 (9)	1.000
Hypergammaglobulinemia, *n* (%)	249 (16)	30 (12)	1 (6)	29 (12)	0.701
HbA1c > 6.5%, *n* (%)	247 (16)	6 (2)	0	6 (3)	1.000

N, number of patients (whole population) with available data; N aPL+, number of patients aPL+ with available data; ANA, antinuclear antibody; anti–topo I, anti–topoisomerase I antibody; ACA, anticentromere antibody; Anti-RNA pol III, anti-RNA polymerase III antibody; Anti-U1RNP, Anti-U1RNP antibody; CRP, C-reactive protein; DVT, Deep venous thrombosis; PE, pulmonary embolism; mRSS, mean Rodnan skin score. Values in bold are significant p < 0.05.

while tobacco use was less frequent (13% vs. 42%, $p = 0.032$). No difference was found regarding disease subtype, ILD, DU, autoantibodies status, CRP elevation, HbA1c > 6.5%, as well as hypergammaglobulinemia.

In univariate analysis, aPL positivity was associated with an increased risk of VT (OR = 3.91; 95%CI [0.98–13.53]; $p = 0.027$). No association was found between aPL positivity and arterial thrombosis, miscarriage, PAH, ILD, DU, and renal crisis. When adjusted on sex, age at aPL testing, disease duration at aPL testing, disease subtype, tobacco use, BMI, systemic hypertension, dyslipidemia and ACA positivity, aPL positivity was significantly associated with VT (OR = 6.25 [1.18–33.00]; $p = 0.028$) and miscarriage (OR = 5.43; [1.31–22.13]; $p = 0.017$) (**Table 3**).

We then focused on aPL titers and their clinical associations. Distribution of aCL and anti-β2GpI are shown in **Figure 1** and **Supplementary Figure 1**. In multivariate analysis (model adjusted on sex, age, disease subtype, tobacco use, follow-up, gammaglobulin level, and anti-U1RNP positivity), there was an association between aCL titers (≥5 UGPL/mL) and VT (OR 3.69; [0.98–12.9]; $p = 0.043$) as well as with PAH (OR 6.35; [0.99–41.1]; $p = 0.043$). Anti-β2GpI titer ≥10 UA/mL

TABLE 2 | Prevalence of aPL in this cross-sectional SSc study and frequencies of LA, aCL, and anti-β2GpI in SSc patients with aPL ($n = 249$, single testing).

	Prevalence of aPL in this study (% and 95% CI)	Frequencies of LA, aCL and anti-β2GpI in SSc patients with aPL (%)
≥ 1 aPL	6.4 (3.8–10.4)	–
LA	1.6 (0.4–4.1)	25.0
aCL	1.2 (0.3–3.5)	18.8
Anti-β2GpI	4.4 (2.3–8.0)	68.8

was associated with an increased risk of miscarriage (OR 5.19; [0.99–28.4]; $p = 0.049$) in multivariate analysis (model adjusted on sex, age, disease subtype, tobacco use, dyslipidemia, BMI, gammaglobulin level, ACA positivity, and anti-topo I positivity) (**Table 4**, **Supplementary Tables 3, 4**).

As a sensitivity analysis, we then focused on patients who were tested twice for LA, aCL, and anti-β2GpI (repeat testing, $n = 213$). The time interval between the two assessments of aPL were 13.2 ± 6.3 months. Seven patients were found with persistent aPL corresponding to a prevalence of 3.3% (1.5–6.9). The prevalence of patients with persistent LA, aCL, and anti-β2GpI were 0.9% (0.1–3.4), 0 and 2.4% (0.9–5.7), respectively. Patients with persistent aPL had higher BMI (33.3 ± 8.6 vs. 24.9 ± 5.4, $p < 0.001$), and higher rates of VT (57 vs. 11%, $p = 0.006$) and miscarriage (67 vs. 21%, $p = 0.023$) than patients with aPL negative or non-persistent aPL. In univariate and multivariate analysis, persistent aPL were associated with venous thrombosis (multivariate OR 7.93 [1.38–53.40]; $p = 0.022$) and miscarriage (multivariate OR 18.35 [2.83–163.34]; $p = 0.003$) (**Supplementary Tables 5–7**).

TABLE 3 | Univariate and multivariate comparisons of associations between aPL positivity in SSc patients and clinical manifestations.

	Univariate OR (95% CI)	p	Multivariate OR (95% CI) *	p
Arterial or venous thrombosis	2.92 (0.82–9.51)	0.086	**5.21 (1.18–23.20)**	**0.027**
Arterial thrombosis	2.56 (0.43–10.55)	0.160	2.50 (0.31–13.90)	0.323
Venous thrombosis	**3.91 (0.98–13.53)**	**0.027**	**6.25 (1.18–33.00)**	**0.028**
Miscarriage	2.84 (0.67–11.11)	0.136	**5.43 (1.31–22.13)**	**0.017**
Digital ulceration	0.32 (0.03–1.47)	0.150	0.48 (0.07–2.29)	0.400
Pulmonary arterial hypertension	0.97 (0.02–7.26)	1	0.72 (0.03–6.37)	0.790

**OR adjusted for sex, age at aPL testing, disease duration at aPL testing, disease subtype, tobacco use, BMI, systemic hypertension, dyslipidemia, ACA positivity. Values in bold are significant p < 0.05.*

Systematic Review and Meta-Analysis

Studies Included

One thousand and two hundred and ninety-one references were retrieved as result of search (575 articles in Pubmed and 716 in Embase). Seventy-nine articles were included for full text review after reading the titles and abstracts. Of these articles, 30 were assessed for eligibility. Six articles were further excluded (duplicate studies) (**Figure 2**). Finally, 24 studies (23 + our study) were included in the meta-analysis, representing a total population of 3,036 adult patients with SSc (8–10, 12, 13, 21–38) (**Supplementary Table 8**). One study (26) provided the prevalence of LA, aCL, and anti-β2GpI but did not provide the global prevalence of aPL. This study was therefore included in the meta-analysis excepted for the calculation of the global prevalence.

Prevalence of APL

The overall pooled prevalence of aPL was 14% (95%CI [9-20]), with a high degree of heterogeneity ($I^2 = 94\%$, $P < 0.0001$). The overall pooled prevalence of LA, aCL, and anti-β2GpI were 1% (0–3), 9% (6–13), and 9% (3–18), respectively. There was a high degree of heterogeneity ($I^2 = 81\%$, $P < 0.0001$ for LA, $I^2 = 87\%$, $P < 0.0001$, for aCL, and $I^2 = 95\%$, $P < 0.0001$ for anti-β2GpI). Pooled prevalence stratified by continent are shown in **Table 5** and **Figure 3**.

We assessed whether the characteristics of the studies included could explain the observed heterogeneity. **Table 6** summarizes the results of all meta-regression analyses. Meta-regression revealed a significant association between the sex ratio and the prevalence of aPL positivity ($p = 0.0265$). However, when sex ratio was entered as a variable in the model, residual heterogeneity between studies remained significant ($P < 0.0001$, $I^2 = 91.8\%$). Meta-regression did not find any other factors significantly associated with aPL prevalence: continent, disease subtype, disease duration, age, proportion of patients with ILD, DU, SRC, PAH, ACA, and anti-topo I. Meta-regression revealed a significant association between prevalence of LA and disease duration ($p < 0.0001$), between prevalence of aCL and proportion of patients with ACA ($p = 0.0055$), and between prevalence of anti-β2GpI and continent ($p = 0.0040$), age ($p = 0.0333$), sex ratio ($p = 0.0469$), respectively. Yet, excepted for disease duration and prevalence of LA (residual heterogeneity $p = 0.428$, $I^2 = 0\%$), residual heterogeneity was still significant ($P < 0.0001$) after inclusion of these factors in the models. The tested isotype of aCL and anti-β2GpI were not associated with the prevalence of their respective antibodies (**Table 6**).

Since quality bias is an important concern in meta-analyses, we assessed the quality of the studies using the QUADAS-2 tool (**Figure 4** and **Supplementary Table 8**). Meta-regression did not find any association between the risk of bias and the prevalence of aPL (**Table 6**). Moreover, the aforementioned analyses were performed in duplicate for the five studies for which both LA, aCL and anti-β2GpI were tested [(8, 10, 22, 26), in this study]. The prevalence of aPL, LA, aCL, and anti-β2GpI were 24% (7–47), 1% (0–4), 11% (4–19), and 10% (1–27), respectively. There was still a high degree of heterogeneity ($I^2 = 98$, 84, 93, and 97% for aPL, LA, aCL, and anti-β2GpI prevalence respectively, $P < 0.0001$).

TABLE 4 | Multivariate comparisons of associations between aCL and anti-β2GpI titers and clinical manifestations.

	aCL titers (UGPL/mL)[a]					Anti-β2GpI titers (UA/mL)[b]							
	[0,1[	[1,5[		[5,20]		[0,1[	[1,5[		[5,10[		[10,100]		
	Ref	Adjusted OR (CI)	*p*	Adjusted OR (CI)	*p*	Ref	Adjusted OR (CI)	*p*	Adjusted OR (CI)	*p*	Adjusted OR (CI)	*p*	
Arterial or venous thrombosis		1.12 (0.50–2.44)	0.780	2.66 (0.83–8.10)	0.088		0.55 (0.21–1.37)	0.211	0.62 (0.16–1.96)	0.443	3.27 (0.77–13.6	0.101	
Arterial thrombosis		0.84 (0.28–2.34)	0.739	1.52 (0.30–6.17)	0.576		0.54 (0.13–1.90)	0.356	0.58 (0.08–2.54)	0.511	3.76 (0.61–2.08)	0.131	
Venous thrombosis		1.71 (0.67–4.36)	0.257	**3.69 (0.98–12.9)**	**0.043**		0.79 (0.26–2.29)	0.669	0.55 (0.08–2.33)	0.466	3.44 (0.72–15.3)	0.106	
Miscarriage		1.43 (0.64–3.19)	0.381	0.62 (0.09–2.62)	0.557		1.04 (0.41–2.58)	0.933	0.56 (0.11–2.04)	0.413	**5.19 (0.99–28.4)**	**0.049**	
Digital ulceration		0.66 (0.33–1.28)	0.226	1.85 (0.64–5.39)	0.253		0.47 (0.21–1.02)	0.06	0.78 (0.31–1.85)*		0.573*		
PAH		3.65 (0.94–17.9)	0.074	**6.35 (0.99–41.1)**	**0.043**		0.98 (0.27–3.42)	0.977	0.25 (0.01–1.76)*		0.237*		

[a]*Adjusted on sex, age, disease subtype, tobacco use, follow-up, gammaglobulin level, anti-U1RNP positivity.*

[b]*Adjusted on sex, age, disease subtype, tobacco use, dysplidemia, BMI, gammaglobulin level, ACA positivity, anti-topo I positivity; Ref: class reference; * class: [5,100]. Values in bold are significant p < 0.05.*

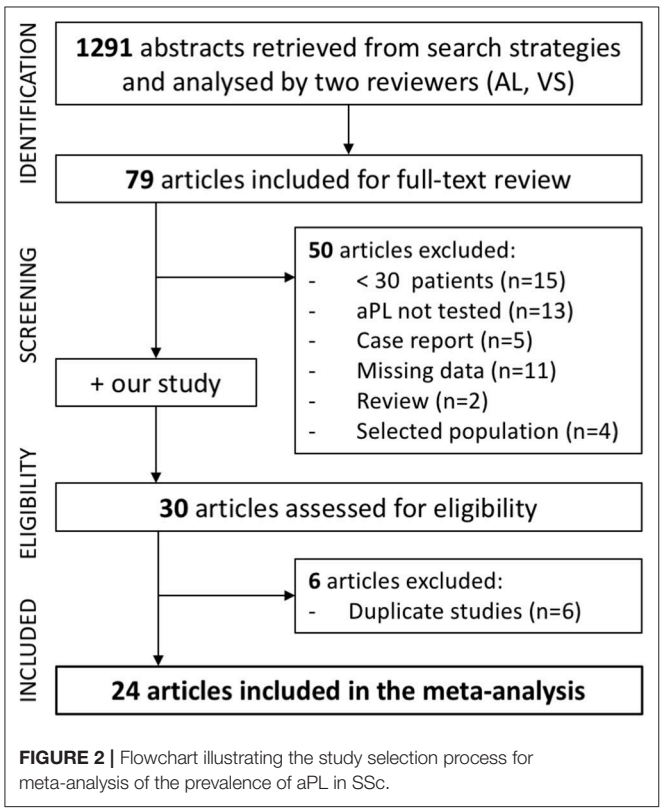

FIGURE 2 | Flowchart illustrating the study selection process for meta-analysis of the prevalence of aPL in SSc.

DISCUSSION

The main results of our study were as follows: (1) the prevalence of aPL in this population of SSc patients was 6.4% (1.6, 1.2, 4.4% for LA, aCL, and anti-β2GpI, respectively) and the overall pooled prevalence of aPL in SSc was 14% (9–20) by meta-analysis, (2) there was an association between VT and miscarriage and aPL positivity, (3) higher levels of aCL were associated with a higher risk of PAH and VT, and higher levels of anti-β2GpI with a higher risk of miscarriage.

The prevalence of aPL in SSc in our study was 6.4% (3.8–10.4), which is rather low compared to previous reports. Although the prevalence of aPL ranges from 0 to 57% in the literature (8, 10, 12, 13, 21–27, 29–40), most of the studies reported an overall prevalence higher than 10% and the overall pooled prevalence of aPL in SSc was 14% (9–20) as found by our meta-analysis. The important variation in the prevalence of aPL reported in literature was associated with a high heterogeneity among studies included in our meta-analysis. The first hypothesis to explain this heterogeneity was the influence of methodological differences: different positivity cutoff, different type of aPL tested and subgroups analysis. One of the most important factors which may induce variations of prevalence of aPL was the aPL isotypes tested. Indeed, some studies only analyzed IgG-aPL isotype, other analyzed IgG and IgM-aPL isotype, and even some analyzed IgG, IgM, and IgA isotypes of aPL. IgG is the most prevalent isotype among patients with thrombosis and fetal loss in APS, and the only one associated with these events (39). Our meta-regression analysis did not find any association between isotype and aCL and anti-β2GpI prevalence. Disease duration was associated with LA prevalence, but this should be interpreted with caution given the small number of studies included (5) and the low prevalence of LA (between 0 and 4% in these studies). The observed heterogeneity could also be explained by the geographic origin of the studies, because of potential genetic or environmental factors. Indeed, Touré et al. reported the highest prevalence in the review of literature (57%), with an African ethnicity cohort (22) while studies from North and South America found a prevalence range of 2.2–12.2% (13, 36–38). However, meta regression did not show an association between continent and aPL positivity ($p = 0.8529$), but there

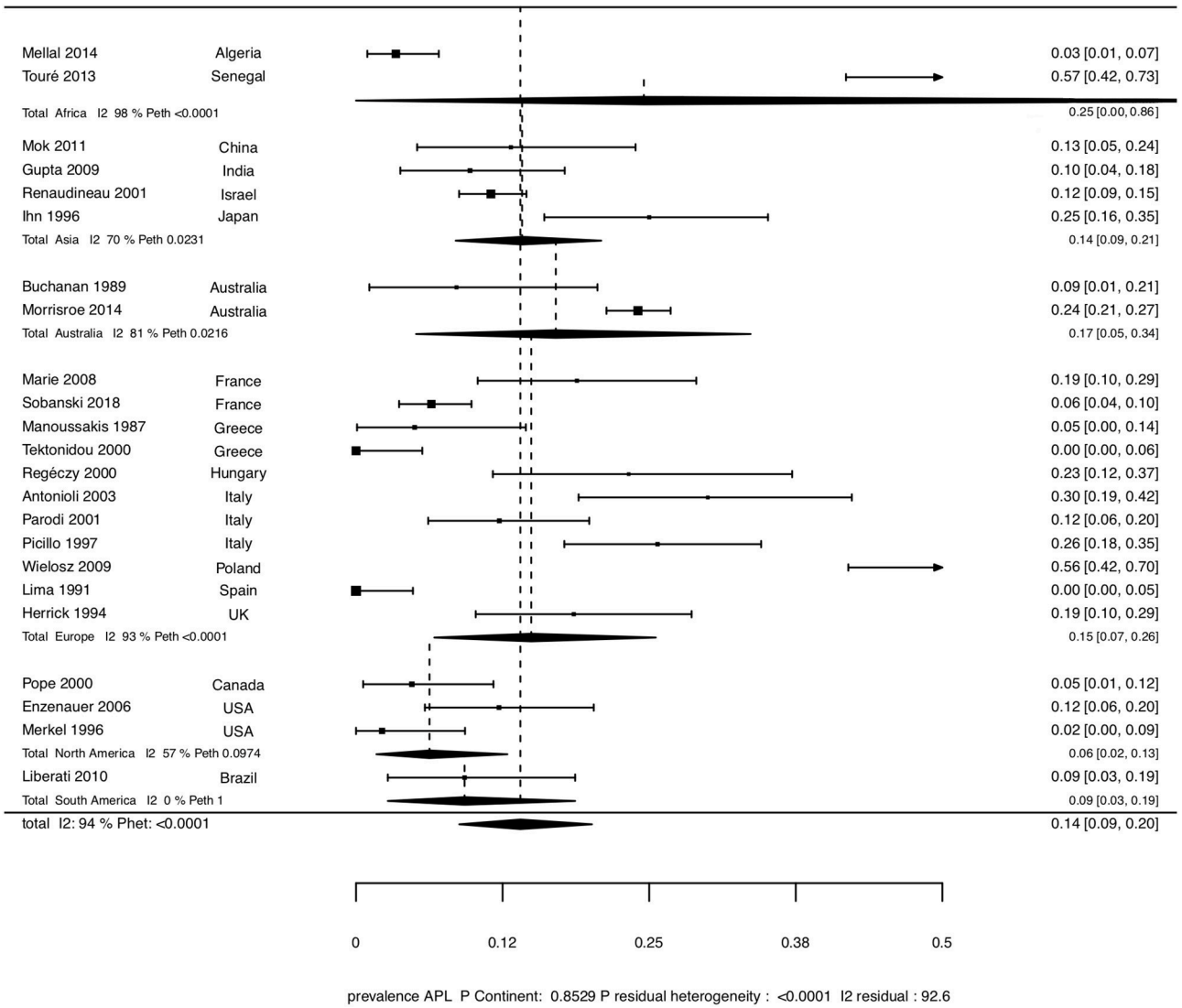

FIGURE 3 | Forest plot showing the pooled prevalence of aPL in the whole population of SSc patients, and stratified by continent.

was an association between continent and anti-β2GpI positivity ($P = 0.0040$). It has been reported that aPL positivity and APS were associated with various HLA alleles (41). Interestingly, gender was associated with aPL and anti-β2GpI prevalence. Age of patients was also associated with anti-β2GpI prevalence. Yet, these results explained only very partially the marked heterogeneity among studies.

We found an association between aPL positivity and VT in univariate and in multivariate analysis. This association is not common in studies of SSc patients and most of them did not find an increased risk of VT in case of aPL positivity (8, 10, 12, 13, 22). Yet, Antonioli et al. reported an association between thrombosis (arterial or venous) and aPL positivity (30). Interestingly, SSc has been recently reported to be associated with a higher risk of VT when compared to non-SSc individuals with a HR of 2.96 (1.54–5.69), particularly in the first year following the diagnosis of SSc (2). There are a number of possible mechanisms that could account for the increased risk of VTE seen in SSc patients, in particular vasculopathy and low-grade inflammation. Moreover, obesity is a common risk factor for VT. In our study, patients aPL positive had a higher BMI than those who were aPL negative. BMI, tobacco use and disease duration were included in our multivariate analysis.

Our study also showed an increased risk of miscarriage in case of aPL positivity, and this association was significant in multivariate analysis. One of our patients with miscarriage history was known to have an APS. Mean age at onset of the disease was 47.7 years, meaning that most patients had already finished childbearing at that time. In our study, SSc had been diagnosed more than 2 years after the last miscarriage in 25/40 women who had experienced miscarriage and with available

data. Interestingly, we also reported a significant association between higher titers of anti-β2GpI and risk of miscarriage. To our knowledge, there is no previously published data on these findings in SSc. This result might be linked with the pathogenic role of aPL in fetal loss observed in APS. Indeed, aPL (in particular β2GpI-dependant antibodies) bind to human trophoblasts and affect several cell function *in vitro* (42).

Using the manufacturer cut-off levels, we did not show any association between aPL positivity and other clinical manifestations, in particular with PAH and DU. This may be due to a lack of power (small number of events). Regarding the association between aPL positivity and PAH, results in literature are discrepant. Marie et al. reported an increased risk of PAH in case of one or more aPL positivity (8). Antonioli et al., Assous et al., and Morrisroe et al. reported an association between aCL positivity and PAH (10, 26, 30), while Boin et al. found this

TABLE 5 | Prevalence of aPL, LA, aCL, and anti-β2GpI in SSc, stratified by continent.

	aPL (%[95%CI]) *N* = 23	LA (%[95%CI]) *N* = 6	aCL (%[95%CI]) *N* = 21	anti-β2GpI (%[95%CI]) *N* = 9
Africa	25 [0–86]	5 [0–15]	9 [0–27]	50 [35–65]
	N = 2	*N* = 1	*N* = 2	*N* = 1
Asia	14 [9–21]	3 [0–8]	14 [6-24]	10 [4–18]
	N = 4	*N* = 1	*N* = 3	*N* = 1
Oceania	17 [5–34]	0 [0–0]	10 [8–12]	6 [5–8]
	N = 2	*N* = 1	*N* = 2	*N* = 1
Europe	15 [7–26]	1 [0–3]	9 [4–16]	5 [1–11]
	N = 11	*N* = 3	*N* = 10	*N* = 6
North America	6 [2–13]	NA	6 [2–13]	NA
	N = 3		*N* = 3	
South America	9 [3–19]	NA	9 [3–19]	NA
	N = 1		*N* = 1	

N, number of studies with available data; NA, not available.

TABLE 6 | Results of meta-regression analyses of the associations between aPL prevalence and characteristics of the studies included.

Variable	P value for association of variable with prevalence			
	aPL	LA	aCL	anti-β2GpI
Continent	0.8529	0.1964	0.9387	**0.0040**
Disease subtype	0.5226	0.9850	0.1734	0.7101
Disease duration	0.8790	**<0.0001**	0.7068	0.5799
Age	0.7507	0.1755	0.4863	**0.0333**
Gender	**0.0265**	0.1690	0.9489	**0.0469**
ILD	0.2986	0.4585	0.8599	0.2105
DU	0.5581	0.4516	0.3294	0.7851
SRC	0.0809	0.2796	0.6226	0.9635
PAH	0.4690	0.3098	0.2763	0.8342
ACA	0.1795	0.5451	**0.0055**	0.8613
Anti-topo I	0.3705	0.7974	0.2068	0.6560
Tested isotype of aCL	—	—	0.3580	—
Tested isotype of anti-β2GpI	—	—	—	0.2847
Risk of bias (QUADAS-2)	0.1510	0.9881	0.2766	0.8717

Values in bold are significant $p < 0.05$.

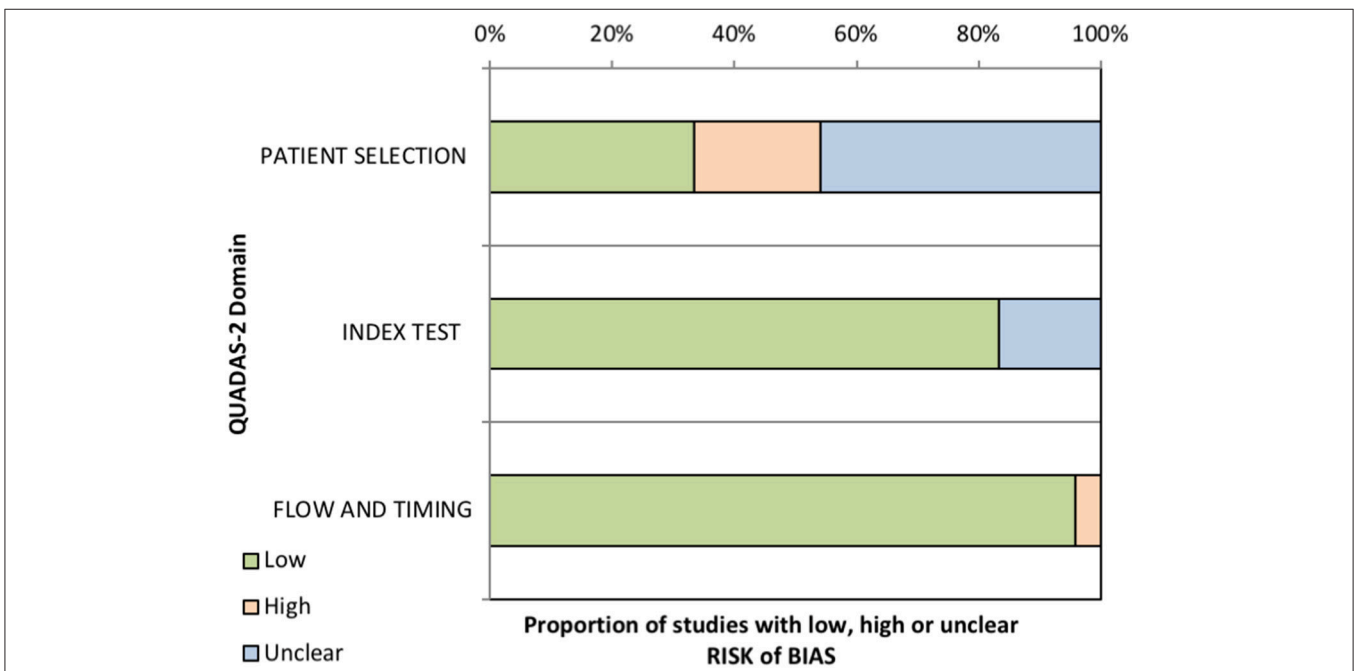

FIGURE 4 | Results of the methodologic assessment using the QUADAS-2 tool showing the proportion of studies with low, high, or unclear risk of bias.

association with anti-β2GpI (11) in a selected population. On the other hand, Gupta et al., Enzenauer et al., did not report this association. Touré et al. found a trend (12, 13, 22). As in our study, studies that did not highlight an association between PAH and aPL positivity had a low prevalence of aPL (9.1–14%) (12, 13). Moreover, we defined PAH by hemodynamics during a right heart catheterization, which is the gold standard and not by echocardiography. We cannot rule out to have overlooked PAH in some patients but, in the same way, some patients diagnosed as having PAH only on echocardiography and not hemodynamics in other studies could also have been misclassified.

Interestingly, considering the titers of aPL rather than the aPL status positivity/negativity, we found that higher titers of aCL ≥5 UGPL/mL were associated with PAH. This is consistent with the existing literature. Morrisroe et al. identified that higher titers of aCL-IgG corresponded with a higher risk of PAH in SSc patients (10). Assous et al. identified a trend toward an association between a higher mean titer of aCL and PAH ($p = 0.06$). They also found an association between patients with PAH and the amount of von Willebrand factor produced (26). It has been shown that endothelial cell injury in SSc patients was accompanied by an elevation in the level of von Willebrand factor (43). This suggests that aCL positivity could be associated with endothelial injury and PAH in SSc. It has also been reported that there was an increased amount of E-selectin in patients with aPL positivity (with or without APS), and an increased amount of P-selectin and sVCAM-1 in patient with APS (44, 45). These three molecules are also involved in pathogenesis of SSc and PAH (1, 46).

Our study has several limitations. First, we chose to quantify only IgG subtype of aCL and anti-β2GpI, because IgG was the most prevalent isotype among patient with thrombosis and fetal loss in APS, and the only one associated with these events (39). However, an overall screening of anti-β2GpI IgG, IgM, and IgA subtype was done. IgM and IgA were often more prevalent in the studies in which they are quantified (10, 11). Secondly, due to the low prevalence of aPL, there might be a lack of power. Moreover, some clinical events were quite rare in aPL positive patients (PAH in one patient, miscarriage in 5) appealing for a cautious interpretation of these data. Thirdly thrombosis history and miscarriage has been collected retrospectively, leading to a potential memorization bias and a selection bias (in classification of APS patients). It is difficult to avoid this bias, because obstetrical events occurred mainly years before the onset of SSc. A prospective study would be needed to avoid these biases.

In conclusion, this study found a prevalence of aPL in SSc of 6.4% (3.8–10.4) and an overall pooled prevalence of 14% (9–20). aPL positivity was associated with VT and miscarriage. These data provide additional insights into the role of aPL in the vasculopathy observed in SSc.

AUTHOR CONTRIBUTIONS

VS, AL-O, SD, and DL: design of study; VS, AL-O, MS, BL, CY, ML, P-YH, EH, SD, and DL: collection of data; JG and LD: statistical analysis; VS, AL-O, JG, LD, SD, and DL: redaction of manuscript; VS, AL-O, JG, LD, MS, BL, CY, ML, P-YH, EH, SD, and DL: critical review of manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02457/full#supplementary-material

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Fibrosis Development in HOCl-Induced Systemic Sclerosis: A Multistage Process Hampered by Mesenchymal Stem Cells

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Objectives: Skin fibrosis is the hallmark of systemic sclerosis (SSc) a rare intractable disease with unmet medical need. We previously reported the anti-fibrotic potential of mesenchymal stem cells (MSCs) in a murine model of SSc. This model, based on daily intra-dermal injections of hypochlorite (HOCl) during 6 weeks, is an inducible model of the disease. Herein, we aimed at characterizing the development of skin fibrosis in HOCl-induced SSc (HOCl-SSc), and evaluating the impact of MSC infusion during the fibrogenesis process.

Methods: After HOCl-SSc induction in BALB/c mice, clinical, histological and biological parameters were measured after 3 weeks (d21) and 6 weeks (d42) of HOCl challenge, and 3 weeks after HOCl discontinuation (d63). Treated-mice received infusions of 2.5×10^5 MSCs 3 weeks before sacrifice (d0, d21, d42).

Results: HOCl injections induced a two-step process of fibrosis development: first, an 'early inflammatory phase', characterized at d21 by highly proliferative infiltrates of myofibroblasts, T-lymphocytes and macrophages. Second, a phase of 'established matrix fibrosis', characterized at d42 by less inflammation, but strong collagen deposition and followed by a third phase of 'spontaneous tissue remodeling' after HOCl discontinuation. This phase was characterized by partial fibrosis receding, due to enhanced MMP1/TIMP1 balance. MSC treatment reduced skin thickness in the three phases of fibrogenesis, exerting more specialized mechanisms: immunosuppression, abrogation of myofibroblast activation, or further enhancing tissue remodeling, depending on the injection time-point.

Conclusion: HOCl-SSc mimics three fibrotic phenotypes of scleroderma, all positively impacted by MSC therapy, demonstrating the great plasticity of MSC, a promising cure for SSc.

Keywords: mesenchymal stem cells, systemic sclerosis, fibrosis, hypochlorite, oxidative stress, scleroderma, cell therapy, autoimmunity

INTRODUCTION

Skin fibrosis is the hallmark of systemic sclerosis (scleroderma, SSc), a rare intractable autoimmune disorder with high morbidity and mortality due to multi-organ involvement (1). Although precise etiology for SSc remains unknown, fibrosis development in the disease may originate from a complex interplay between environmental and intrinsic triggers–including oxidative stress-leading to tissue damage, immune response, endothelial cell and myofibroblast activation. The latter results in fibroblast proliferation and extracellular matrix (ECM) production (2–4). However, fibrogenesis is a dynamic process, with counteracting mechanisms involved to dampen immune activation or up-regulate tissue remodeling. So, different stages of tissue fibrosis may be observed, with variable participation of immune cell activation, angiogenesis or ECM synthesis by fibroblasts. Thus, targeting fibrosis in SSc may be challenged by the complexity of this multistage process, as well as the unpredictability of clinical evolution. Studies are still on going to help defining more precisely the clinical and biological status of SSc patients, hence refining therapy (5, 6).

Among new potential approaches for treating SSc, mesenchymal stem cells (MSCs)-based therapy stands as a promising lead (7). Indeed, these mesodermal multipotent progenitors can be easily isolated and expanded from virtually all tissues, and display both immunomodulatory and anti-fibrotic properties when infused (8). We recently demonstrated the feasibility and the therapeutic benefits of an approach based on MSCs in a preclinical murine model for SSc (9, 10). In this model, fibrosis is induced by repeated exposure to hypochlorite (HOCl), an oxidative agent administered by daily intra-dermal injections during 6 weeks. This model, mimicking early diffuse cutaneous SSc, is particularly useful to evaluate new therapeutic approaches. However, we still lack data on the development and the spontaneous evolution of fibrosis in HOCl-induced SSc (HOCl-SSc), as well as the impact of MSCs-based therapy along the process.

In this study, we first precisely characterized the fibrotic process induced by HOCl injections, as well as the evolution of fibrosis after discontinuation of HOCl challenge. Doing so, we further investigated the impact of MSC infusion, at each stage of the process, on skin fibrosis, inflammation and remodeling.

MATERIALS AND METHODS

Isolation and Culture of Mesenchymal Stem Cells

MSCs from BALB/c mice were isolated from bone marrow (BM). BM was flushed out from long bones and the cell suspension was plated in DMEM supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (Lonza, France). Cells were passaged till obtaining homogeneity for mesenchymal marker expression and lack of hematopoietic markers as analyzed by flow cytometry. They were used between passages 10 and 15.

Flow Cytometry Analysis

Cells were harvested by treatment with 0.05% trypsin and 0.53 mM EDTA, and resuspended in PBS containing 0.1% BSA and 0.01% sodium azide. Cells were incubated on ice with conjugated antibodies against CD11b, CD44, CD45, CD73, and Sca1 (BD Pharmingen, France) or conjugated isotypic controls. Samples were analyzed on the FACS Canto II and analysis performed using the BD FACSdiva software (BD Pharmingen).

Differentiation of MSCs

Differentiation of MSCs was induced as reported elsewhere (11). In brief, for adipogenesis, MSCs were plated at 10^4 cells/cm^2 in inductive medium and adipocytes characterized by presence of lipid droplets as visualized by Oil red O staining and expression of specific markers by RT-qPCR. Chondrogenic differentiation was induced by culture in micropellet and chondrogenesis was assessed by RT-qPCR. Osteogenesis was induced by culture at low density in osteogenic medium. Differentiation was assessed by RT-qPCR quantification of osteoblast markers and ECM mineralization visualized after staining with a 2% Alizarin Red S solution.

T-Cell Proliferation Assay

For T-cell proliferative experiments, 10^5 splenocytes were stimulated with 1 µg/ml concanavalin A (conA; Sigma-Aldrich, France) in presence of different ratios of MSCs as already reported (11). After 3 days, cell proliferation was measured using the CellTiter-Glo™ luminescent cell viability assay (Promega, France).

HOCl Preparation

HOCl was generated extemporaneously by adding NaClO (9.6% as active chlorine) to KH_2PO_4 solution (100 mM, pH: 6.2), usually using a 1:100 ratio. The right amount of NaClO was adjusted to obtain the desired HOCl concentration, defined by the absorbance of the mixture at 292 nm (optical density between 0.7 and 0.9 read on a Nanodrop spectrophotometer, Thermoscientific). Stock solutions were stored at 4°C in the dark and NaClO was replaced every 3 weeks.

Experimental Design and Animals

Six-week-old female BALB/c mice purchased from Janvier were housed and cared for according to the Laboratory Animal Care guidelines. Approval was obtained from the Regional Ethics Committee on Animal Experimentation before initiation of the study (approval APAFIS#5351-2016050919079187). All experiments were performed after final approval given by the French Ministry for Education, Higher Education and Research. Mice had their backs shaved the day before disease induction. Skin thickness was assessed with a caliper before disease induction and every week during the whole experiment by a blinded experimenter. As previously described, a total amount of 300 µl of freshly prepared HOCl was injected in two sites into the backs of the mice with a 29 G needle, 5 days a week for 6–9 weeks [d0 to d42 or day 63; (12)]. Control mice received PBS in the same conditions. MSCs-treated SSc-HOCl mice received an infusion of MSCs (2.5×10^5 cells in 100

µl PBS), in the tail vein of the mice at indicated time points (d0, d21, d42). Groups of 7 to 10 mice were made for each condition (PBS-, HOCl- and MSCs-treated HOCl-mice). Three weeks after MSCs infusion, and after a 2-day recovery time without HOCl injections, animals were sacrificed, at indicated time points (d21, d42, or d63). Skin biopsies (6 mm punches) were taken on the backs of mice. Samples were stored at −80°C for RT-qPCR, ELISA and collagen content determination or fixed in 4% formaldehyde for histopathological analysis. Overall experimental scheme is shown in **Figure 1**, and representative pictures of mice during experimental procedure are shown in **Supplementary Figure 1**.

Histopathology

Samples were embedded in paraffin and 5 µm thick sections were stained with Masson trichrome or immunostained with antibodies for α-sma (ab5694, Abcam, 1/100) TGFβ (ab66043, Abcam, 1 /100), Ki67 (SP6, VP-RM04, Vector laboratories, 1/200), CD3-epsilon (M-20, sc−1127, Santa Cruz Biotechnology, 1/250), F4/80 (MF4800, Invitrogen, 1/50), Pax5 (C-20, sc−1974, Santa Cruz Biotechnology, 1/250). Histological slides were scanned using Nanozoomer (Hamamatsu) or Pannoramic 250 Flash II (3DHistech) for immunofluorescence. Quantification of immunostaining was made using Definiens Tissue Studio software.

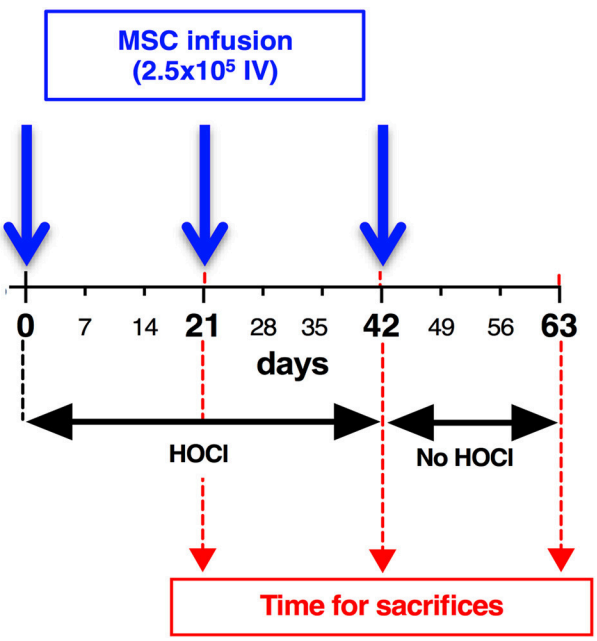

FIGURE 1 | Development of skin fibrosis in HOCl-mice: experimental scheme. To induce systemic sclerosis, BALB/c mice underwent 6 weeks of daily HOCl intradermal injections, while control mice received PBS injections. Skin thickness was measured weekly during the experiment. After d42 (6 weeks), HOCl injections were abrogated, and groups of mice were kept in order to investigate skin thickness evolution till d63. Groups of mice were sacrificed at indicated time points (d21, d42, d63).

RT-qPCR Analysis

Samples (cells or skin biopsies) were crushed in RLT-buffer and total RNA was extracted using the RNeasy mini kit and Qiacube robotic workstation (Qiagen, France). 1 µg RNA was reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen, France). qPCR was performed on 20 ng cDNA using LightCycler 480 SYBRGreen I Master mix and real-time PCR instrument (Roche Applied Science, France). The following conditions were used: 95○C for 5 min; 40 cycles at 95○C for 15 s; 64○C for 10 s and 72○C for 20 s in a LightCycler 480 system (Roche diagnostics, France) and analyzed with the dedicated software. Primers were designed using the web-based applications Primer3 and BLAST (**Table 1**). Samples were normalized to mRNA expression of TATA binding protein *(Tbp)* gene for tissue samples or GAPDH for MSCs, and results were provided either as relative expression to the housekeeping gene using the formula $2^{-\Delta Ct}$ or as fold change using the formula $2^{-\Delta\Delta Ct}$.

Collagen Content in Skin

Collagen content assay was based on the quantitative dye-binding Sircol method (Biocolor, Ireland). Skin biopsies were suspended in 2 ml of a 0.5 M acetic acid—pepsin (2.5 mg/ml) solution and dissociated using UltraTurrax (vWR, France). Collagen extraction was performed overnight at 4°C under stirring. Suspension was then centrifuged at 12,000 g for 10 min and 20 µl of each sample were added to 1 ml of Syrius red reagent. Tubes were rocked at room temperature for 30 min and centrifuged at 12,000 g for 10 min. The supernatants were discarded and tubes washed with 750 µl of ice-cold salt acid. After another 12,000 g centrifugation for 10 min, the collagen-dye pellets were suspended in 1 ml of 0.5 M NaOH. Optical Density (OD) was read at 555 nm on a microplate reader (Varioskan Flash, Thermo scientific) vs. a standard range of bovine collagen type I concentrations (supplied as a sterile solution in 0.5 M acetic acid). Results were expressed as collagen content in µg/mm^2 of skin.

Anti-topoisomerase 1 Antibody Elisa

Anti-topoisomerase 1 or anti-scl-70 antibodies were detected using scl-70 Ig ELISA kit (Abnova, Taiwan). In brief, 200 µl of 1:5 diluted sera were dispensed into scl-70 pre-coated wells and incubated at room temperature for 90 min. Goat total anti mouse IgG antibody, HRP conjugate (BD Biosciences, France), diluted 1:1,000 was then incubated for 60 min at room temperature. TMB substrate was incubated for 5 min and stopped with an equivalent amount of sulfuric acid. Absorbance was read at 450 nm under Varioskan Flash and results expressed as arbitrary unit (AU) for optical density (OD).

Statistical Analysis

All quantitative data were expressed as mean +/- SEM. Gaussian distribution of values was tested using the Shapiro-Wilk normality test. Data were then compared using Mann-Whitney's test for nonparametric values or Student's *t*-test for parametric values. When analysis included more than two groups, one-way ANOVA was used. All statistical analyses

TABLE 1 | List of primers designed and used in RT-qPCR experiments.

Gene abbreviation	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Ap	GTGGTGGACGGTGAACGGGA	TCCACCGTGGGTCTCATGGC
αSma	AAGGCCAACCGGGAGAAAAT	AGCCAAGTCCAGACGCATGA
Col1	TGTTCAGCTTTGTGGACCTC	TCAAGCATACCTCGGGTTTC
Col2B	CTGGTGCTGCTGACGCT	GCCCTAATTTTCGGGCAT
Col3	CGGTGAACGGGGCGAAGCTGGTT	GACCCCTTTCTCCTGCGGCTCCT
Col10	TGCTGCCTCAAATACCCTTT	CAGGAATGCCTTGTTCTCCT
Fabp4	CGTAAATGGGGATTTGGTCA	TCGACTTTCCATCCCACTTC
Gapdh	GGTGCTGAGTATGTCGTGGA	GTGGTTCACACCCATCACAA
Il1b	TTTGACAGTGATGAGAATGACCTGTTC	TCATCAGGACAGCCCAGGTCAAAG
Il6	TGGGACTGATGCTGGTGACA	TTCCACGATTTCCCAGAGAACA
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Lpl	TTTGGCTCCAGAGTTTGACC	GTCTTGCTGCTGTGGTTGAA
Mmp1	TTCAAAGGCAGCAAAGTATGGGCT	CCAGTCTCTTCTTCACAAACAGCAGCA
Oc	GCGCTCTGTCTCTCTGACCT	GCCGGAGTCTGTTCACTACC
Pparγ	AAGAGCTGACCCAATGGTTG	GGATCCGGCAGTTAAGATCA
Runx2	ACAGTCCCAACTTCCTGTGC	ACGGTAACCACAGTCCCATC
Sox9	AGGAAGCTGGCAGACCAGTA	CTCCTCCACGAAGGGTCTCT
Tbp	GGGAGAATCATGGACCAGAA	CCGTAAGGCATCATTGGACT
Tgfβ1	TGCGCTTGCAGAGATTAAAA	CTGCCGTACAACTCCAGTGA
Timp1	CTCCGCCCTTCGCATGGACATT	GGGGGCCATCATGGTATCTGCTCT
Tnfα	AGCCCACGTCGTAGCAAACCA	TGTCTTTGAGATCCATGCCGTTGGC

Ap, alkaline phosphatase; aSma, alpha, smooth muscular actin; Col, collagen; Fabp, fatty acid binding protein; Gapdh, glyceraldehyde-3-phosphate deshydrogenase; Hmox, heme oxygenase; Il, interleukin; Lpl, lipoprotein lipase; Mmp, metalloprotease; Oc, osteocalcin; Ppar, peroxisome proliferator-activated receptor; Runx, Runt related transcription factor; Sox, SRY (sex determining region Y)-related HMG (high mobility group)-box gene; Tbp, TATA binding protein; Timp, tissue inhibitor of metalloproteinase; Tnf, tumor necrosis factor.

were performed using Prism 6 GraphPad software for Mac OS (California, United States). A $P < 0.05$ was considered significant.

RESULTS

Induction of Skin Fibrosis Under HOCl Challenge Is a Two-Step Process With Early Proliferative and Inflammatory Stage and Late Constitution of ECM Deposition

As previously shown, the induction of skin fibrosis by daily HOCl injections was clinically characterized by progressive thickening of skin from d7 to d42 when compared with control PBS-mice (**Figure 2A** and **Supplementary Figure 1**). At d42, histology disclosed strong collagen deposition in skin from HOCl-mice compared with PBS mice, as shown by sirius red coloration (**Figure 2B**). Histological examination from sequential sacrifices revealed a discontinued process with two main stages. First, in the third week of experiment (d14–d21), we observed transparietal polymorphous cellular infiltrates; second, in the last week of experiment (d35–d42), these cellular infiltrates were gradually replaced by ECM deposition, resulting in disorganization of dermis and complete loss of hypodermic adipose tissue (**Figure 2C**). During the process, we noted a strong and steady expression of myofibroblastic markers *αSma* and *Tgfβ1* in dermis from SSc mice, with no obvious difference between d21 and d42 (**Figure 2D**).

While comparing these two main time points (i.e., d21 and d42), we observed a higher number of proliferative cells in skin tissues at d21 compared with d42, as shown by Ki67 staining (**Figures 3A,B**). Looking at cytokine mRNA expression within the tissue, we noted a stronger expression of pro-inflammatory cytokines *Il1β* and *Tnfα* at d21 compared with d42, together with a lower expression of the anti-inflammatory cytokine *Il10* (**Figure 3C**). We therefore focused on d21, and further characterized these cellular infiltrates by immunostaining. We observed high number of CD3+ T-lymphocytes, of F4/80+ macrophages, but no Pax5+ B cells (**Figure 4**). Of note, we also noticed some cells co-expressing CD3 and Ki67 (data not shown).

Altogether, the analysis of skin fibrosis kinetics during the induction of HOCl-SSc revealed a two-step process, with an early cellular phase made of highly proliferative T-lymphocytes, macrophages and myofibroblasts, culminating at d21, and a late evolution toward paucicellular matricial fibrosis mainly characterized by ECM deposition at d42. Conversely, as previously reported, lung fibrosis development in this model is a continuous and progressive process, leading to tissue fibrosis at the end of the 6-wk experiment [(9) and **Supplementary Figure 2**]. For these reasons and because skin fibrosis is the hallmark of SSc, we kept focused on skin fibrosis development in the present study.

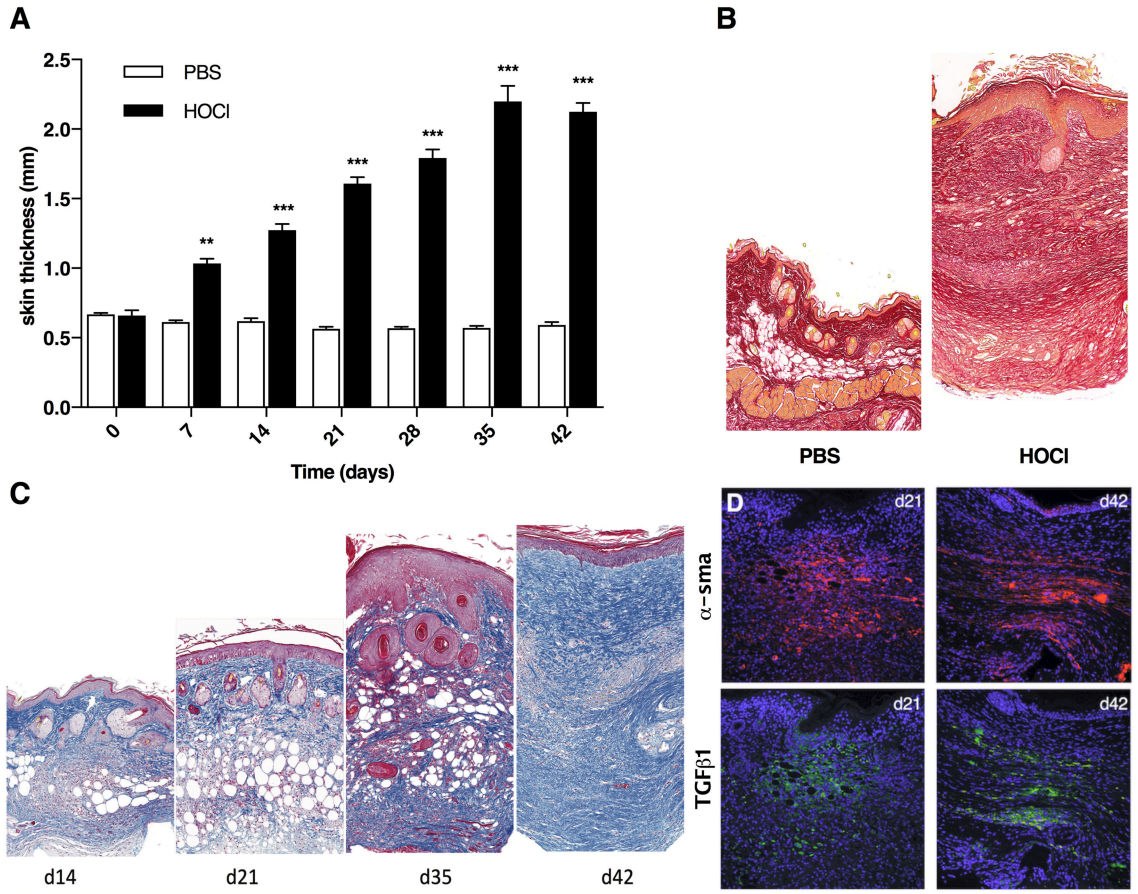

FIGURE 2 | Development of skin fibrosis in HOCl-mice. **(A)** Skin thickness from PBS or HOCl-injected mice at different time-points during the induction of HOCl-SSc (d0 to d42) ($N = 8$ per group) $^{**}P < 0.001$, $^{***}P < 0.001$. **(B)** Representative skin sections of PBS and HOCl mice at d42 (original magnification 10x; Sirius Red staining). **(C)** Representative skin sections at different time-points during the induction of HOCl-SSc (original magnification 10x; Masson Trichrome staining). **(D)** Immunostaining for αSma (in red) and Tgfβ1 (in green) in representative skin sections at d21 and d42.

MSCs-Based Treatment Efficiently Reduces Inflammation During the First Stage of Fibrosis Constitution in HOCl-SSc

In a first experiment, we aimed at evaluating the anti-inflammatory impact of MSCs-based treatment administered during the first stage of fibrosis induction in the model (d0–d21). Therefore, we used MSCs isolated from BALB/c mice in a syngeneic approach. These cells were characterized by the expression of CD44, CD73, CD29, CD105, CD106, and stem cell antigen 1 (Sca1), and the absence of expression of the haematopoietic markers CD45, CD3, CD19, CD31, CD11b, and HLA-DR by cytometry analysis (**Figures 5A,B**). MSCs exerted immunosuppressive properties on the mitogen-induced proliferation of T-lymphocytes (**Figure 5C**) and were able to differentiate into adipocytes expressing lipoprotein lipase *(Lpl),* peroxisome proliferator-activated receptor *(Pparγ),* fatty acid binding protein *(Fabp4)* (**Figure 5D**), chondrocytes expressing SRY (sex determining region Y)-related HMG (high mobility group)-box gene *(Sox9), Col2B, Col10* (**Figure 5E**) and osteoblasts expressing osteocalcin *(Oc),* alkaline phosphatase *(Ap), Col1,* Runt related transcription factor (*Runx2;* **Figure 5F**).

A unique dose of 2.5×10^5 MSCs was administered intravenously before HOCl induction at d0, and mice were sacrificed 3 weeks later. A significant reduction of skin thickness was obtained at d21 in MSCs-treated SSc mice compared with untreated SSc mice (**Figure 6A**). Because inflammation was the highest at d21, we analyzed the effect of MSCs on inflammatory mediators in skin. MSCs treatment was associated with a significant decrease of *Il1β, Tnfα, Il6,* and *Il10* expression compared with untreated SSc mice (**Figure 6B**). Histological analysis revealed overall less cellular infiltrates in MSCs-treated mice, together with less Ki67 staining, strong decrease in CD3 staining and almost abolition of F4/80 staining (**Figures 6C,D**). MSC infusion also prevented specific anti-scl70 autoantibody production as detected in the serum (**Supplementary Figure 3**). On the whole, MSCs treatment during the first phase of HOCl-induced fibrosis prevented skin inflammation

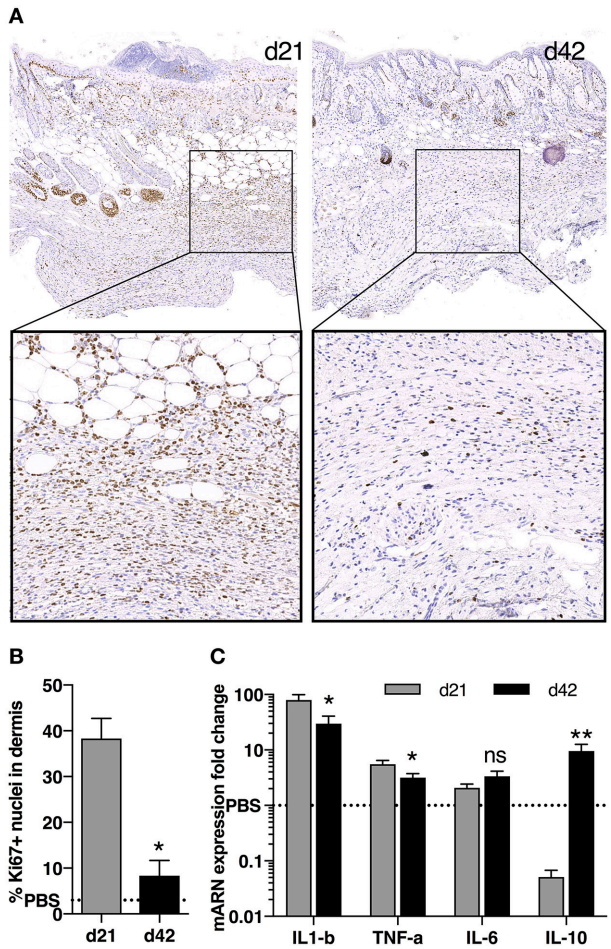

FIGURE 3 | Cell proliferation and cytokine production in skin during fibrosis induction in HOCl-mice. **(A)** Immunostaining for nuclear Ki67 in representative skin sections at d21 and d42 (top panels: magnification 10x, bottom panels: top image box, 30x). **(B)** Percentage of Ki67$^+$ proliferative cells in dermis as quantified on immunostaining in **A** using Definiens Tissue Studio software ($n = 3$ per group, $^*P < 0.05$, $^{**}P < 0.01$). **(C)** mRNA expression of *Il1b, Tnfa, Il6* and *Il10* in skin sections from HOCl-SSc mice at d21 and d42. Results are given as fold-change *vs.* control PBS-mice normalized at 1. $N = 8$ per group.

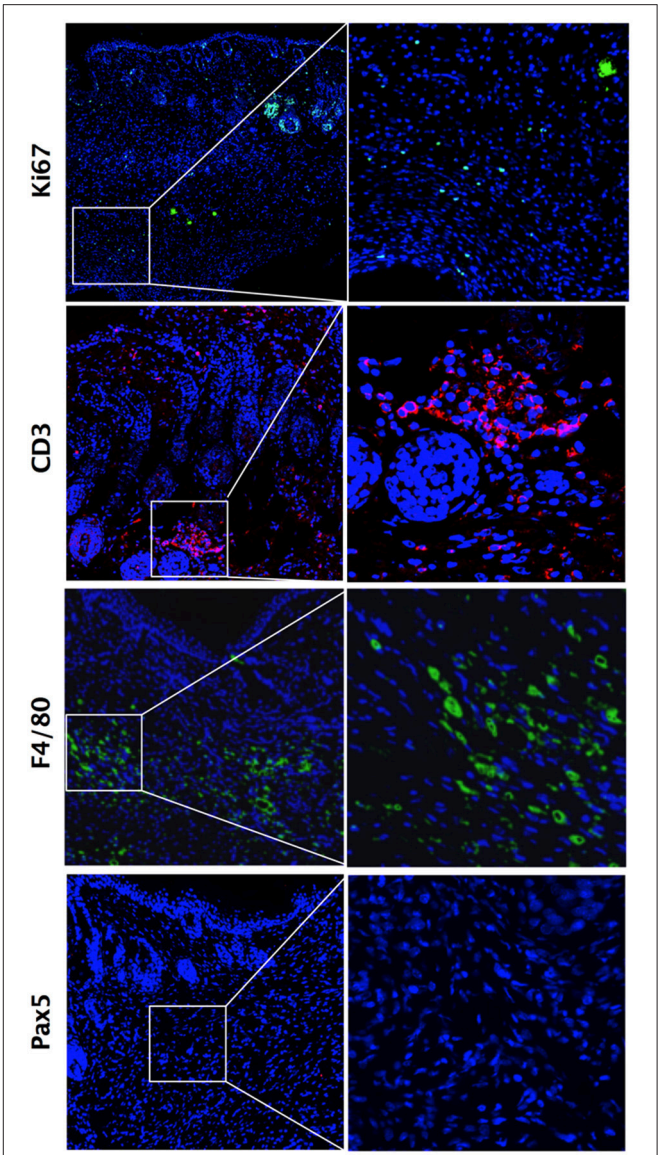

FIGURE 4 | Inflammatory cell infiltrates within dermis during fibrosis induction in HOCl-mice. Immunostaining for Ki67$^+$ proliferative cells, CD3$^+$ T-lymphocytes, F4/80$^+$ macrophages and Pax5$^+$ B lymphocytes in representative skin sections at d21 from HOCl mice (original magnification 10x; enlargement 60x).

by down-regulating T-cell and macrophage immune response resulting in reduced cytokine and autoantibody production.

MSCs Treatment Reduces Myofibroblastic Activation and Collagen Deposition During the Second Stage of Fibrosis Constitution in SSc Mice

We then investigated the effect of a single infusion of 2.5×10^5 MSCs administered at d21 on the second phase of HOCl-SSc induction (d21–d42). MSCs treatment completely inhibited skin thickening during the whole period, resulting in significantly reduced skin thickness at d42 (**Figure 7A** and **Supplementary Figure 1**). At the end of the experiment, MSCs-treated mice exhibited reduced skin fibrosis characterized by less ECM deposition in Masson Trichrome staining (**Figure 7B**) and reduced total collagen content in skin (**Figure 7C**), almost reaching the level of control PBS mice. Immunostaining for α-Sma and Tgfβ confirmed less myofibroblastic activation in MSCs-treated mice compared with untreated mice (**Figures 7D,F**). Of note, fewer F4/80+ macrophages were also noted in skin from MSCs-treated mice compared with SSc mice, whereas no difference could be detected concerning CD3+ lymphocyte infiltrates, since there was almost none in SSc mice at d42 (**Figures 7E,F**). However, in this setting, we did

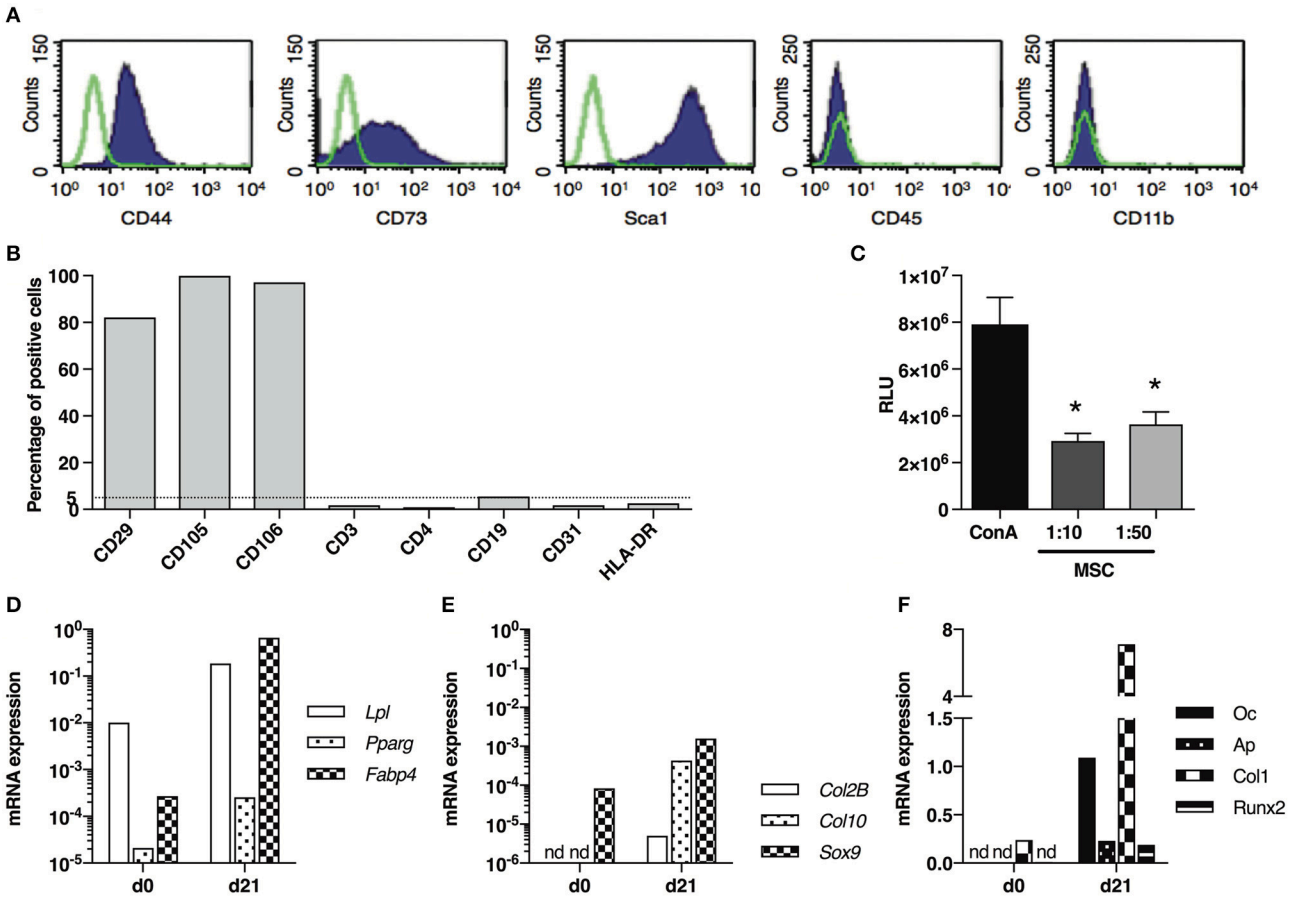

FIGURE 5 | Phenotypical and functional characterization of BM-derived BALB/c MSCs. **(A)** Representative graph of flow-cytometry analysis of MSCs stained with antibodies against CD44, CD73, Sca1, CD45 and CD11b. **(B)** Percentage of MSCs stained with antibodies against CD29, CD105, CD106, CD3e, CD4, CD19, CD31 and HLA-DR in flow-cytometry analysis. **(C)** T cell proliferation assay expressed as relative luminescent units (RLU) for splenocytes stimulated with concanavalin A (ConA) and cultured in presence of MSCs (ratio 1:10 or 1:50; $n = 3$ per group) $^*P < 0.05$. **(D)** mRNA expression of *Lpl, Pparg* and *Fabp4* in MSCs, normalized to *Gapdh* expression, before (d0) and after (d21) adipogenesis induction. **(E)** mRNA expression of *Col2B, Col10* and *Sox9* in MSCs, normalized to *Gapdh* expression, before (d0) and after (d21) chondrogenesis induction. **(F)** mRNA expression of *Oc, Ap*, *Col1* and *Runx2* in MSCs, normalized to *Gapdh* expression, before (d0) and after (d21) osteogenesis induction. Data are presented as average ± SEM. nd: not detected. *Ap,* alkaline phosphatase; *Col,* collagen; *Fabp,* fatty acid binding protein; *Lpl,* lipoprotein lipase; *Oc,* osteocalcin; *Ppar*, peroxisome proliferator-activated receptor; *Runx,* Runt related transcription factor*; Sox,* SRY (sex determining region Y)-related HMG (high mobility group)-box gene.

not observe a reduction of anti-scl70 antibody production (**Supplementary Figure 3**).

MSCs Treatment After HOCl Discontinuation Activates Tissue Remodeling Toward Fibrosis Clearance

In a last experiment, we aimed at investigating both the evolution of HOCl-SSc after HOCl discontinuation and the effect of MSCs treatment in this condition. Therefore, we administered a single infusion of 2.5×10^5 MSCs at d42, stopped HOCl injections and sacrificed the mice 3 weeks later (d63). We first observed a spontaneous decrease in skin thickness in untreated HOCl-induced mice, significant in the last week of experiment (**Figure 8A**). MSCs-treated mice exhibited an earlier and stronger decrease in skin thickness compared with untreated HOCl-induced mice, significant in the last 2 weeks (**Figure 8A**). Histological analysis confirmed the strong anti-fibrotic effect of MSCs treatment, with an almost normal aspect of skin compared with untreated HOCl-induced mice (**Figure 8B**). Immunostaining for α-SMA and TGF-β corroborated these observations (**Figures 8C,E**), while mRNA expression for the main fibrotic markers confirmed a significantly reduced expression of *Col1, Col3, Tgfβ1*, and α-*Sma* in MSCs-treated mice compared with untreated HOCl-induced mice (**Figure 8F**). Notably, levels of these four markers were found below the levels of control PBS-mice. Interestingly, untreated HOCl-induced mice also exhibited lower levels of α-*Sma* in comparison with control PBS mice. Concerning tissue inflammation, we noted residual F4/80 macrophage infiltrates in SSc mice, which was reduced in MSCs-treated mice (**Figures 8D,E**). In MSCs-treated mice we also observed a reduced expression of *Il1β,* and *Tnfα,* while *Il6* was increased

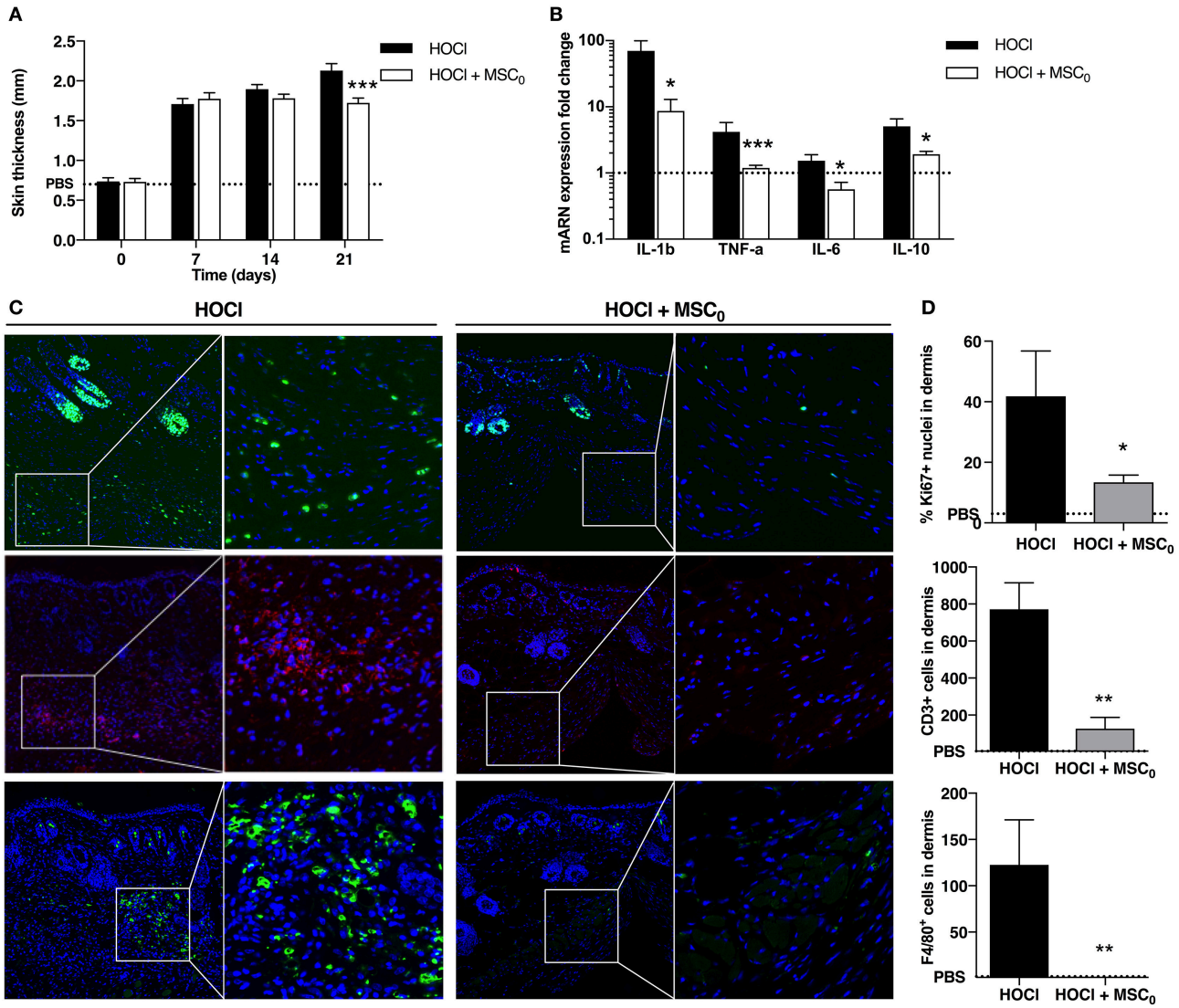

FIGURE 6 | Anti-inflammatory effects of MSC treatment during fibrosis induction in SSc mice at day 21. **(A)** Skin thickness measured from d0 to d21 in control PBS mice, non-treated SSc mice and SSc mice treated with 2.5×10^5 MSCs at d0 (MSC_0). **(B)** mRNA expression of *IL1b, TNF-a, IL-6* and *IL-10* in skin at d21. Results are given as fold-change vs. control PBS-mice normalized to 1. **(C)** Nuclear immunostaining for proliferative cells using Ki67 (top panels, in green), CD3+ T-lymphocytes (middle panels, in red), and F4/80+ macrophages (bottom panels, in green), in representative skin sections at d21 from HOCl-SSc mice and MSCs-treated HOCl-SSc mice. Left: original magnification 10x; middle: part of left image 40x. **(D)** Quantification of immunostainings in **C** using Definiens Tissue Studio IF software (top: percentage of Ki67+ nuclei among all nuclei observed; middle and bottom: absolute number of CD3+ or F4/80+ cells; $n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, data are presented as mean ± SEM. $N = 8$ for HOCl-mice, $N = 7$ for MSCs-treated mice.

and *Il10* remained unaffected (**Figure 8F**). Looking for changes in tissue remodeling, we noted a positive effect of MSCs treatment, associated with significant increased expression of metalloproteinase 1 (MMP1) and decrease of its main inhibitor tissue inhibitor of metalloproteinase 1 (TIMP1) vs. untreated HOCl-induced mice, resulting in a favorable MMP1/TIMP1 ratio in tissue (**Figure 8F**). Of note, untreated mice also exhibited a significantly higher ratio in comparison with PBS mice, or SSc mice sacrificed at d42 (data not shown). On the whole, HOCl discontinuation was responsible for tissue remodeling activation leading to progressive fibrosis clearance, a phenomenon significantly improved by MSCs therapy.

DISCUSSION

This study provides original data regarding skin fibrosis development in a preclinical inducible model for diffuse SSc. In this murine model, we demonstrated a multi-stage process leading to skin fibrosis under repeated exposure to HOCl.

First, during the first 3 weeks of the experiment, daily injections of HOCl were shown to trigger inflammation and led to transparietal cellular polymorphous infiltrates, with high proliferative rate, culminating at d21. These cellular infiltrates were mostly made of T-lymphocytes and macrophages,

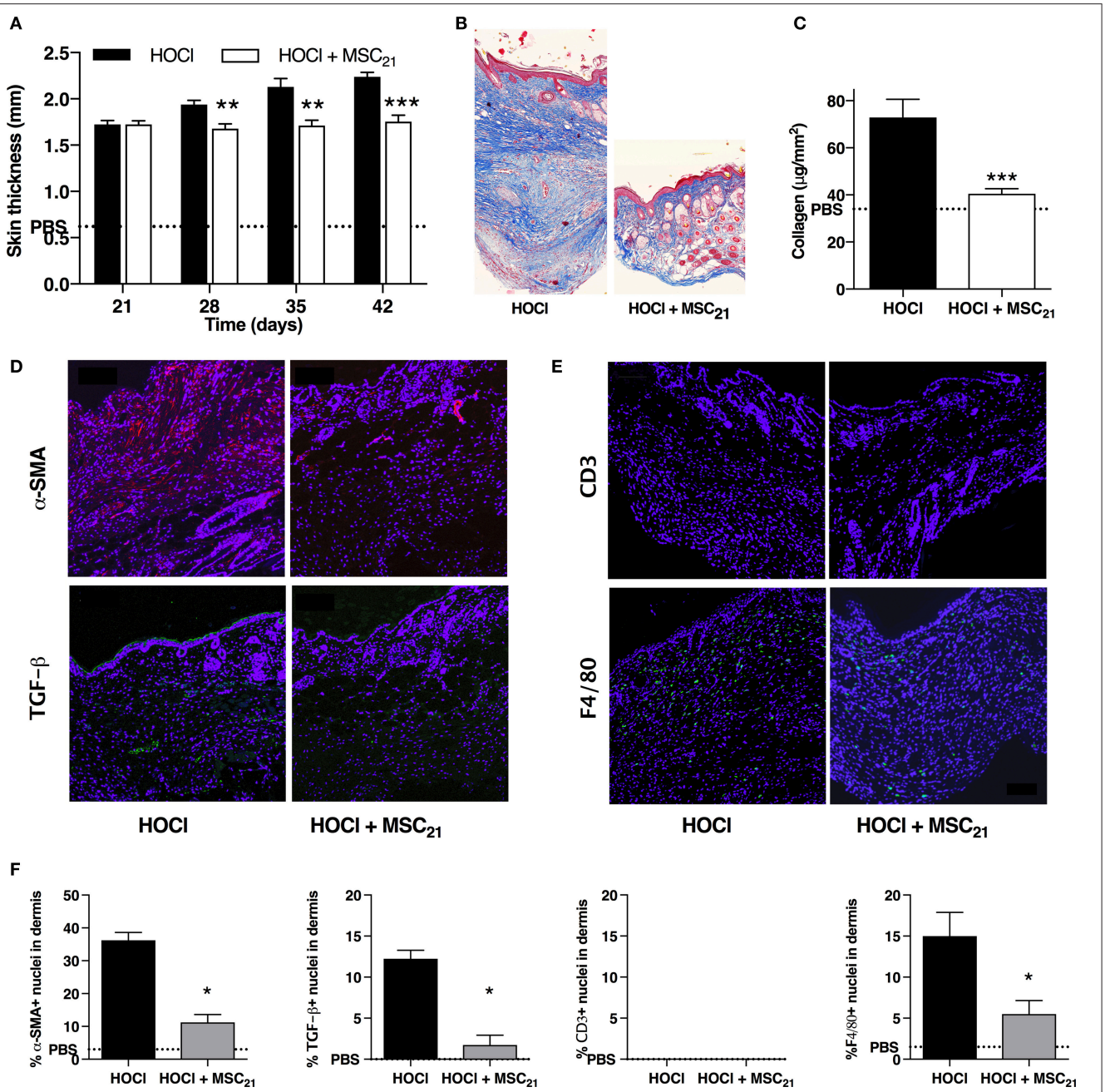

FIGURE 7 | Anti-fibrotic and anti-inflammatory effects of MSCs treatment on skin fibrosis at day 42. **(A)** Skin thickness from d21 to d42 in HOCl-SSc mice, HOCl-SSc mice treated with 2.5×10^5 MSCs at d21 (MSC_{21}), or control PBS mice. $N = 8$ per group. **(B)** Representative skin sections at d42 from HOCl-SSc mice and MSCs-treated HOCl-SSc mice (original magnification 10x; Masson trichrome staining). **(C)** Collagen content in skin from HOCl-SSc mice and MSCs-treated HOCl-mice (MSC_{21}). Mean level for control PBS mice are represented by a discontinued line. $N = 8$ per group. **(D)** Immunostaining with a-SMA (in red) and TGF-b1 (in green) in skin sections from HOCl-SSc mice (left) and MSCs-treated HOCl-SSc mice (MSC_{21}, right) at d42. **(E)** Immunostaining with CD3 (in red) and F4/80 (in green) in skin sections from HOCl-mice (left) and MSC-treated HOCl-mice (MSC_{21}, right) at d42. **(F)** Quantification of immunostainings in D and E (percentage of positive cells among all cells observed; $n = 4$ per group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HOCl-mice; data are presented as mean ± SEM.

but also myofibroblasts. These observations are consistent with those made in SSc patients, notably during oedematous scleroderma, an inflammatory phenotype associated with early rapidly progressive diffuse SSc, where incisive treatments may be required (13, 14). In this condition, concordantly with what we observed, immune cells in skin tissue mainly include CD4+ T-lymphocytes and macrophages, with sparse B lymphocytes (15, 16). Macrophages are a well-known source of TGFβ secretion, this soluble mediator being the most critical trigger of fibroblast activation leading to ECM synthesis (3). Regarding cytokine

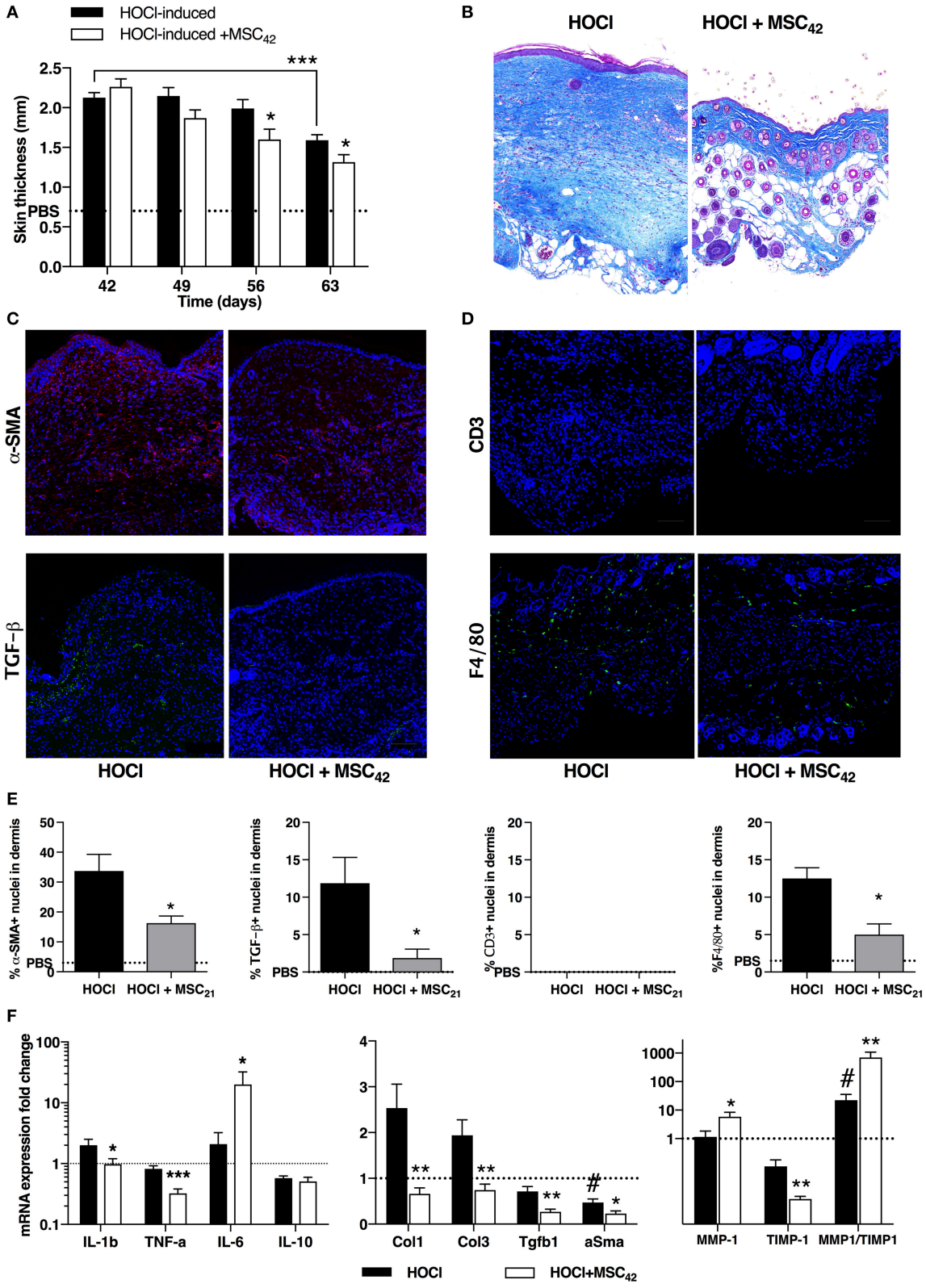

FIGURE 8 | Tissue remodeling after fibrosis induction in HOCl-mice and effects of MSCs treatment at day 63. **(A)** Evolution of skin thickness after discontinuation of HOCl challenge in HOCl-induced mice, HOCl-SSc mice treated with 2.5 × 10^5 at d42 (MSC_{42}), or control PBS mice. **(B)** Representative skin sections from HOCl-SSc mice and MSCs-treated HOCl-mice (MSC_{42}) (original magnification 10x, Masson trichrome staining). **(C)** Immunostaining with a-SMA (in red) and

(Continued)

FIGURE 8 | TGF-b1 (in green) in skin sections from HOCl-induced mice (left) and MSCs-treated HOCl-induced mice (right). **(D)** Immunostaining with CD3 (in red) and F4/80 (in green) in skin sections from HOCl-induced mice (left) and MSCs-treated HOCl-induced mice (right). **(E)** Quantification of immunostainings in C and D (percentage of positive cells among all cells observed; $n = 4$ per group). **(F)** mRNA expression of cytokines (*IL-1b, TNF-a, IL-6, IL-10,* left panel) fibrotic markers (*Col-1, Col-3, TGF-b1, a-SMA,* middle panel), and remodeling parameters (*MMP-1* and *TIMP-1,* right panel) in skin sections from HOCl-induced mice compared with HOCl-mice treated with 2.5×10^5 MSCs. Mean levels for control PBS-mice are represented by a discontinued line. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HOCl-mice; $\#P < 0.05$ vs. PBS-mice; data are presented as mean ± SEM. $N = 8$ per group.

expression, while IL1β and TNFα are associated with cell-mediated immunity, IL6 is also known to promote differentiation of fibroblasts into myofibroblasts (17) and to trigger collagen production (18). Moreover, at d21, we previously showed in this model upregulation of pro-inflammatory metalloproteases (MMP2 and MMP9, also known as gelatinases) and vascular endothelial growth factor [VEGF; (9)], which may activate angiogenesis and epithelial/endothelial-mesenchymal transition, contributing to myofibroblast activation and proliferation (19, 20). On the whole, the *primum movens* of fibrogenesis in HOCl-SSc is greatly related to inflammatory activation of skin tissue, leading to fibroblast proliferation, and preceding ECM synthesis.

In a second phase of HOCl-SSc induction, lasting from day 21 to day 42, we observed the progressive development of "matrix fibrosis," characterized at d42 by paucicellular skin tissue, with panparietal ECM deposition made of disorganized collagen fibers. At the end of the 6-wk experiment, skin fibrosis in SSc mice was characterized by strong ECM deposition made of disorganized collagen fibers, along with strong thickening of epidermis and dermis, destructuring all skin layers. This state of "established fibrosis," previously described in this model (9, 12) and responsible for skin thickening and stiffness, is close to what is usually observed in human SSc. Notably, as previously reported, tissue remodeling is low at d42, as indicated by decreased MMP1/TIMP1 ratio in tissue, contributing to impaired degradation of ECM components (9). Looking at the immune response, when comparing d42 to d21, we previously showed less inflammation and proliferation of cells, lower levels of inflammatory cytokines–except for IL-10 whose level increases during the 6-wk experiment. Of particular interest, dermal and hypodermal adipose tissue progressively disappeared during the whole process, as reported in the bleomycin model (21) and in human SSc (22), where it has been speculated that adipocyte attrition could even contribute to fibrogenesis through a putative adipocyte-to-fibroblast switch under PPAR-γ deregulation (23).

A spontaneous remission of SSc was observed once HOCl injections ceased, consistent with tissue remodeling activation and up-regulated MMP1/TIMP1 ratio. This third phase of fibrogenesis in HOCl-SSc had not been described before, and represents an original and interesting study model for SSc. As a matter of fact, clinical evolution in human scleroderma may be unpredictable, with patients first presenting with early and rapidly progressive diffuse SSc, but who may secondarily evolve with spontaneous improvement of skin fibrosis due to natural remodeling. Hence, this can somehow be disconcerting when considering therapeutic aspects, notably in the design of clinical trials (24, 25). Therefore, preclinical models reflecting disease heterogeneity and mimicking the different stages of fibrosis development are needed. In that perspective, HOCl-SSc, which reproduces three distinct phenotypes of scleroderma (i.e., early inflammatory phase, established matrix fibrosis, and spontaneous remodeling of tissue), seems particularly helpful to study new therapeutic approaches.

We previously reported the therapeutic benefits of a single IV infusion of 2.5×10^5 MSCs, capable of preventing fibrosis development in a preventive approach, or hampering fibrosis extension in a curative approach. In the present study, considering the three steps of fibrosis development and receding in the model, we focused on the effects obtained 3 weeks after MSCs infusion (i.e., d21, d42, d63). We demonstrated beneficial effects of MSCs treatment for each therapeutic strategy, in terms of skin thickness and histological lesions improvement. However, benefits were associated with more specific mechanisms. In the first setting, when infused at d0, MSCs exhibited a pre-eminence of immunomodulatory properties and were able to efficiently prevent immune response, resulting in an extinction of cellular inflammatory infiltrates together with a strong decrease in cytokine production at d21. These immunomodulatory capacities of MSCs principally involve paracrine mechanisms with the secretion of various soluble factors (i.e., IDO, iNOS, PGE2) down-regulating the immune system (8). In the second setting, when infused at d21, MSCs treatment was associated with less myofibroblast activation, and less ECM deposition at d42. These effects were previously shown to depend primarily on TGFβ signaling abrogation by MSCs, resulting in lower expression of TGFβ, TGFβRII and phospho-SMAD in the tissue (9). In the last setting, when infused at d42, after discontinuation of HOCl injections, MSCs were shown to enhance tissue remodeling, leading to enhanced MMP1/TIMP1 ratio and a more rapid clearance of fibrotic lesions. Interestingly, lost adipose tissue seemed to be partially restored during the process, suggesting regenerative potential of MSCs. Of note, MSCs were also able to prevent autoantibody production when infused at d0, indicating a specific inhibition of B-cell activation toward plasma cells. However, we could not observe any effect when mice were treated at d21, maybe because of the *in vivo* half-life of IgG (about 3–4 weeks).

Altogether, we observed benefits of MSCs treatment whatever the setting of infusion, in three distinct conditions of fibrosis development, through immunosuppressive, trophic or regenerative properties. This indicated that not only do MSCs possess wide and pleiotropic capabilities, but they also show adaptability and versatility depending on the surrounding pathological environment. Actually, it has been shown that according to the signals in the vicinity (hypoxia, ischemia, cytokine secretion…), MSCs would be primed differently, and polarized into a specific phenotype [i.e., immunosuppressive, trophic…; (8, 26)]. This plasticity in response to specific injury

makes MSCs-based therapy particularly interesting in treating SSc, considering the heterogeneity of the disease and the unpredictability of its evolution.

AUTHOR CONTRIBUTIONS

AM participated in the design of the study, acquisition, analysis and interpretation of data, manuscript redaction and final approval. KT, MM, PR, and M-CV participated in acquisition and analysis of data, manuscript proofreading and final approval. AL and CJ participated in the design of the study, interpretation of data, manuscript preparation and final approval. DN and PG carried out the conception and design of the study, participated in analysis and interpretation of data, manuscript redaction and final approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02571/full#supplementary-material

Supplementary Figure 1 | (A) Skin thickness measurement during experiment (PBS mouse). **(B)** Skin at injection site on the mouse back during experiment (d34), in PBS injected-mice, HOCl-injected mice, HOCl-injected mice receiving MSC infusion at d21 (from left to right).

Supplementary Figure 2 | (A) Representative lung sections at different time points during the induction of HOCl-SSc (Masson trichrome staining). **(B)** Lung sections from PBS mice and HOCl-SSc mice at d42 (red sirius staining).

Supplementary Figure 3 | Levels of anti-scl70 antibodies in sera from PBS-healthy mice, HOCl-SSc mice and HOCl-SSc mice treated with MSC at d0 **(A)** or at d21 **(B)**. $^{**}P < 0.01$, $^{***}P < 0.001$; data are presented as mean ± SEM. $N = 8$ per group.

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REVIEW
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Unraveling SSc Pathophysiology; The Myofibroblast

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Systemic sclerosis (SSc) is a severe auto-immune disease, characterized by vasculopathy and fibrosis of connective tissues. SSc has a high morbidity and mortality and unfortunately no disease modifying therapy is currently available. A key cell in the pathophysiology of SSc is the myofibroblast. Myofibroblasts are fibroblasts with contractile properties that produce a large amount of pro-fibrotic extracellular matrix molecules such as collagen type I. In this narrative review we will discuss the presence, formation, and role of myofibroblasts in SSc, and how these processes are stimulated and mediated by cells of the (innate) immune system such as mast cells and T helper 2 lymphocytes. Furthermore, current novel therapeutic approaches to target myofibroblasts will be highlighted for future perspective.

Keywords: myofibroblast, systemic scleroderma, immune system, treatment, cytokine

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INTRODUCTION

Systemic sclerosis (SSc) is a rare but severe auto-immune disease characterized by inflammation, vasculopathy and excessive fibrosis of connective tissues. Its incidence worldwide is on average an estimated 13 people per 1 million per year, with a prevalence of ∼200 people per 1 million (1). Risk factors include genetic predisposition (2), female sex (3), and exposure to environmental cues such as chemicals like silica or solvents (4), but its etiology remains poorly understood. The excessive fibrosis characteristic for SSc typically starts distally in the skin of the extremities and moves upwards toward and trough the trunk until it greatly negatively affects the function of many organs like the gastro-intestinal tract and lungs. SSc is therefore accompanied by a high morbidity and patients often require extensive medical care with a (severely) reduced quality of life (5). Mortality is also increased in SSc patients. On average, the standard mortality rate of all causes is 2.7, with lung involvement being the major cause of death (6). Furthermore, the estimated loss of life-expectancy for patients is more than 15 years (7). Unfortunately, to date, no targeted disease-modifying therapy is available, resulting in a large unmet medical need. Because of this need, SSc has been designated an orphan disease to support research and development of a treatment.

The lack of targeted therapy for SSc is partly due to a lack of understanding of its pathophysiology. Its pathophysiology is a complex interplay between endothelium, the innate and acquired immune system, target organs and connective tissue which culminates in excessive fibrosis of e.g., skin and internal organs. A key cellular player in many fibrotic conditions such as keloid formation, Dupuytren's contracture and post-operative scarring is the myofibroblast, which is a special type of fibroblast. In this review we will discuss the role of myofibroblasts in SSc, their formation and how these cells are at the center of SSc pathophysiology, by regulating many of this disease's aspects.

ON THE MYOFIBROBLAST AND ITS BIOLOGICAL FUNCTION

Myofibroblasts were first identified in granulation tissue during open wound healing, as cells that resembled fibroblasts but contained microfilaments in their cytoplasm similar to those of smooth muscle cells (8, 9). Subsequently, it was demonstrated that these cells have contractile properties and are key in open wound closure (9). Myofibroblasts facilitate wound healing in several ways (**Figure 1**); First, they are capable of producing large amounts of extra cellular matrix (ECM) molecules such as collagen type I, collagen type III and fibronectin to replace lost ECM. Secondly, myofibroblasts are contractile. Their microfilaments (also known as stress fibers) consist of alpha smooth muscle actin (αSMA) and non-muscle myosin type II (10) and can contract in typical actin-myosin fashion, albeit rather slowly compared to muscle actin myosin filaments. Thirdly, myofibroblasts strongly connect physically to their environment; via integrin-mediated focal adhesions and cadherin-mediated adherens junctions their actin cytoskeleton is strongly anchored to their surrounding ECM and neighboring cells, respectively (11). The combination of this strong connection to the environment with their ability to contract allows myofibroblasts to exert tension on their surroundings and contract (damaged) tissue. This contraction decreases wound size and is crucial for open wound healing. Long term wound healing is further supported by myofibroblasts via their ability to strengthen the ECM; myofibroblasts express several protein and collagen crosslinking enzymes such as protein-glutamine gamma-glutamyltransferase 2 (= transglutaminase 2), protein-lysine 6-oxidase (LOX), and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) (12). These enzymes help strengthen e.g., fibrillar collagen bundles by post-translationally modifying collagen molecules, which results in increased crosslinking of these molecules in collagen networks during the maturation phase of wound healing. These crosslinks increase this networks' strength and prevents enzymatic degradation and thus strengthen the (scar) tissue.

Myofibroblasts also secrete and/or activate various autocrine and paracrine mediators to facilitate wound healing. For example, myofibroblasts produce vascular endothelial growth factor (VEGF) (13). This polypeptide growth factor is key in the formation of new blood vessels. Furthermore, myofibroblasts produce endothelin 1, a potent vasoconstrictor but also a factor which stimulates the formation of new myofibroblasts (14) and enhances their function in regard to collagen production and contractile properties (15). Myofibroblast function is also enhanced by their production of connective tissue growth factor (CTGF), a matricellular protein which stimulates e.g., their formation and collagen type I production. A key growth factor which is produced (13) and potently activated by myofibroblasts is transforming growth factor β (TGFβ) (16). This polypeptide growth factor is strongly pro-fibrotic and stimulates myofibroblast formation and activity. TGFβ is produced in latent form [bound by latency associated peptide (LAP) and latent TGFβ binding proteins (LTBP)] but can efficiently be activated by myofibroblasts via an integrin-mediated process (16, 17). Of note, TGFβ induces the expression of ET-1, CTGF, and VEGF in myofibroblasts, indicating that this growth factor lays at the heart of the expression of these factors. In addition, myofibroblasts can produce a range of various cytokines and chemokines to aid in the recruitment and facilitate the function of (innate) immune cells (13). Most notably, they produce interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), and monocyte chemoattractive protein 1 (MCP-1) (13).

Together these abilities make myofibroblasts well suited to facilitate wound healing.

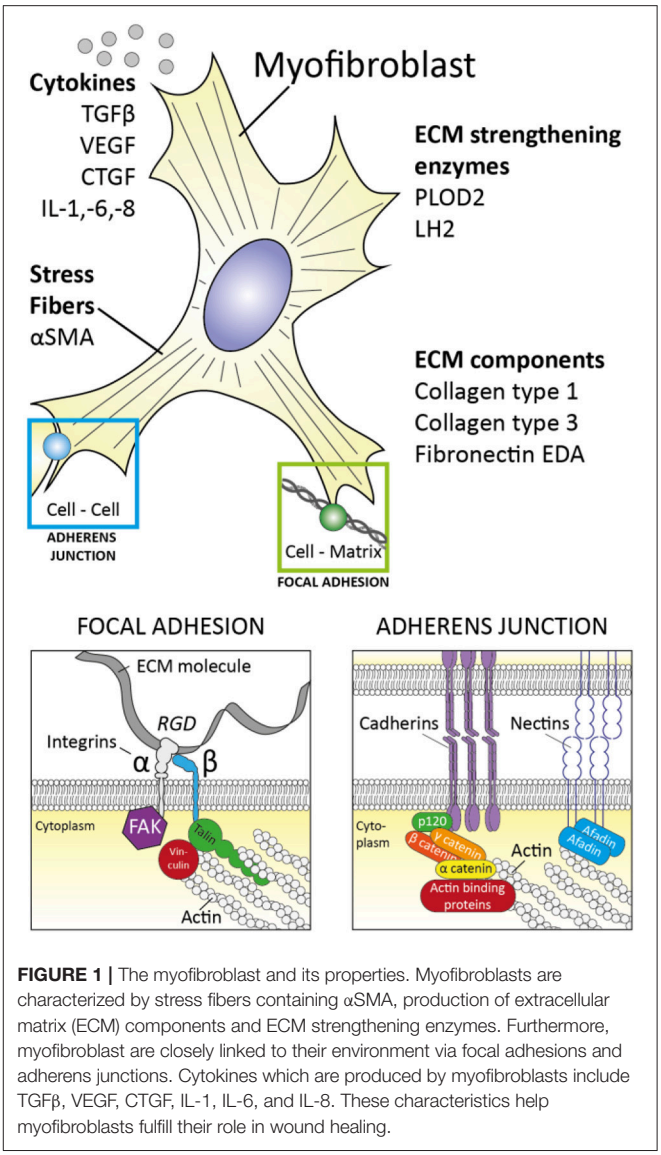


FIGURE 1 | The myofibroblast and its properties. Myofibroblasts are characterized by stress fibers containing αSMA, production of extracellular matrix (ECM) components and ECM strengthening enzymes. Furthermore, myofibroblast are closely linked to their environment via focal adhesions and adherens junctions. Cytokines which are produced by myofibroblasts include TGFβ, VEGF, CTGF, IL-1, IL-6, and IL-8. These characteristics help myofibroblasts fulfill their role in wound healing.

ON THE PRESENCE OF MYOFIBROBLASTS IN SSC

Myofibroblasts have long been associated with SSc pathophysiology (18). Already in 1972 it was identified that

fibroblasts obtained from SSc skin have a pro-fibrotic phenotype and produce more collagens than control fibroblasts (19). In 1990 it was confirmed using immunohistochemistry that fibroblasts of SSc patients near lesional areas in skin, esophagus, and lungs contain alpha smooth muscle actin (20) and are thus myofibroblasts.

In skin, the presence of myofibroblasts correlates with the amount of (hyalinized) collagen and skin parameters related to fibrosis such as tightness, hardness and stiffness, and does so more significantly than inflammation (21–23), supporting for a role of myofibroblasts in the pathogenesis of these clinical signs. This skin thickening and hardening can occur to such extent that it impairs movement of e.g., fingers. Furthermore, excessive matrix deposition leads to loss of tissue architecture such as sweat glands and hair follicles.

In lungs of SSc patients, the presence of myofibroblasts in the interstitial space can already be observed early during the fibrotic process (24), and with progression of interstitial lung disease they can ultimately also be observed in bronchoalveolar lavage liquid of SSc patients (25). The presence of pathological myofibroblasts greatly negatively affects lung function. Their matrix producing ability destroys alveolar architecture and increases interstitial space thickness, which both hamper respiration. Furthermore, the presence of myofibroblasts can induce stenosis; the abnormal narrowing of bloodvessels, and blood vessel narrowing is further enhanced by myofibroblasts' expression of ET-1, a potent vasoconstrictor. This hampers pulmonary blood flow, and as a consequence induces strain on the right heart ventricule.

Another location where myofibroblasts can be detected in SSc is in the esophagus and gastric wall of patients with severe fibrosis (26). Here, myofibroblast presence results in loss of muscle function, making these tissues unable to contract. As a consequence, gastric acid can flow into the esophagus, causing gastro-oesophageal reflux disease.

Together, these observations place myofibroblasts in the various organs that can be affected by SSc. In addtion, organs such as kidney, intestine and myocard can also be affected by myofibroblast-driven fibrosis in SSc (18). However, of note, in late stage fibrotic atrophic SSc skin these cells can no longer be detected (27). **Figure 2** gives an overview of the location of myofibroblasts in SSc.

In healthy tissues, the presence of myofibroblasts is (very) rare due to the tendency of myofibroblasts to undergo apoptosis when they are no longer needed for the healing process (28, 29). However, a putative resident type of myofibroblast can be found in lung alveolar ducts, where they help regulate alveolar function. In contrast, in SSc their presence is unwanted and attributed to a lowered susceptibility of myofibroblasts to undergo apoptosis and to increased formation.

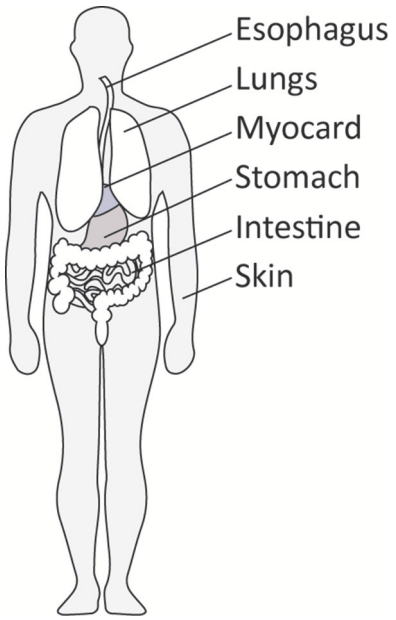


FIGURE 2 | Organs commonly affected by diffuse cutaneous SSc.

DECREASED APOPTOSIS OF MYOFIBROBLASTS IN SSC

Two major pathways govern cellular apoptosis; the intrinsic and extrinsic pathway. The extrinsic pathway is induced by activation of fas cell surface death receptor (Fas). Fas is a membrane spanning receptor of the TNF receptor superfamily and can, upon binding of Fas ligand, trigger the formation of a death-inducing signaling complex (DISC). This complex subsequently activates apoptosis-initiator caspase 8 to start a caspase pathway ultimately culminating in activation of caspase-3 and apoptosis (**Figure 3**). The intrinsic pathway is triggered by release of cytochrome c from mitochondria, which is subsequently incorporated into apoptosomes, cellular structures which activate the apoptosis-initiator caspase-9 to initiate apoptosis (30). A key protein in release of cytochrome c from mitochondria is BCL2-associated X protein (BAX), which, upon oligomerization, forms pores in the mitochondrial membrane through which cytochrome c can leak (31). Two important inhibitors of BAX are BCL2 and BCL2-X_L (also known as BCL2L1), which both prevent oligomerization of BAX and are thus anti-apoptotic. Of note, the extrinsic and intrinsic pathways are not fully discrete but linked, for example via BH3 interacting domain death agonist (BID), a protein which is activated by caspase 8 and subsequently forms mitochondrial membrane pores in cooperation with BAX (32). Ultimately, whether cells like myofibroblasts undergo apoptosis is determined by the ratio of activity between pro-apoptotic mitochondrial membrane pore forming proteins (e.g., BAX) and their anti-apoptotic inhibitors (e.g., BCL2). Pro-survival signaling can skew this balance in favor of anti-apoptotic proteins.

In systemic sclerosis, myofibroblasts are less prone to undergo apoptosis for several reasons. To begin, it has been observed that, in quiescent state, SSc myofibroblasts express less pro-apoptotic BAX compared to myofibroblasts of control subjects (33). A possible cause for this is increased activity of tyrosine-protein kinase ABL1 (c-Abl). Silencing of c-ABL enhances apoptosis in both healthy and SSc skin fibroblasts by increasing the

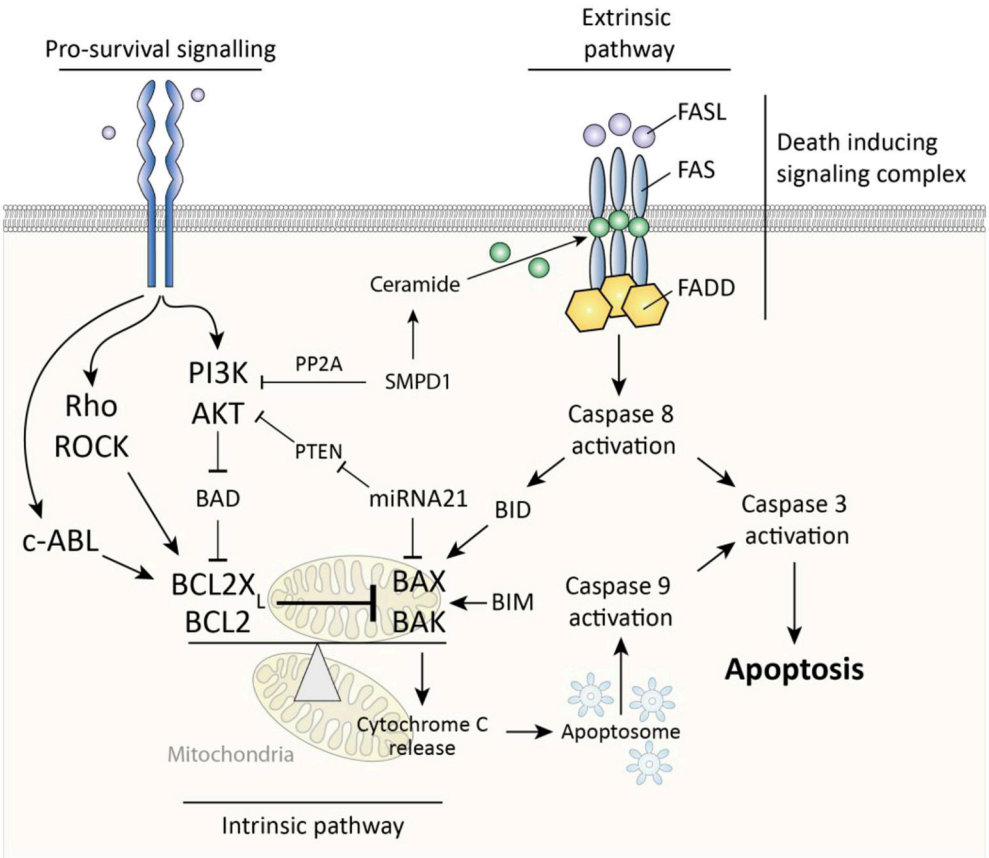


FIGURE 3 | Caspase-dependent apoptosis pathways in myofibroblasts. The extrinsic pathway is activated via death inducing signaling complex and results in caspase 8-mediated caspase 3 activity which results in apoptosis. The intrinsic pathway is triggered by cytochrome c release from mitochondria which results in caspase 9-mediated caspase 3 activity. This cytochrome c release is governed by the ratio between pro-apoptotic BAX/BAK and BCL2(X_L). Pro-survival signaling affects this ratio in favor of BCL2(X_L).

BAX/BCL2 ratio toward pro-apoptotic BAX (34). An example of how c-ABL can be activated is via TGFβ signaling; in idiopathic pulmonary fibrosis, c-Abl is activated by TGFβ (35), and silencing of c-Abl inhibits the pro-survival effects of TGFβ on myofibroblast apoptosis (34).

Secondly, in fibrotic tissues, extracellular matrix stiffness is increased compared to healthy tissue. This increased stiffness is an important survival signal for myofibroblasts; via mechanosensing such stiffness results in intracellular activation of Rho and Rho-associated kinase (ROCK) whose activity increases BCL2-X_L expression (36). Importantly, this increased, stiffness-induced, BCL2-X_L expression is needed to counteract the function of the pro-apoptotic protein BIM (36). BIM is an activator of BAX and accumulates in myofibroblasts exposed to a stiff matrix. This accumulation primes the cells to undergo apoptosis (36), and only the continued presence of BCL2-X_L prevents this. This balance between BCL-2 and BIM serves a role during normal wound healing; once the matrix softens during the final wound remodeling stage, pro-surivival ROCK signaling drops, resulting in loss of BCL-2 expression, and rapid BIM-mediated apoptosis of myofibroblasts (36). Recently, it has been shown that pharmacological inhibition of BCL2-X_L can mimic this process and induce targeted BIM-mediated apoptosis in myofibroblasts and even revert established (murine) fibrosis (36).

In addition, in SSc skin, phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) signaling (37) is increased. This pathway facilitates myofibroblasts survival by inhibiting the activity of BAX. It does so by inactivating bcl2-associated agonist of cell death (BAD) via phosphorylation, after which this protein can no longer inhibit the function of anti-apoptotic proteins such as BCL2-X_L. Many growth factors can induce PI3K/AKT signaling, including TGFβ. TGFβ signaling is increased in skin of SSc patients, and TGFβ has been demonstrated to induce AKT signaling in dermal fibroblasts to lower myofibroblasts' sensitivity for Fas-mediated apoptosis (34, 37, 38). Furthermore, TGFβ signaling also lowers expression of acid sphingomyelinase (SMPD1) (39). This enzyme induces the activation of protein phosphatase 2 (PP2A), i.e., an inhibitor of AKT signaling, and a reduction in SMPD1 thus enhances pro-survival AKT signaling. Additionaly, SMPD1 facilitates Fas-dependent apoptosis via its product; i.e., the lipid ceramide, which helps cluster Fas at the cell membrane, thus facilitating

the formation of death inducing signaling complexes (40). In SSc fibroblasts, it has been shown that TGFβ lowers Fas-mediated apoptosis and that overexpression of SMPD1 prevented this effect, indicating its importance (39).

Finally, a role for micro RNAs (miRNA) in protecting myofibroblasts against apoptosis has been described in SSc. miRNAs are small non coding RNA molecules that can bind messenger RNAs and induce their degradation via an RNA-induced silencing complex (RISC). In SSc skin, expression of miRNA21 is increased, and this miRNA targets and degrades pro-apoptotic BAX mRNA (41). Additionally, miRNA21 targets phosphatase and tensin homolog (PTEN), which is an inhibitor of AKT signaling, as this phosphatase lowers intracellular PIP_3 levels, the activator of AKT signaling (38). Via these mechanisms, presence of this miRNA lowers cellular sensitivity to apoptosis. Notably, TGFβ induces expression of miRNA21 in fibroblasts (38).

Together these mechanisms protect myofibroblasts from apoptosis in SSc which, in contrast to their final loss during wound healing, ensures their continued presence (long) after their formation.

ON THE FORMATION OF MYOFIBROBLASTS IN SSC: PATHWAYS

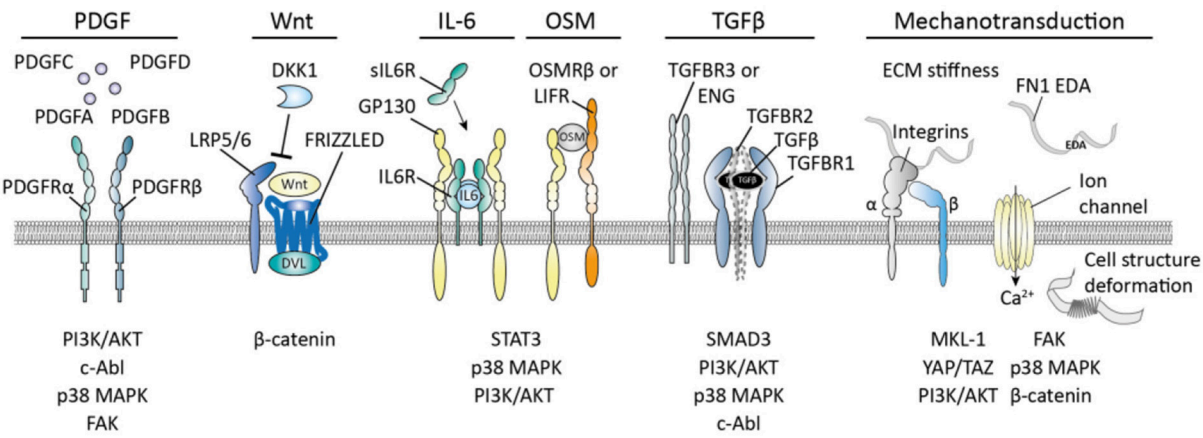
In SSc, not only the apoptosis of myofibroblasts is decreased but also their formation is increased. Myofibroblasts can originate in several ways, including the differentiation of fibroblasts toward myofibroblasts. This process is key in normal wound healing and facilitated by growth factors such as TGFβ, Wnts, damage associated molecular patterns such as fibronectin cloths, and tissue stiffness; the stiffer the matrix the more prone fibroblasts are to become myofibroblasts (42). In **Figure 4** several intracellular pathways are listed that are involved in the transition of fibroblasts to myofibroblasts.

To begin, a key growth factor for myofibroblast formation is TGFβ; this growth factor directly induces extracellular matrix production and αSMA expression in fibroblasts. TGFβ activity is increased in skin of SSc patients, just as expression of its activating integrin αVβ5 (43, 44). This integrin can recognize latent TGFβ via its RGD domain and can mechanically separate the latency conferring peptides from the active peptide (42). The importance of integrin-mediated TGFβ activation is illustrated by the observation that inhibition of integrin αVβ5 by the use of antibodies or antisense RNA inhibits myofibroblasts formation (43, 44). Various intracellular pathways play a role in establishing the effects of TGFβ, in particular: SMAD3, PI3K/AKT, p38 MAPK, and c-ABL. Overexpression of SMAD3 enhances, whereas knockdown inhibits αSMA and extracellular matrix production in fibroblasts (45–48). Furthermore, fibroblast-specific deletion of SMAD3 reduces αSMA production and myofibroblast phenotype (49–52), for example, loss of SMAD3 lowers the number of activated myofibroblasts in cardiac fibrosis *in vivo* and reduces extracellular matrix production by myofibroblasts (47). Inhibition of PI3K/AKT signaling inhibits TGFβ-mediated myofibroblast formation, whereas overexpression of a constitutively active form of AKT1 enhances myofibroblasts development. The use of p38 MAPK inhibitors also lowers TGFβ-induced collagen type I and αSMA production and prevents TGFβ-induced AKT signaling (53–55). Additionally, this pathway alters cellular energy metabolism in such a way that is facilitates cellular contraction (56). Finally, in fibroblasts lacking c-ABL the expression of extracellular matrix molecules and αSMA is reduced in response to TGFβ. Of note, TGFβ can also negatively affect myofibroblasts. For example, SMAD3 can inhibit cellular proliferation via lowering the expression of c-myc and preventing the progression of cell division from G1 to S phase (57). Furthermore, pre-treatment of granulation tissue (myo) fibroblasts with TGFβ enhances their sensitivity to undergo bFGF-mediated apoptosis (58). This last observation illustrates that cellular context, e.g., the presence of bFGF, can greatly impact TGFβ signaling outcome.

Importantly, TGFβ facilitates the function of various other growth factors in fibroblasts. In SSc skin fibroblasts, TGFβ makes fibroblasts more sensitive to anabolic stimulation with platelet derived growth factor (PDGF), via induction of its receptor (PDGFR) (59). This growth factor induces extracellular matrix production and proliferation via the activation of PI3K/AKT, p38 MAPK, c-ABL, and focal adhesion kinase (FAK) pathways. In addition, this last pathway regulates PDGF-induced migration of myofibroblasts which recruits myofibroblasts to fibrotic areas (60). TGFβ and PDGF can work in concert, for example, in mouse corneal stromal fibroblasts co-stimulation of fibroblasts with TGFβ and PDGF greatly enhances myofibroblast formation compared to TGFβ alone (61).

Another pathway enhanced by TGFβ in SSc is canonical Wnt signaling. TGFβ signaling via p38 MAPK lowers dickkopf-1 (DKK1) expression (62), which is an inhibitor of canonical Wnt signaling via β-catenin. In skin and fibroblasts of SSc patients, decreased DKK1 expression is observed (62), together with increased β-catenin accumulation (63), and increased expression of Wnt signaling-related genes (62, 64). Stimulation of fibroblasts with canonical Wnts such as Wnt-1 or Wnt3a upregulates collagen type 1 and αSMA expression, and does so to a similar extent as TGFβ. Furthermore, mice with continuous fibroblast-specific Wnt signaling by artificial β-catenin stabilization rapidly develop skin fibrosis whereas fibroblast-specific deletion of β-catenin protects mice from bleomycin-induced skin fibrosis (63). Notably, Wnt signaling can induce autocrine TGFβ signaling (64) and overexpression of DKK1 protects mice even against TGFβ-receptor mediated skin fibrosis, indicating that both pathways are closely interwoven and interdependent.

Apart from the abovementioned effects, TGFβ can induce the expression of fibronectin 1 extra domain A (FN1 EDA) in (myo) fibroblasts. FN1 EDA is a splice variant of fibronectin which contains the so-called EDA domain. Normally, FN1 EDA is not expressed in healthy tissues but its expression is induced during wound healing (65) Fibroblasts can detect FN1 EDA via membrane bound receptors such as α4 containing integrins or toll like receptor 4 (TLR4), and its presence is a prerequisite for TGFβ-mediated myofibroblast formation; its expression precedes αSMA expression, and mice that lack their FN1 EDA domain are unable to produce myofibroblasts during injury (65–67). In turn,



Molecule	Effect	References
β-catenin	Enhances pro-fibrotic gene expression, proliferation, migration and contractile properties of fibroblasts Mice with fibroblast-specific stabilization of β-catenin rapidly develop fibrosis Fibroblast-specific deletion of β-catenin significantly reduces bleomycin-induced dermal fibrosis Enhances the function of MRTF by inhibiting the inhibitory role of SMAD3 on MRTF	[62; 63; 71]
c-Abl	In fibroblasts lacking c-Abl, the expression of collagen type 1, fibronectin, αSMA, and CTGF is reduced in response to TGFβ c-Abl silencing decreases the anti-apoptotic effect of TGF-β1 on BAX to BCL-2 ratio	[33; 207 208; 209]
FAK	Pharmacological inhibition of FAK lowers TGFβ1 induced αSMA expression FAK activation is a critical step in mediating fibroblast migration over fibronectin in response to PDGF-BB The absence of FAK leads to unregulated myofibroblast differentiation due to loss of FGFR signaling	[59; 96; 198; 210]
MKL-1	Knockdown of MKL-1 lowers αSMA expression in cells grown on a stiff matrix Constitutively-active MKL-1 increases αSMA expression in cells grown on soft matrix Overexpression of MKL-1 induces cellular contraction and collagen type 1 and αSMA expression Knockout of MKL-1 lowers TGFβ1-mediated SMAD3-induced gene expression epigenetically	[67-70; 75]
p38 MAPK	p38 inhibitors lower TGFβ-induced collagen type 1, fibronectin, and αSMA production p38 MAPK activity lowers DKK1 expression, enhancing Wnt signaling Overexpression of a dominant negative p38 MAPK form inhibits myofibroblast proliferation p38 MAPK inhibition or expression of kinase-deficient p38 inhibits TGFβ-induced AKT signaling p38 MAPK enhances energy metabolism which facilitates cellular contraction and αSMA expression	[52 - 55; 61; 116; 211]
PI3K/AKT	Inhibition of PI3K inhibits TGFβ-mediated myofibroblast differentiation Expression of a dominant-negative form of AKT1 inhibits TGFβ-mediated myofibroblast differentiation Constitutively active AKT1 stimulates αSMA expression and myofibroblast differentiation Protects against apoptosis via inhibition of BAD PI3K/AKT/MTOR inhibition bleomycin-induced fibrosis lowers fibroblast proliferation and collagen type 1 production	[48 - 51; 212]
SMAD3	Fibroblast-specific Smad2/3 deletion in activated fibroblasts reduces fibrosis and ECM-related gene expression SMAD3 binds to αSMA promotor and overexpression enhances αSMA whereas knockdown decreases αSMA expression Overexpression of SMAD7 or dominant negative SMAD3 decreases αSMA expression SMAD3 interacts with SRF to induce myofibroblast gene expression Cardiac fibroblasts of SMAD3 null mice show reduced myofibroblast phenotype but have increased proliferation Directly interacts with MKL-1, which inhibits MKL-1 function on αSMA Can inhibit proliferation via inhibition of cMyc transcription and CDK2 and CDK4 activity	[44 - 47; 56; 71; 213]
STAT3	Inhibition of STAT3 (siRNA, pharmacological, dominant negative) inhibits collagen type 1 production, cell proliferation, cell migration and cell contraction STAT3 blockade lowers fibroblast responsiveness to exogenous TGFβ1 STAT3 blockade decreases resistance to apoptosis by lowering BCL-2 Overexpression of constitutively activated STAT3 protects fibroblasts against apoptosis Antisense oligonucleotides to STAT3 inhibit IL-6-induced BAX expression in healthy fibroblasts IL-6 and OSM downregulate TGFβ1–induced αSMA expression in a STAT3-dependent mechanism	[80; 81; 214 - 217]
YAP/TAZ	On stiff matrixes, knockdown of YAP and TAZ lowers: collagen type 1 synthesis, proliferation, contractile force and increases pro-apoptotic caspase3/7 activity Overexpression of dominant negative YAP inhibits TGFβ-mediated myofibroblast formation YAP1 knockdown lowers TGFβ-mediated αSMA expression, cell contraction and collagen type 1 production TAZ mitigates nuclear accumulation of MRTF Increases matrix stiffness by induction of serpine1 expression, an inhibitor of plasmin	[72 -75]

FIGURE 4 | Continued.

FIGURE 4 | Stimuli for myofibroblast formation and their intracellular pathways. The four variants of platelet-derived growth factor (**PDGF**) can interact with homo- or heterodimers of PDGF receptor alpha (PDGFRα) and beta (PDGFRβ) to induce signaling by: phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT), p38 mitogen-activated protein kinases (p38 MAPK), focal adhesion kinase (FAK) and tyrosine-protein kinase ABL1 (c-ABL). Canonical **Wnt** signaling is activated via formation of a wnt/frizzled/LRP5/6 complex which recruits disheveled (DVL) to the plasma membrane. This inhibits β-catenin degradation, leading to the accumulation of this protein and subsequent signaling. Interleukin 6 (**IL-6**) signaling uses a complex of membrane-bound or soluble IL-6 receptor (IL-6R) and glycoprotein 130 (gp130) to activate PI3K/AKT, p38MAPK and signal transducer and activator of transcription 3 (STAT3) signaling. Oncostatin M (**OSM**) also uses gp130, but together with oncostatin M receptor beta (OSMRβ) or leukemia inhibitory factor receptor (LIFR). Transforming growth factor beta (**TGFβ)** induces heterotetramerization of TGFβ-receptor type I (TGFBR1) and II (TGBR2) and results in intracellular activation of SMAD3, p38 MAPK, PI3K/AKT c-ABL. TGFβ-receptor type III receptors such as betaglycan (TGFBR3), and endoglin (ENG) guide TGFβ availability and receptor complex formation. **Mechanotransduction** can occur via mechanosensitive ion channels, leading to e.g., calcium ion (Ca^{2+}) influx, integrin complexes and deformation of cellular structures, leading to activation of myocardin-like protein 1 (MLK1), β-catenin, FAK, p38 MAPK, PI3K/AKT, and yes-associated protein 1 (YAP)/WW domain-containing transcription regulator protein 1 (TAZ). The effects of each of these pathways are listed in the table. Note that not all intracellular pathways are listed for each stimulus, only those connected to myofibroblast formation.

FN1 EDA facilitates the mechanical activation of TGFβ because it binds the latent form of TGFβ and presents this to integrins.

Next to these aforementioned stimuli, cellular mechanosensing is another crucial element in the transition of fibroblasts to myofibroblasts. Via for example intergrins, mechanosensitive ion channels, and cell structure deformation, fibroblasts can sense mechanical cues such as matrix stiffness. This mechanosensing results in activation of various intracellular pathways such as FAK, PI3K/AKT, p38 MAPK, and β-catenin, and activation of transcription activators such as myocardin-like protein 1 (MKL-1) and transcriptional coactivator YAP1 (YAP1) and WW domain-containing transcription regulator protein 1 (TAZ). Both MKL-1 and YAP/TAZ directly regulate myofibroblast phenotype. Knockdown of MKL-1 lowers αSMA expression in cells grown on a stiff matrix whereas overexpression of a constitutively active form of MKL-1 increases αSMA expression in cells grown on a soft matrix (68, 69). MKL-1 also activates collagen type 1 expression in lung fibroblasts (70). Furthermore, MKL-1 interacts with SMAD3 to bind the promoters of collagen type I and ASMA, and knockdown of MKL-1 lowers SMAD3-dependent gene expression (71). However, this interaction with SMAD3 can result in more rapid degradation of MKL-1, leading to repression of MKL-1-dependent genes (72). β-catenin has been shown to counteract this effect of SMAD3 (72), indicating that MKL-1 function depends on the integration of various pathways. Knockdown of YAP/TAZ in fibroblasts that are grown on stiff matrixes lowers proliferation, collagen type 1 synthesis, contractile force and increases pro-apoptotic caspase3 and caspase 7 activity. Furthermore, knockdown of YAP or overexpression of a dominant negative form lowers TGFβ-mediated myofibroblast formation (73–76). Notably, YAP/TAZ influence matrix stiffness by directly inducing serpine1 expression (73). Serpine1 inhibits the activation of plasmin, a protease which degrades extracellular matrix molecules such as fibrin and fibronection and can activate collagenases. Plasmin activity thus degrades and softens the extracellular matrix, but YAP/TAZ activity counteracts this (73) of note, serpine1 expression can also be rapidly and highly induced by TGFβ (77), and mechanical activation of TGFβ is enhanced in stiffer matrixes (42). Both YAP/TAZ and TGFβ activity can thus result in a feed forward loop in which tissue stiffness results in tissue stiffness-enhancing activity. Such a mechanism can explain continued fibrosis in absence of a exogenous stimulus.

Finally, the transition of fibroblasts to myofibroblasts is also facilitated by intracellular STAT3 signaling. STAT3 is induced by various cytokines such as interleukin 6 (IL-6) and oncostatin M (OSM). IL-6 expression is strongly expressed in SSc skin fibroblasts (78), and *in vitro*, stimulation of SSc skin fibroblasts with IL-6 results in collagen and αSMA expression (78–80). Furthermore, in the murine bleomycin model for skin fibrosis, knockout of IL-6 reduces skin pathology, as does administration of an anti-IL-6 receptor antibody (MR16-1) (79). In SSc skin, STAT3 signaling is activated (81) resulting in pro-fibrotic gene expression in fibroblasts; for example, STAT3 regulates collagen type I expression in SSc skin fibroblasts (82). However, of note, in lungs of SSc patients no enhanced STAT3 activation can be observed (82). Importantly, in both bleomycin induced skin and lung fibrosis in mice, knockout or pharmacological inhibition of STAT3 ameliorates fibrosis (83) (81). Furthermore, in both models, STAT3 was shown to be downstream of TGFβ signaling, as inhibition of STAT3 prevented TGFβ-induced myofibroblasts formation (81, 83).

Together these pathways can mediate the transition of fibroblasts to myofibroblasts and direct myofibroblasts activity after formation but cellular context plays an important role in guiding the outcome.

ON THE FORMATION OF MYOFIBROBLASTS IN SSC: CELLS

Apart from the transition of fibroblasts to myofibroblasts, an important source of myofibroblasts in SSc is the transdifferentiation of other cell types (**Figure 5**).

To begin, one cell type that can function as a source of myofibroblasts is the pericyte. These contractile cells surround endothelial cells in the microvasculature and regulate blood flow. Pericytes already express αSMA, and can become myofibroblasts if they leave their cellular niche and start to express proteins such as collagen type I and FN1-EDA. That this process occurs in SSc is suggested by a study that shows that pericytes in SSc skin, but not in healthy skin, express FN1-EDA and other myofibroblast markers (27). Furthermore, using lineage tracing it has elegantly been demonstrated that perivascular cells end up in skin scars as myofibroblasts (84). In addition, this transition is also observed in lung, liver, and kidney fibrosis (85), indicating that pericyte to myofibroblast transition is a common aspect of many fibrotic

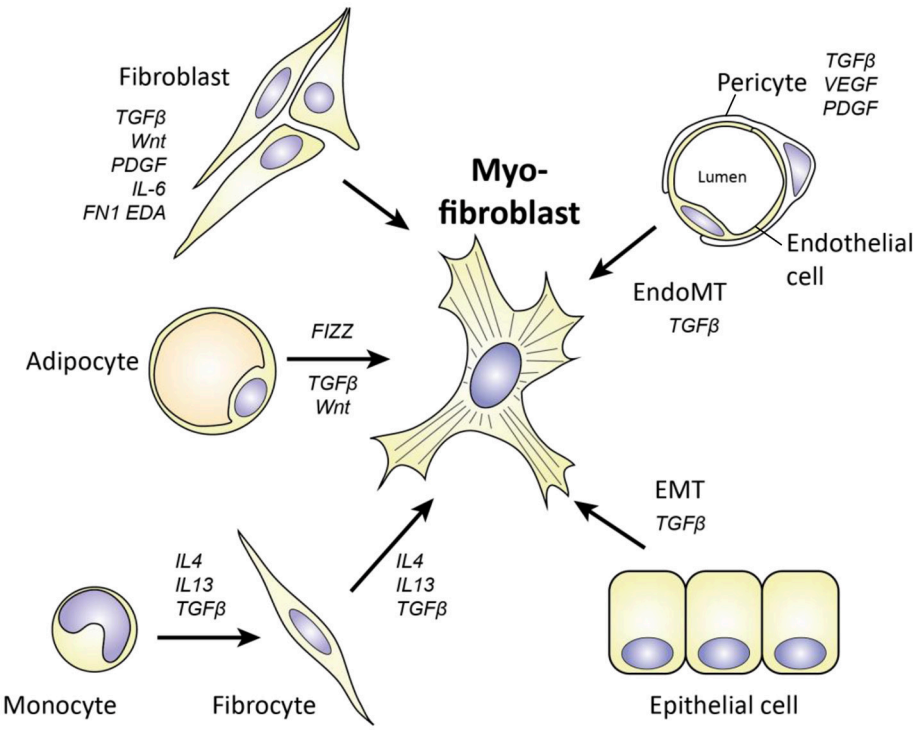


FIGURE 5 | Cellular origins of myofibroblasts in SSc. Myofibroblasts can originate from various cell types, including fibroblasts, adipocytes, monocytes/fibrocytes, pericytes, endothelial cells, and epithelial cells. Key molecules for each transition are depicted. For epithelial cells to become myofibroblasts, they have to undergo epithelial to mesenchymal transition (EMT). For endothelial cells a similar process is needed, called endothelial to mesenchymal transition (EndoMT).

disorders. Putative drivers of this transition are VEGF, PDGF, and TGFβ.

Another cell type which can give rise to myofibroblasts is the fibrocyte. Fibrocytes are circulating cells of myeloid origin with stem cell like characteristics. These cells were first identified as the myeloid cells that rapidly invade wounds and, in contrast to other myeloid cells, produce ECM molecules. Their migration to wounds is guided by damage associated molecular patterns (DAMPs) and chemokines such as Chemokine (C-C motif) ligand 21 (CCL21) (86), and after arrival, these cells start differentiating into a myofibroblast-like phenotype under the influence of factors such as TGFβ (86). Of note, fibrocytes can originate from monocytes, and, importantly, SSc monocytes display increased maturation toward myofibroblasts as indicated by αSMA expression when compared to monocytes from healthy controls (87). Furthermore, fibrocyte presence and involvement in pulmonary fibrosis can readily be detected in SSc (87). Paradoxically, fibrocyte numbers in blood are lower in SSc patients than in healthy controls. Possibly, these cells are recruited out of the blood compartment into affected areas which would explain their lower numbers in blood.

In addition to the abovementioned cells, adipocytes, i.e., fat cells, are another source of myofibroblasts in SSc. Via the process of adipocyte to myofibroblast transition these cells can become myofibroblasts. In SSc skin, subcutaneous fat disappears over the course of the disease (88). With the use of adiponectin-lineage tracking, it has been demonstrated in the murine bleomycin model of skin fibrosis that adipocytes can lose their adipocyte-related gene expression and start expressing αSMA to become myofibroblasts (88). Importantly, in this model of skin fibrosis the loss of fat tissue precedes fibrosis (88) indicating that this process can underlie the fibrotic process. Adipocyte to myofibroblast transition is strongly driven by TGFβ (88), Found in inflammatory zone 1 (FIZZ1) and possibly Wnt signaling (89). *In vitro*, FIZZ1 suppresses adipogenesis and stimulates myofibroblast differentiation via Notch1 signaling. Furthermore, mice lacking FIZZ1 retain more fat and develop less fibrosis in response to bleomycin skin injury (90). Of note, FIZZ1 has also been attributed a role in lung fibrosis, by recruiting bone marrow derived stem like cells like to damaged lung tissue (91), and its levels are increased in serum of SSc patients (90).

Finally, two important sources of myofibroblasts in SSc are epithelial to mesenchymal transition (EMT) and endothelial to mesenchymal transition (EndoMT). In both processes, respectively epithelial and endothelial cells lose their phenotype and become myofibroblasts. Both processes can be observed in SSc. EndoMT can be identified using immunohistochemistry by observing endothelial cells with both endothelial (cluster of differentiation (CD31, and VE-cadherin) and myofibroblast markers (αSMA), and has been observed in skin and in lungs of SSc patients (92, 93). Furthermore, EndoMT has been linked to endothelial dysfunction as a cause for pulmonary arterial hypertension, a major complication in SSc (94). Notably, endothelial cells that undergo EndoMT produce more IL-6, IL-8

and TNFα compared to normal endothelial cells (94). EMT is an important driver of lung fibrosis, in which alveolar epithelial cells become myofibroblasts (95). This was demonstrated using alveolar specific lineage tracking, which visualized that alveolar cells started to express αSMA upon overexpression of TGFβ1 (95). The role of EMT in skin fibrosis is less clear. In SSc skin, expression of the key EMT inducing transcription factor SNAI1 can be observed in keratinocytes, but not loss of their epithelial E-Cadherin marker (96). Possibly, the EMT process is therefore only partially evoked here.

In conclusion, myofibroblasts can originate from many sources in SSc. Possibly, their origin has an effect on their phenotype and function, yet little is known if this is the case.

ON INCREASED ACTIVITY OF MYOFIBROBLASTS IN SSC

Because of reduced apoptosis and increased formation, myofibroblasts numbers are increased in SSc. However, also their activity is markedly increased in SSc. For example, skin (myo) fibroblasts of SSc patients show more activation of focal adhesion kinase (FAK) *in vitro* than those of controls (97). This focal adhesion kinase is a key component of integrin signaling, and regulates fibroblast migration, survival and growth. Furthermore, *in vitro*, (myo)fibroblasts obtained from SSc patients produce more extracellular matrix molecules such as collagen type I than those of healthy controls, and their migratory and contractile properties are also increased (19, 98). Because the activated phenotype of SSc (myo) fibroblasts persists *ex vivo*, e.g., during cell culture, epigenetic changes most likely play an important role in this phenotype. For example, recent research has shown that in SSc skin fibroblasts, expression of the histone demethylase Jumonji domain-containing protein 3 (JMJD3) is increased (99). This histone demethylase removes the so-called H3K27me3 mark from histones, and this mark can repress expression of pro-fibrotic genes such as collagen type I in fibroblasts (100). Furthermore, pharmacological inhibition of H3K27 trimethylation induces skin fibrosis and aggravates pathology in bleomcyin induced skin fibrosis (100). A key target which is activated by JMJD3 is Fos-related antigen 2 (Fra-2) (99). This transcription factor has been identified as an important regulator of extracellular matrix production in skin fibroblasts; transgenic overexpression of Fra-2 results in increased dermal thickness and myofibroblast formation and is a mouse model for SSc (101), whereas knockdown of Fra-2 reduces both TGFβ- and PDGF-induced collagen production in primary skin fibroblasts of SSc patients (102).

Next to epigenetic changes, several cytokines can enhance the formation and function of myofibroblasts. In **Table 1** an overview is given of how various cytokines affect myofibroblasts activity. As already mentioned TGFβ, PDGF, Wnts, IL-6, and OSM are key cytokines for myofibroblasts formation and activity. In addition to these factors, both IL-4 and IL-13 are pro-fibrotic (150). Both cytokines induce αSMA expression in primary lung fibroblasts in a dose- and time-dependent manner (105, 150), and enhance the production of collagen type I in normal fibroblasts (108). IL-22 has been described to have similar effect (118). Less clear is the role of IL-1 and Tumor necrosis factor α (TNFα). Of these factors both inhibitory and stimulatory effects on (myo) fibroblasts have been described. In atrial and intestinal myofibroblasts TNFα induces proliferation and collagen synthesis (119, 120). However, in dermal fibroblasts TNFα can inhibit αSMA expression by inhibiting TGFβ signaling (124). Interleukin 1 can not only induce, but also inhibit, collagen production, proliferation and myofibroblasts formation in dermal and lung fibroblasts by inhibition of TGFβ signaling (103, 104). Apart from these stimulatory cytokines, several signaling molecules inhibit myofibroblast formation and activity. For example, interferon γ (IFNγ) inhibits collagen synthesis, sensitizes dermal fibroblast to Fas-mediated apoptosis (125, 126) and inhibits IL-4 effects (125). Prostaglandin E2 has similar effects on formation and apoptosis in lung and keloid fibroblasts (145–147). The role of basic fibroblast growth factor (FGF2) is less clear, as it can inhibit TGFβ-mediated myofibroblast formation (140), but can also increase myofibroblast proliferation (151).

The increased presence and activity of myofibroblasts in SSc results in various deleterious effects. First of all, their excessive matrix production and remodeling capabilities can destruct organ architecture leading to loss of function like in lung fibrosis. Furthermore, deposition of extracellular matrix molecules such as collagens in the interstitial space of lung tissue inhibits gas exchange, greatly lowering lung function and resulting in interstitial lung disease. In skin excessive matrix deposition increases stiffness, increases hardness, and leads to loss of cutaneous tissues like, fat tissue, sweat glands, hair follicles, and sebaceous glands (152). In the gastro-intestinal tract, myofibroblast-induced fibrosis negatively affect motility, digestion, absorption, and excretion (153).

Blood vessel function is also impacted by myofibroblasts. To begin, myofibroblasts produce endothelin-1 (15). Endothelin 1 is a potent vasoconstrictor, leading to increased blood pressure. Notably, endothelin 1 also stimulates the formation of new myofibroblasts. Furthermore, myofibroblasts also produce VEGF (154), e.g., during wound healing, and can also express angiopoietin 1 and 2, both of which stimulate the formation of new blood vessels (155). As mentioned, myofibroblasts also produce and activate TGFβ. VEGF, angiopoietins, and TGFβ are all key regulators of endothelial homeostasis, and normally these factors are well balanced to maintain this homeostasis. However, this balance can be disturbed by the myofibroblast's production of these factors, leading to aberrant vascular remodeling. For example, uncontrolled VEGF signaling has been suggested to be a cause for capillary malformations in SSc (154).

Myofibroblast also have an immunomodulatory role. As mentioned, they express for example interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractive protein 1 (MCP-1) (13). Both IL-8 and MCP-1, also known as CCL2, are chemokines, attracting neutrophils, monocytes and T cells and in this way facilitate inflammation. Both IL-1 and IL-6 can enhances pro-inflammatory gene expression in immune cells. Furthermore, both factors can participate in the differentiation of monocytes toward

TABLE 1 | Influence of various cytokines on myofibroblast biology.

Signal molecule	Type of (myo)-fibroblasts	Observations	Effect	References	Remarks
IL-1	Dermal, Lung	Stimulates collagen type 1 production	+	(103)	Can inhibit TGFβ effects
		Stimulates proliferation	+	(103)	
		Inhibits collagen type 1 production	–	(103, 104)	
		Reduces formation and proliferation	–	(103, 104)	
IL-4	Lung	Increases formation (αSMA expression)	+	(105)	Stimulates Th2 formation and alternative activation of macrophages
	Lung	Increases proliferation	+	(106, 107)	
	Keloid, Dermal	Increases collagen type 1 production	+	(108)	
IL-6	Lung	Inhibition of sIL6R signaling lowers myofibroblasts numbers	+	(109)	sIL6R signaling enhances TGFβ signaling (110)
	Lung	Inhibition of sIL6R signaling lowers collagen and fibronectin deposition	+	(109)	
	Dermal	Increases collagen type I and αSMA expression	+	(78, 110)	
IL-10	Dermal, cardiac	Reduces collagen type I production	–	(111–113)	Induces TGFβ production by macrophages
	Dermal	Reduces TGFβ and TNFα induced proliferation	–	(114)	
	Dermal	Lowers sensitivity to FAS-induced apoptosis	+		
IL-13	Lung	Increases αSMA expression	+	(105)	
	Lung	Increases proliferation	+	(105, 107)	
	Keloid & Dermal	Increases collagen type 1 production	+	(108)	
IL-17	Dermal	Inhibits collagen type 1 production	–	(115, 116)	Induces IL-6 production and immune cell attraction in fibroblasts
	Lung	Stimulates collagen, TGFβ and IL-6 production	+	(117)	
IL-22		Induces differentiation	+	(118)	Enhances fibroblast response to TNFα
		Induces collagen type 1 production	+		
		No effect on collagen production	=		
TNFα	Intestinal	Induces collagen accumulation via TNFR2	+	(119)	Alters PDGF signaling (121)
	Intestinal, Dermal, cardiac	Induces proliferation via TNFR2	+	(119–121)	
		Induces myofibroblasts formation	+	(119)	
	Palmar dermal	Induces apoptosis via TNFR1	–	(122)	
	Dermal	Sensitizes fibroblasts to FAS-induced apoptosis	–	(114, 123)	
	Lung, Dermal				
	Dermal	Suppresses αSMA expression and TGFβ effects	–	(124)	
IFNγ	Dermal	Inhibits collagen synthesis	–	(125, 126)	Antagonizes IL-4 (125) and TGFβ (127)
	Dermal	Sensitizes to FAS-induced apoptosis	–	(114)	
	Dermal	Inhibits proliferation in fast dividing cells, stimulates proliferation in slowly dividing cells	?		
OSM	Lung	Increases αSMA expression and contraction	+	(128)	OSM signaling is augmented by IL4 or IL13 (132)
	Lung, Dermal	Increases proliferation	+	(129, 130)	
	Lung, Dermal	Increases collagen production	+	(117)(131)	
	Lung	Increases cell survival	+	(117)	
CCL2	Lung	Inhibits apoptosis via production of IL-6	+	(133)	Chemoattractant of monocytes Stimulates IL-4 production in Th2 cells
TGFβ	Lung, Dermal, cardiac, keloid	Increases αSMA expression	+	(38, 45–48, 134, 135)	Works in concert with Integrin-FAK
		Stimulates collagen type 1 production	+		Enhances Wnt signaling in SSc by downregulating DKK1 via p38 MAPK (62)
		Stimulates proliferation	+	(136)	
		Increases contraction	+	(58)	
		Inhibits apoptosis	+	(57)	Causes hyperactivation of STAT3 in SSc (81)
		Stimulates apoptosis	–		
		Inhibits proliferation	–		Counteracted by bFGF signaling
CTGF	Corneal	Facilitates TGFβ effects	+	(137)	

(Continued)

TABLE 1 | Continued

Signal molecule	Type of (myo)-fibroblasts	Observations	Effect	References	Remarks
PDGF	Corneal, Dermal, Lung	Increases αSMA expression Stimulates collagen type 1 production Stimulates proliferation	+ + +	(61, 138, 139)	TGFβ stimulates PDGFR expression (59)
FGF2 (bFGF)	Dermal Dermal	Inhibits TGFβ-induced myofibroblasts formation Increases fibroblast proliferation Stimulates apoptosis	– 	(140) (140) (58)	
Wnt		Canonical Wnt signaling induces fibroblast proliferation and migration, collagen gel contraction, and myofibroblast differentiation	+	(62–64, 89)	Induces TGFβ production (64)
Histamine	Lung Dermal Dermal	Enhances proliferation via a H2R Increases αSMA expression Inhibits TGFβ-induced αSMA expression via H1R	+ + –	(141) (142) (143)	
Leukotriene D4	Lung	Enhances TGFβ-induced collagen synthesis	+	(144)	
PGE2	Lung Keloid Lung	Induces apoptosis Inhibits migration, contraction and TGFβ-induced collagen synthesis Inhibits myofibroblasts formation	– – –	(145) (146) (147)	
Serotonin	Lung Lung	5-HT_{2B} receptor antagonists reduce myofibroblast differentiation Induces extracellular matrix synthesis	+ +	(148) (149)	Effects depend on TGFβ signaling (149)

macrophages and play a role in the differentiation of naive T-cells toward an effector subtype (156).

ON THE ROLE OF THE (INNATE) IMMUNE SYSTEM IN MYOFIBROBLAST FORMATION AND FUNCTION

Myofibroblast survival, formation, and function are all increased in SSc. The (innate) immune system plays an important role in this. In **Figure 6** an overview is given of how.

One immune cell which can induce myofibroblasts formation and activity is the mast cell. Mast cells are part of the innate immune system and well known for their role in allergy. However, they have already been implicated in SSc pathophysiology for a long time (157), because they can produce several mediators which stimulate fibrosis (158). One such factor is Platelet-activating factor, which stimulates platelet aggregation and degranulation. Platelet degranulation releases many (growth) factors, including TGFβ, PDGF, and fibronectin, all of which are factors which stimulate myofibroblasts formation and function. Another product of mast cells and platelets is serotonin. Serotonin has long been implicated in fibrotic disorders; already in 1958 it was demonstrated that subcutaneous injections of serotonin induce skin fibrosis (159). More recently, it was demonstrated that serotonin directly increases extracellular matrix production in primary skin fibroblasts (149). This effect runs via the 5H-T_{2b} receptor; inhibition of this receptor with terguride decreases collagen and fibronectin production by fibroblasts. Importantly, mice that lack this receptor (5H-$T_{2b}^{-/-}$) are protected against bleomycin-induced skin fibrosis, just as mice in which the 5H-T_{2b}, receptor is pharmacologically inhibited (149). Mast cells also produce tryptase, a serine proteinase, which, remarkably, stimulates fibroblast proliferation and collagen production (142, 160, 161), and histamine, which also induces (lung) fibroblast proliferation (141). Next to these factors, mast cells also produce a large array of pro-fibrotic cytokines; IL-4, IL-6, IL-13 TNF-α, TGFβ, and PDGF (158) which directly stimulate the formation and activity of myofibroblasts. Interestingly, mast cells can directly interact with skin (myo) fibroblasts, and this facilitates their role in fibrosis. This interaction was shown to be serpine1 dependent. Apart from the aforementioned role as inhibitor of plasmin activation, this protein is a chemotactic for mast cells and induces the expression of intercellular adhesion molecule 1 (ICAM1) in fibroblasts, which is needed for mast cells to adhere to fibroblasts (162). Of note, serpine1 is a downstream target of TGFβ signaling in many cell types, including fibroblasts.

Another innate immune cell which can have a pro-fibrotic role is the neutrophil. Like mast cells, neutrophils produce various pro-fibrotic cytokines including: TGFβ, IL-6, and VEGF (163). Furthermore, activated neutrophils release reactive oxygen species (ROS) (164). Reactive oxygen species activate fibroblasts and stimulate fibrosis (165). In part, this effect is due to the

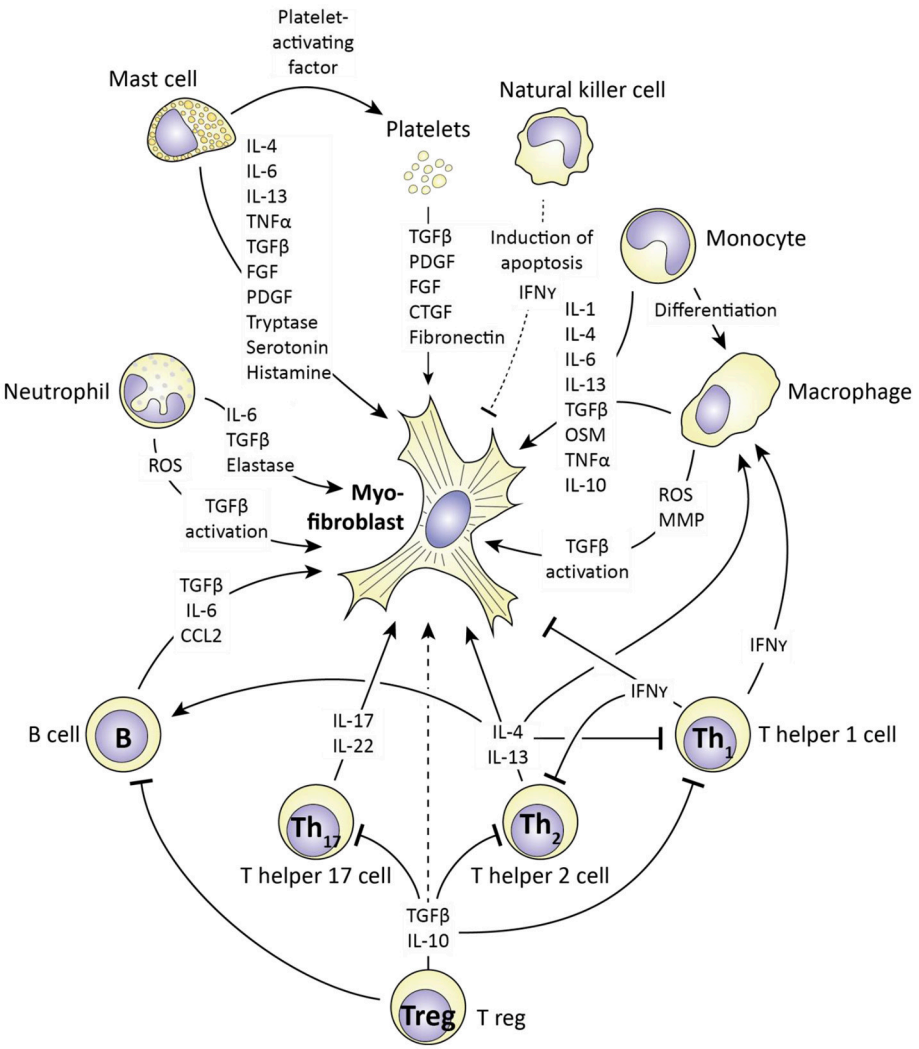


FIGURE 6 | The influence of immune cells on myofibroblast formation and function. Immune cells produce various mediators (also see **Table 1**) that influence myofibroblast formation and function. For each cell type (and platelets) the corresponding mediators are depicted. Cells which stimulate myofibroblast function include mast cells, monocytes/macrophages and T helper 2 lymphocytes via e.g. production of IL-4, IL-13, and TGFβ. In contrast, T helper 1 cells can negatively affect myofibroblast function via production of interferon gamma (IFNγ). Importantly, the ultimate outcome of an immune response on myofibroblast function depends on the interplay between immune cells, as this interplay regulates the production of the mediators the affect myofibroblast function.

activation of TGFβ. Chemical reaction of reactive oxygen species with latent TGFβ disrupts the quaternary protein structure of latent TGFβ, and results in release of active TGFβ (165). Of note, neutrophils of SSc patients release more ROS than neutrophils of healthy controls when challenged with TNFα (164). Recently, it was also demonstrated that neutrophil elastase, a serine proteinase, can induce myofibroblasts formation (166). Mice lacking this enzyme are protected against asbestos-induced lung fibrosis, and *in vitro* neutrophil elastase directly stimulates myofibroblasts formation, proliferation, and contractility (166). Furthermore, pharmacological inhibition of neutrophil elastase by sivelestat protects mice from bleomycin induced lung fibrosis (167), demonstrating that at least in lungs, neutrophil elastase is pro-fibrotic.

Next to mast cells and neutrophils, also macrophages can stimulate the formation and activity of myofibroblasts. To begin, macrophages, and their precursor the monocyte, can produce large amounts of TGFβ, for example during bleomycin induced lung fibrosis in rats (168). Apart from TGFβ, macrophages produce many cytokines with pro-fibrotic effects, including IL-4, IL-6, and IL-13 (156). Especially alternatively activated macrophages, also known as M2 macrophages, are associated with production of pro-fibrotic cytokines. These cells have a less pro-inflammatory and more repair oriented phenotype than classically activated macrophages, i.e., M1 macrophages (156). Macrophages, like neutrophils, also produce reactive oxygen species which enhance fibrosis. The importance of macrophages in regulating fibrosis is demonstrated by the observation that in

mice, deletion of lung macrophages using liposomal chlodronate reduces bleomycin induced lung fibrosis, and a similar effect is obtained if circulating monocytes are depleted using liposomal chlodronate (169).

A cell of the innate immune system with a possible anti-fibrotic role is the natural killer (NK) cell. In liver fibrosis, this cell type can recognize myofibroblasts and stimulate them to undergo apoptosis (170). Furthermore, NK cells produce IFNγ a strong inhibitor of myofibroblasts formation and function (171). However, in SSc, both the killing ability and stimulation-dependent IFNγ production of NK cells has been reported to be reduced (171).

In addition to the cells of the innate immune system, cells of the acquired immune system also play a role in fibrosis. A cell type particularly associated with fibrosis in SSc is the T helper 2 cell (Th_2). These cells produce the pro-fibrotic cytokines IL-4, IL-5, and IL-13, which directly stimulate fibroblasts but also induce the formation of alternatively activated macrophages (172, 173). SSc is characterized by Th_2 polarization, i.e., a Th_2 cytokine profile in blood, and importantly, in SSc, the extent of Th_2 polarization directly positively correlates with active interstitial lung disease (i.e., lung fibrosis), supporting for a role of Th_2 cells in this process (132). Also T helper 17 cells (Th_{17}) can play a role in fibrosis, in part via their production of IL-17 and IL-22, which can stimulate collagen, TGFβ and IL-6 production in pulmonary fibroblasts (117, 118). In contrast to these two T helper subtypes, T helper 1 (Th_1) cells are more associated with inhibition of myofibroblast function, for example in pulmonary fibrosis (174). This effect of Th_1 cells is attributed to their production of IFNγ, which directly inhibits myofibroblast formation and function, but also directs macrophage polarization away from the pro-fibrotic, alternatively activated (M2) phenotype. The role of regulatory T cells (T_{reg}) in fibrosis and myofibroblast activity is less clear. These cells produce TGFβ and IL-10 which can directly regulate myofibroblast function, but also affect the activity of Th_1, Th_2, and Th_{17} cells. Because these effector T cells have different functions on myofibroblasts, the end effect of T_{reg} activity is difficult to predict. Finally, B lymphocytes have also been demonstrated to be able to promote fibrosis; co-culture of B cells with skin fibroblasts induced the expression of collagen and αSMA by the latter (175). For this effect cell-cell contact was required, as the use of a transwell system negated the stimulatory effect of B cells on fibroblast activity (175). However, B lymphocytes are able to produce TGFβ, CCL2, and IL-6, which enhance myofibroblast activity without the need for cell contact.

ON THERAPEUTIC TARGETING OF MYOFIBROBLASTS IN SSC

In view of the role of myofibroblasts in SSc, inhibiting their formation or function makes an excellent option for targeted therapy. Several compounds that have been investigated in, or are currently under investigation in clinical trials are listed in **Tables 2**, **3,** respectively. Whether these compounds truly target myofibroblasts is up for debate, yet they do target cellular processes important for myofibroblast formation and function.

To begin, one compound that is currently under investigation is tocilizumab. Tocilizumab is a humanized antibody directed against the IL-6 receptor and currently enrolled in a phase III trial for SSc therapy (98). In an initial phase 2 double-blind, placebo controlled study tocilizumab did not significant reduce skin thickening (189), but the open label extension phase of this study did show encouraging protective effects on skin thickening and loss of forced vital capacity in SSc patients (190). Interestingly, skin biopsies were collected from enrolled patients before and after treatment and analyzed for fibroblast phenotype. Treatment with tocilizumab for 24 weeks decreased fibroblast protein production, migration and contractility compared to baseline (98). Furthermore, a large effect between the tocilizumab and placebo treated groups was observed on gene expression profile; in the placebo group, gene expression was not significantly altered over 24 weeks, whereas in the tocilizumab treated group 2,136 genes were significantly differentially expressed. Strikingly, many TGFβ signaling related genes, together with cell contractility pathways, were downregulated to a level similar to normal expression levels (98). This study thus demonstrates that tocilizumab is a serious candidate for targeting (myo-) fibroblasts in SSc.

In view of these results with tocilizumab, the results of tofacitinib in SSc treatment will be of interest. Tofacitinib is a small molecule JAK1 and JAK3 kinase inhibitor downstream of IL-6 signaling which can be used for the treatment of rheumatoid arthritis. Because JAK1 and JAK3 both activate STAT3 this compound can be expected to inhibit myofibroblast function. Currently, tofacitinib is under investigation in a small double-blinded phase I/II trial for safety and efficacy in SSc.

Another compound of interest for treatment of fibrosis in SSc is pirfenidone. Pirfenidone is used for the treatment of idiopathic pulmonary fibrosis and is a pyridone derivative. Dietary intake of this compound was shown to inhibit bleomycin-induced lung fibrosis in hamsters (191). Furthermore, this compound reduces fibroblast proliferation and attenuates TGFβ-induced αSMA and collagen production in primary skin fibroblast (192, 193). In lung fibroblast of SSc patients with interstitial lung disease (ILD), treatment with pirfenidone lowered αSMA and fibronectin expression (194). However, in an open label phase 2 study with 63 SSc patients with ILD, no beneficial effects of pirfenidone were observed on disease outcomes (187).

Nintedanib is a small molecule kinase inhibitor of platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and fibroblast growth factor receptor (FGFR), which has been approved for the treatment of interstitial lung disease, and which can possibly be used for the treatment of (ILD in) SSc. For this latter application, it was recently granted a fast track designation by the U.S. Food and Drug Administration (FDA). In lung fibroblasts *in vitro*, nintedanib inhibits proliferation and motility as induced by FGF and PDGF, but also inhibits TGFβ-induced collagen deposition (195). *In vivo*, nintedanib protects mice and rats against bleomycin-induced lung fibrosis (195, 196), and lowers the amount of lymphocytes and neutrophils but not macrophages

TABLE 2 | Clinical trials conducted with putative anti-fibrotic agents in SSc.

	Target	Type of trial	Phase	Duration (months)	Number of patients	Type of patients	Result	References
Abatacept	CD80/ CD86	Randomized, double-blind, placebo-controlled	I/II	6	10	dcSSc	Five out of seven patients (71%) randomized to abatacept and one out of three patients (33%) randomized to placebo experienced ≥30% improvement in skin score	(176)
Bovine Collagen type I		Randomized, double-blind, placebo-controlled	II	12–15	168	dcSSc 6 months stable mRSS of ≥16	No significant differences in the mean change in MRSS or other key clinical parameters between the CI and placebo treatment groups at 12 or at 15 months	(177)
C-82 topical gel	CBP β catenin	Randomized, double-blind, placebo-controlled	I/Ii	1	17	dcSSc ≤3 years, increase in mRSS ≥ 5 in 6 months	No detected result in clinically efficacy on mRSS	(178)
Dasatinib	PDGFR c-ABL	Single-arm, open label	I/II	9	31	dcSSc ≤ 3 years, mRSS ≥15	No significant clinical efficacy on mRSS or pulmonary function test	(179)
Fresolimumab	TGFβ	Single-arm Open label	I	6	15	dcSSc ≤ 2 years mRSS ≥15	Improved mRSS Reduced TGFβ dependent gene expression in skin biopsies	(180)
Imatinib	PDGFR c-ABL	Single-arm Open label	IIa	6	24	dcSSc	Improved skin morphology and mRSS compared to baseline	(181)
		Randomized Double-blind Placebo-controlled	II	6	28	mophea >20% or SSc with mRSS >20/51	This study failed to demonstrate the efficacy of imatinib on mRSS	(182)
		Randomized Double-blind Placebo-controlled	II	6	10	active dcSSc	Imatinib was poorly tolerated; only 10 of 20 patients included	(183)
		Single-arm Open-label	II	6	26	SSc patients with active pulmonary involvement and unresponsive to cyclophosphamide	Stabilized lung function, no effect on skin	(184)
Metelimumab (CAT 192)	TGFβ	Randomized Double-blind Placebo-controlled	I/II	6	45	SSc duration of <18 months	No evidence of a treatment effect	(185)
Nilotinib	PDGFR c-ABL	Single-arm Open label	IIa	6 and 12	10	dcSSc ≤3 yr mRSS ≥16	Significant MRSS improvement	(186)
Pirfenidone	?	Randomized Open-label	II	4	63	SSc <7 years	No clinically relevant differences on skin on FVD	(187)
Relaxin	Relaxin receptor	Randomized Double-Blind Placebo-controlled	II	6	231	dcSSc ≤5 years mRSS ≥16	Recombinant relaxin was not significantly better than placebo in improving total skin score, pulmonary function, or functional disability in	(188)
Tocilizumab	IL6 receptor	Double-blind, placebo-controlled	II	12	87	progressive SSc ≤5 yr 15 ≥ mRSS ≤40	Not associated with a significant reduction in skin thickening	(189)
		Open label extension phase		24	51		Skin score improvement and FVC stabilization	(190)

TABLE 3 | Clinical trials currently underway with putative anti-fibrotic agents in SSc.

Compound	Target	Type of trial	Phase	Duration (months)	Number of patients	Type of patients	Identifier
Dabigatran	Thrombin	Single-arm open label	I	6	15	SSc <7 yr with ILD	NCT02426229
GSK2330811	OSM	Randomized Double-blind Placebo-controlled	II	3	40	active dcSSc <5 years 10 ≥ mRSS ≤35	NCT02453256
Lenabasum (CT-101)	CBr2	Randomized double-blind Placebo-controlled	III	12	354	dcSSc <6 yr	NCT03398837
Nintedanib	PDGFR/VEGFR/FGFR	Randomized double-blind Placebo-controlled	III	12 up to 24	580	SSc <7 yr with ILD	NCT02597933
SAR156597	IL4-13	Randomized double-blind Placebo-controlled	II	6	94	dcSSc	NCT02921971
Tofacitinib	JAK1/JAK3 kinase	Randomized double-blind Placebo-controlled	III	12 (+ 12 open label)	212	dcSSc <5 years 10 ≥ mRSS ≤35	NCT02453256

in bronchoalveolar lavage liquid in this model. In SSc skin fibroblasts, nintedanib also prevents proliferation and motility and lowers the expression of αSMA (197). Furthermore, it lowers the myofibroblasts count and skin fibrosis in bleomycin induced skin fibrosis in mice (197). In two phase 3 trials with patients suffering from interstitial lung disease, nintedanib profoundly lowered the decline in forced vital capacity (195, 198). Currently a phase 3 trial is underway to test its safety and efficacy in SSc.

PDGFR signaling is also targeted by nilotinib. This small molecule kinase inhibitor inhibits both PDGFR signaling and c-ABL signaling. In dermal fibroblasts, nilotinib inhibits TGFβ- or PDGF-induced collagen production in a dose dependent manner (199). Furthemore, this compound strongly lowered myofibroblasts formation and dermal thickness in bleomycin induced skin fibrosis in mice (199). Nilotinib has been tested in a small open label trial with 10 SSc patients (186), and has shown promising results on the modified Rodnan skin score after 6 and 12 months of treatment. A compound similar to nilotinib is imatinib, which also targets PDGFR and c-ABL signaling (200) This compound also reduces collagen production in skin fibroblasts, and also protects mice against bleomycin induced fibrosis via reduction of myofibroblasts formation and matrix deposition (200). Several clinical phase 2 trials have been conducted with imatinib in SSc. In an open label, single-arm clinical trial a positive effect of imatinib on skin thickening was reported after 6 months of treatment (181). However, in another placebo controlled, double blinded phase 2 study no efficacy on modified Rodnan skin score was reported after 6 months of treatment (182). Furthermore, a single center randomized, double-blind, placebo-controlled phase 2 trial warned for poor drug tolerance in SSc patients (183). A low dose of imatinib has also been tested in an open label study for treatment of intestitial lung disease in SSc patients unresponsive to cyclophosphamide (184), and was reported to stabilize lung function but again had no effect on skin.

In view of its pro-fibrotic effects, TGFβ has also been targeted in SSc. Currently, several TGFβ signaling targeting drugs are under clinical development for e.g., cancer treatment (201), but no trials for SSc are currently reported. In a small open label single center study, Fresolimumab (180), a high affinity TGFβ inactivating monoclonal antibody was recently tested in SSc patients, and reduced TGFβ dependent gene expression in skin biopsies and improved modified Rodnan skin score (180). In the past, a low affinity antibody had no such effect (185). Interestingly, several compounds that inhibit TGFβ activation by integrins are under development for various diseases. In a genetic mouse model for SSc (characterized by a mutation in fibrilin) antibodies against integrin α5β1 and integrin α5β3 inhibit skin fibrosis (202). The effects of these antibodies are mimicked by a TGFβ inhibiting antibody, illustrating that these effects possibly run via inhibition of TGFβ activation. In addition, a monoclonal antibody targeting integrin αVβ6 has been shown to protect mice from radiation induced fibrosis (203).

Currently also under development for treatment of SSc is lenabasum (CT-101). Lenabasum is a canabinoid type 2 receptor (CBr2) agonist and is currently being tested in a phase 3 trial for its efficacy and safety in treatment of SSc. Skin fibroblasts express CBr2, and this expression is increased in SSc (204). Stimulation of SSc skin fibroblasts with the synthetic cannabinoid WIN55,212-2 lowers matrix production, myofibroblast formation, and production of TGFβ, CTGF, and IL-6 (204). Furthermore, addition of this compound to mice inhibits bleomycin induced

skin fibrosis by lowering fibroblast to myofibroblast transition and TGFβ, CTGF, and PDGF production (205). In addition, in bleomycin lung fibrosis activation of canabinoid receptor type 2 signaling by JWH133 lowered both the inflammatory response and extracellular collagen deposition, which was accompanied by reduced levels of TGFβ in blood (206). These observations make lenabasum a promising compound.

Finally, a therapy currently under investigation in a phase 2 trial as targeted therapy for SSc is the use of abatacept. Abatacept is a fusion protein consisting out of an IgG1 Fc tail fused with the extracellular part of CTL4 and is currently in use for the treatment of rheumatoid arthritis. Abatacept targets and prevents the function of CD80/CD86 molecules of professional antigen producing cells. This prevents these antigen presenting cells from activating T cells, as CD80/CD86 provide the co-stimulatory signal required in addition to MHCII binding to initiate T (helper) cell differentiation. Early SSc skin is characterized by perivascular T cell infiltrates (172), and (late stage) SSc patients have increased T_{h2} cell activation (T_{h2} polarization) and these cells express the pro-fibrotic cytokines IL-4, IL-5, IL-6, and IL-13 (172). In bleomycin induced skin fibrosis in mice, abatacept lowers the influx of monocytes, T cells and B cells into lesional areas, lowers IL-6 and IL-10 levels and lowers skin fibrosis (207). Importantly, abatacept does not affect skin fibrosis in the murine Tsk1 model of SSc which is less dependent on inflammation nor in bleomycin induced skin fibrosis in SCID mice which lack T cells. In a very small double-blind placebo controlled trial of 10 patients, abatacept improved the mRSS of patients. Especially patients with an inflammatory gene expression profile in their blood responded well to abatacept (176). These results indicate that abatacept can possibly eleviate inflammation driven fibrosis, but not by directly targeting myofibroblasts.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we have addressed the role of myofibroblasts in SSc pathophysiology. The presence and formation of these cells are increased in SSc, giving rise to pathology due to their ability to produce excessive amounts of extracellular matrix molecules like collagen type I, their ability to affect vascular biology by production of e.g., VEGF and ET1, but also due to their immuno-modulatory effects via production of IL-6 and TGFβ. Targeting these cells is therefore a feasible strategy to get to a targeted therapy for SSc. Currently multiple drugs doing just that are in phase 3 trials, giving hope for the future of SSc treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Anti-Ro60 Seropositivity Determines Anti-Ro52 Epitope Mapping in Patients With Systemic Sclerosis

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Epitope mapping of anti-Ro52 antibodies (Abs) has been extensively studied in patients with Sjögren's syndrome (SjS) and systemic lupus erythematosus (SLE). Comprehensive epitope mapping in systemic sclerosis (SSc), where anti-Ro52 antibodies are also frequently detected, has not been performed. The aim of the present study was to fully characterize Ro52 epitopes in anti-Ro52-positive SSc using Ro52 fragments spanning the full antigen. Further analysis was made according to anti-Ro60 status. Epitope mapping was performed in 43 anti-Ro52-positive SSc patients. Seventy eight anti-Ro52-positive pathological controls, including 20 patients with SjS, 28 patients with SLE, 15 patients with dermatomyositis (DM), and 15 patients with primary biliary cholangitis (PBC), and 20 anti-Ro52-negative healthy individuals as normal controls were also tested. Five recombinant Ro52 fragments [Ro52-1 (aa 1-127), Ro52-2 (aa 125-268), Ro52-3 (aa 268-475), Ro52-4 (aa 57-180), and Ro52-5 (aa 181-320) were used to test reactivity by line-immunoassay and *in house* ELISA. Anti-Ro60 reactivity was tested by ELISA. All anti-Ro52 positive sera reacted with Ro52-2; none recognized Ro52-3. Antibodies against Ro52-1 were less frequently found in SSc than in SjS/SLE (11.6 vs. 41.7%, $p = 0.001$); and antibodies against Ro52-4 were less frequently found in SSc than in SjS/SLE (27.9 vs. 50%, $p = 0.03$). In SSc patients, reactivity against Ro52-1 was more frequent in anti-Ro52+/anti-Ro60+ than in anti-Ro52+/anti-Ro60-patients (33.3 vs. 0%, $p = 0.003$). In this comprehensive analysis of Ro52 epitope mapping in SSc, the coiled coil domain remains the predominant epitope on Ro52. Contrary to SjS and SLE, patients with SSc fail to identify epitopic regions within the N-terminus of the protein, especially if they lack con-current anti-Ro60 reactivity.

Keywords: autoantibody, autoimmunity, autoimmune rheumatic diseases, epitope, SS-A

Abbreviations: Ab, antibody; AutoAb, autoantibody; ARD, autoimmune rheumatic diseases; PBC, primary biliary cholangitis; Sjögren's syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

INTRODUCTION

Anti-Ro52 antibodies (Abs), along with anti-Ro60 or in isolation, are frequently found in patients with autoimmune rheumatic diseases (AIRDs) (1–4). These autoantibodies (autoAbs), originally described in patients with Sjögren's syndrome (SjS), systemic lupus erythematosus (SLE), are detected in other ARDs, as well as in other organ and non-organ specific autoimmune diseases (1–3, 5–7). For instance, we and others reported the presence of anti-Ro52 Abs in ∼20–30% of patients with systemic sclerosis (SSc), making it the third most common antibody (Ab) in this disease (8–10). Ro52, originally considered as potential part of the ribonucleoprotein complex, is now well established as member of the tripartite TRIM family (TRIM21). It has been shown that Ro52 (TRIM21) is a cytosolic Fc receptor, bound with high affinity preferentially to IgG, but also to IgA and IgM intra-cytoplasmic receptor of IgG (11, 12). This ability of Ro52 (TRIM21), for simplicity there after mentioned as Ro52, along with its pleiotropic immunomodulatory properties have led us to appreciate the important role of this antigen in regulation of immune-mediated inflammation and regulation of autoreactive immunity (11, 12).

The exact epitopic regions on Ro52 targeted by antigen-specific autoAbs have been extensively studied in SjS and SLE (13–19), but their characterisation in patients with SSc is ill-defined. In SjS and SLE, anti-Ro52 autoAbs mainly target large polypeptidyl sequences in the coiled coil region of the protein (13–19). Linear short sequences within the corresponding epitopes are subdominantly recognized (20). A recent study by Infantino et al (21), using a set of 5 epitopic regions overlapping the whole sequence, has demonstrated reactivity mainly to aa 125-268. Having access to these Ro52 constructs, we considered that it is worth investigating the B-cell epitopes of Ro52 in patients with SSc. Our findings neither refute nor agree with those obtained in SjS and SLE. When anti-Ro52 Ab-positive SSc patients were divided according to con-current anti-Ro60 Abs, different patterns of epitope recognition were found.

MATERIAL AND METHODS

Material

A total of 121 anti Ro52 Ab-positive patients with various autoimmune diseases were analyzed, including 43 patients with SSc (41 females; mean age ±SD: 57.83 ± 12.58 years; disease duration 9.72±7.4 years; 41 ANA positive, median titre 1/160, range 1/80-1/5,120) as the study group (**Table 1**), 20 patients with SjS (18 females; mean age ±SD: 52 ± 11.2 years), 28 patients with SLE (all females; mean age ±SD: 45 ±14.3 years), 15 with dermatomyositis (DM) (9 females; mean age ±SD: 62.13 ±11.3 years); and 15 patients with primary biliary cholangitis (PBC) (22) (13 females; mean age ±SD: 47.2 ± 13.4 years), as pathological controls. All patients were regularly followed up at the Out-patient Clinic, Department of Rheumatology and Clinical Immunology, University General Hospital of Larissa, in Larissa, Greece (9, 23, 24). A cohort of 10 additional anti-Ro52 Ab-positive SSc patients were also included; these patients were followed up at two other Greek University Hospitals, University of Athens and University of Patras. Diagnosis of SSc was based on the 2013 ACR/EULAR Criteria for the Classification of SSc (25); diagnosis of SjS on the 2016 ACR/EULAR Classification Criteria for primary SjS(26), diagnosis of SLE was based on the 2012 SLICC Criteria (27), and diagnosis of DM was based on the Bohan and Peter Criteria for Polymyositis and Dermatomyositis (28, 29). Diagnosis of PBC was based on the internationally accepted criteria for PBC (22, 30).

Fifty anti-Ro52 Ab-negative patients with various AIRDs and other autoimmune diseases, including 12 patients with SSc,10 with SjS, 12 with SLE, 5 with DM, and 11 with PBC, were tested as anti-Ro52 Ab-negative disease controls.

TABLE 1 | Clinical and immunological characteristics of SSc patients.

	SScpatients ***n* = 35**
SSctype	
lcSSc (n,%)	23 (65.7)
dcSSc (n,%)	12 (34.3)
Rodnan skin score (mean ± SD)	7.24 ± 8.3
Pulmonary fibrosis (n,%)	12 (34.3)
Pulmonary arterial hypertension (n,%)	2 (5.7)
Ulcers (n,%)	12 (34.7)
GI INVOLVEMENT	
Upper (n,%)	20 (57.1)
Lower (n,%)	0
Both (n,%)	1 (2.9)
Arthritis (n,%)	13 (37.1)
Serositis (n,%)	4 (11.4)
Telangiectasia (n,%)	18 (51.4)
Calcinosis (n,%)	2 (5.7)
Renal crisis (n,%)	0
Overlap syndrome/MCTD (n,%)	8 (25.0)
Dry mouth (n,%)	13 (37.5)
Dry eyes (n,%)	8 (22.9)
Rash (n,%)	7 (20.0)
Acro-osteolysis (n,%)	5 (14.3)
AUTOANTIBODIES	
-Scl-70 (n,%)	5 (14.3)
-CENPA (n,%)	14 (40.0)
-CENPB (n,%)	14 (40.0)
-RP11 (n,%)	1 (3.1)
-RP155 (n,%)	3 (9.4)
-Fibrillarin (n,%)	1 (3.1)
-NOR90 (n,%)	3 (9.4)
-Th/To (n,%)	0 (0)
-PM-Scl 100 (n,%)	0 (0)
-PM-Scl 75 (n,%)	2 (6.3)
-Ku (n,%)	4 (12.5)
-PDGFR (n,%)	0 (0)
-Ro52 (n,%)	35 (100)
-Ro60 (n,%)	13 (37.1)
-La (n,%)	5 (14.3)

Twenty healthy individuals (all anti-Ro52 Ab-negative) were also tested as normal controls (NCs) (18 females; mean age ±SD: 52.8 ± 10.9 years).

The presence of anti-Ro52 Abs was initially assessed by a line immunoassay (Euroimmun, Lübeck, Germany) and confirmed by an anti-Ro52 specific ELISA (Inova Diagnostics, San Diego, CA).

A written informed consent was obtained by all patients and controls. The study was performed in accordance with the declaration of Helsinki. Patients and NCs participated in the study after approval of the research protocol by the Ethical Committee of the University General Hospital of Larissa, Faculty of Medicine, University of Thessaly, Greece.

Methods

Epitope mapping was performed using a specifically-designed line immunoassay containing five recombinant Ro52 fragments expressed in *E coli* [Ro52-1 (aa 1-127), Ro52-2 (aa 125-268), Ro52-3 (aa 268-475), Ro52-4 (aa 57-180), and Ro52-5 (aa 181-320) (**Figure 1**), as described before (21). Ro-52 full-antigen, expressed with the baculovirus system in insect cells, was used as positive control. Titration experiments were performed to establish optimal conditions of experiments. The final concentration of each fragment was established based on ROC curves using four different concentrations (1, 5, 25, 100 μg/ml) tested in 20 anti-Ro52-positive SSc and 20 anti-Ro52-negative NCs. The final concentration of each fragment that gave specificity up to 94% was as follows: 100 μg/ml for Ro52-1 and 25 μg/ml for all other fragments. The specifically designed line strips were incubated with sera (1:100 dilution) on a rocking platform at room temperature for 30 min (21). After the aspiration of the liquid the strips were washed three times in 1.5 ml wash buffer (Euroimmun) for 5 min. Then strips were incubated in alkaline phosphate-labeled anti-human IgG conjugate (Euroimmun) for 30 min, followed by three 5 min washes (21). Finally, strips were incubated in 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate solution (Euroimmun) for 10 min and then washed with distilled water. After being dried, strips were evaluated by the use of EUROLineScan software (Euroimmun) and results were expressed in arbitrary units (AU/ml), as previously described in detail (9, 21, 31). To determine the cut off values of the *in house* line immunoassays, we tested 70 anti-Ro52 Ab-negative serum samples (39 with various AIRDs, 11 with PBC and 20 NCs, see above). For each fragment the chosen cut off value corresponded to mean+2SD. Based on that, the cut off value was 8 AU/ml for Ro52-1, 10 AU/ml for Ro52-2, 4 AU/ml for Ro52-3, 5 AU/ml for Ro52-4, and 8 AU/ml for Ro52-5 (**Supplementary Table 1**).

The validity of Ab reactivity to fragments by a line immunoassay was also assessed by an *in house* ELISA using the same Ro52 fragments testing 32 serum samples (12 randomly selected anti-Ro52 Ab positive SSc patients and 20 anti-Ro52 negative SSc patients), as previously described with slight modifications (32–34). Titration experiments were executed to establish optimal conditions of experiments. Briefly, initially each well was incubated at 20°C for 1 h with 200 μl of blocking buffer (2% BSA in PBS), to block non-specific binding). All reagents were purchased by Sigma Aldrich, until otherwise stated. After a washing step (5 times with PBS-0.1% Tween-20), 100 ml of each Ro52 fragment (final concentration: 25 μg/ml) was added to the wells (diluted in PBS containing 0.1% BSA and 0.1% sodium azide) and incubated at 20°C on shaker for 1 h. Washing was repeated (5x) and following that, 100 μl of each samples at 1/200 dilution (in 2% BSA/PBS containing 0.1% sodium azide) was added and incubated at 20°C on a shaker for 1 h. To ensure consistency, two sera were used as reference controls, including a high titre anti-Ro52 Ab serum from an SLE patient, know to strongly react with the Ro52 full protein, and fragments Ro52-1, Ro52-2, Ro52-4, and Ro52-5, and a NC serum used as negative control totally unreactive against the full Ro52 protein and its fragments. The washing step was repeated and 100 μl of conjugate-1/1000 peroxidase-conjugated goat anti-human (IgG) diluted in 2% BSA/PBS were added to each well and incubated at 20°C for 1 h. After washing steps (5x), 100 μl of TMB substrate (3,3', 5,5;-tetramethylbenzidine) was added and incubated in the dark for 10 min. The reaction was terminated by adding 50 μl of H_2SO_4. Light absorbance (optical density, OD) was measured against blank well at 450 nm (620 nm as reference wavelength). To determine the cut off value, 20 anti-Ro52 Ab-negative patients (10 with SSc and 10 randomly selected, with other ARDs), were tested with individual Ro52 fragments. Reaction for a given construct exceeded was considered positive when the OD reading of the test serum against the construct exceeded the mean+2SD of the absorbance values of the 32 anti-Ro52 Ab negative controls. To utilize a uniform representation of the absorbance values, the absorbance corresponding to the cut off value was defined as 1 RU/ml.

FIGURE 1 | Schematic representation of full length Ro52 antigen and the 5 separate Ro-52 fragments.

Statistical Analysis

All results are expressed as percentages (%). To determine cut off values for the line immunoassay ROC analyses were performed and for each Ro52 fragment ab concentration was chosen for a specificity up to 94%. Mean plus 2SD of values of negative patients were used as cut off for each assay (line immunoblotting, ELISA). Differences between groups were tested by chi-square, two-tailed *t*-test and nonparametric Mann-Whitney test. *p*-values smaller than or equal to 0.05 were considered significant. The statistical calculations were performed with SPSS statistics 22.

RESULTS

All anti-Ro52 Ab-positive patients reacted against the full Ro52 antigen by a line immunoassay without difference in AU/ml among the various diseases (mean ± SD: 76.55 ± 23.13 AU/ml in SSc compared to 79.8 ± 24.3 AU/ml in SjS; 80.39 ± 28.26 AU/ml in SLE; 81.73 ± 16.93 AU/ml in DM and 83.6 ± 35.2 AU/ml in PBC, $p > 0.05$ for all) (**Figure 2**).

Frequency of Ro52 Fragment ab Recognition of SSc and Controls

Results of serum reactivity to Ro52 fragments are summarized in **Tables 2**, **3**. Overall, reactivity against fragments Ro52-1, Ro52-2, Ro52-3, Ro52-4, and Ro52-5 in patients with SSc was 11.6, 100, 0, 27.9, and 41.9%, respectively. The respective results in SjS were 40, 100, 0, 40, and 60%; in SLE were 42.9, 100, 0, 57.1, and 57.1%;in DM were 20, 100, 0, 33.3, and 40% and in PBC 6.7, 100, 0, 6.7, and 40%.

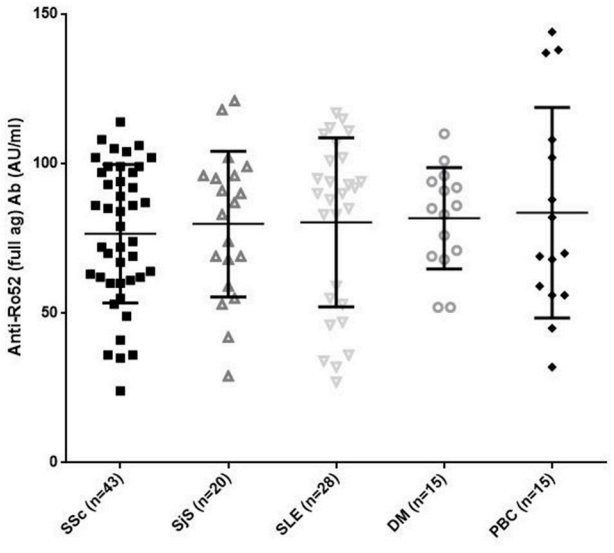

FIGURE 2 | Reactivity in AU/ml against the full length Ro52 antigen in 43 patients with systemic sclerosis (SSc), 20 patients with Sjögren's syndrome (SjS), 28 patients with systemic lupus erythematosus (SLE), 15 patients with DM, and 15 patients with primary biliary cholangitis (PBC). There were no significant differences amongst different diseases. Values are given as symbols. The solid black line at the approximate center of each vertical line is the median. The arms of each line extend with their ends corresponding to 10 and 90% of the values.

According to individual disease, Ab reactivity against fragments Ro52-1, Ro52-4, and Ro52-5 were as follows (SjS and SLE are grouped together as their reactivities to different Ro52 fragments were similar): Abs against Ro52-1 were less frequent in SSc than in SjS/SLE (5/43 [11.6%] vs. 20/48 [41.7%], $p = 0.001$); Abs against Ro52-4 were less frequent in SSc than in SjS/SLE (12/43 [27.9%] vs. 24/48 [50%], $p = 0.03$). In addition, Abs against Ro52-1 were also more frequent in SjS/SLE than in PBC (20/48 [41.7%] vs. 1/15 [6.7%], $p = 0.01$) and Abs against Ro52-4 were more frequent in SjS/SLE than in PBC (24/48 [50%] vs. 1/15 [6.7%], $p = 0.002$) (**Table 2**).

Comparison of reactivities against Ro52 fragments between SSc and separate SLE or SjS patient groups are shown in **Table 3**. In particular, SSc patients were less frequently reactive against Ro52-1 than SjS (5/43 [11.6%] vs. 8/20 [40.0%], $p = 0.0095$) and SLE patients (5/43 [20.9%] vs. 12/28 [42.9%], $p = 0.002$). PBC patients were also less frequently reactive against Ro52-1 than SjS (1/15 [6.7%] vs. 8/20 [40.0%], $p = 0.04$) and SLE patients (1/15 [6.7%] vs. 16/28 [57.1%], $p = 0.02$). Moreover, SSc patients were less frequently reactive against Ro52-4 than SLE (12/43 [27.9%] vs. 16/28 [57.1%], $p = 0.014$) and this was also the case for PBC compared to SjS (1/15 [6.7%] vs. 8/20 [40.0%], $p = 0.025$) and SLE patients (1/15 [6.7%] vs. 16/28 [571%], $p = 0.001$) (**Table 3**).

Ab reactivity to individual Ro52 fragments by line immunoassay correlated with Ab binding of the same fragments when tested by *in house* ELISA ($r = 0.95$, $p < 0.001$ for Ro52-1; $r = 0.783$, $p = 0.003$ for Ro52-2; $r = 0.485$, $p = 0.11$ for Ro52-3; $r = 0.729$, $p = 0.007$ for Ro52-4; $r = 0.784$, $p = 0.003$ for Ro52-5) (**Supplementary Figure 1**). All sera tested negative for Ro52 fragments by line immunoassay were also negative by ELISA.

Magnitude of Ab Reactivity Against Ro52 Fragments in SSc and Controls

The magnitude of Ab reactivity to individual Ro52 fragments is illustrated in **Figure 3**. Ab reactivity to Ro52-2 was lower in SSc compared to SjS (59.62 ± 26.21 AU/ml vs. 78.75 ± 25.66 AU/ml; $p = 0.009$), SLE (79.46 ± 30.92 AU/ml; $p = 0.007$), and PBC (90.06 ± 32.31 AU/ml, $p = 0.004$) patients. Reactivity against Ro52-4 was lower in SSc (4.88 ± 5.75 AU/ml) than in PBC (1.26 ± 2.15 AU/ml; $p = 0.001$) (**Figure 3**).

Ro52 Epitope Recognition in anti-Ro52+/anti-Ro60+ (double Positive) and anti-Ro52+/anti-Ro60- Patients

When anti-Ro52 Ab-positive patients were divided in anti-Ro52+/anti-CEN+ and anti-Ro52+/anti-CEN- no differences were found in epitope recognition patterns. Similarly, comparisons between subgrouping anti-Ro52+/anti-Scl70+ and anti-Ro52+/anti-Scl70- did not reveal statistically significant differences.

When anti-Ro52 positive patients were divided in anti-Ro52+/anti-Ro60+ (double positive) and anti-Ro52+/anti-Ro60–, statistically significant differences amongst diseases were found. In SSc patients ($n = 43$), reactivity against Ro52-1 was

TABLE 2 | Reactivity against full antigen and Ro52 fragments in various autoimmune diseases.

	SSc $n = 43$ (%)	SjS/SLE ($n = 48$) (%)	DM ($n = 15$) (%)	PBC ($n = 15$) (%)	p (SSc vs. SjS/SLE)	p (SSc vs. DM)	p (SSc vs. PBC)	p (SjS/SLE vs. DM)	p (SjS/SLE vs. PBC)	p (DM vs. PBC)
Full antigen	100	100	100	100	NS	NS	NS	NS	NS	NS
Ro52-1	11.6	41.7	20	6.7	**0.001**	NS	NS	NS	**0.01**	NS
Ro52-2	100	100	100	100	NS	NS	NS	NS	NS	NS
Ro52-3	0	0	0	0	NS	NS	NS	NS	NS	NS
Ro52-4	27.9	50.0	33.3	6.7	**0.03**	NS	NS	NS	**0.002**	NS
Ro52-5	41.9	58.3	40	40.0	NS	NS	NS	NS	NS	NS

SSc, systemic sclerosis; SjS, Sjögrens syndrome; SLE, systemic lupus erythematosus; PBC, primary biliary cholangitis. Significant p-values are indicated in bold.

TABLE 3 | Summary of Ab reactivity against the full antigen and the fragments.

	SSc $n = 43$ (%)	SjS ($n = 20$) (%)	SLE ($n = 28$) (%)	DM ($n = 15$) (%)	PBC ($n = 15$) (%)	p (SSc vs. SjS)	p (SSc vs. SLE)	p (SSc vs. DM)	p (SSc vs. PBC)	p (SjS vs. SLE)	p (SjS vs. DM)	p (SjS vs. PBC)	p (SLE vs. PBC)	p (DM vs. PBC)
Full antigen	100	100	100	100	100	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ro52-1	11.6	40.0	42.9	20	6.7	**0.009**	**0.002**	NS	NS	NS	NS	**0.04**	**0.02**	NS
Ro52-2	100	100	100	100	100	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ro52-3	0	0	0	0	0	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ro52-4	27.9	40.0	57.1	33.3	6.7	NS	**0.014**	NS	NS	NS	NS	**0.025**	**0.001**	NS
Ro52-5	41.9	60.0	57.1	40	40.0	NS	NS	NS	NS	NS	NS	NS	NS	NS

SSc, systemic sclerosis; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; PBC, primary biliary cholangitis. Significant p-values are indicated in bold.

more frequent in anti-Ro52+/anti-Ro60+ patients than anti-Ro52+/anti-Ro60– (5/15 [33.3%] vs. 0/28 [0%], $p = 0.003$) (**Table 4**).

Comparing reactivity against various Ro52 fragments in anti-Ro52+/anti-Ro60+ patients among various diseases, no statistically significant difference was detected. On the contrary, the same comparison in anti-Ro52+/anti-Ro60– patients among various diseases showed that reactivity against Ro52-1 was less frequent in SSc compared to SLE (0/28 [0%] vs. 6/10, [60%], $p = 0.001$); Similarly, reactivity against Ro52-4 was less frequent in SSc than in SLE (5/28 [17.9%] vs. 6/10 [60%], $p = 0.019$) patients.

In anti-Ro52-positive SSc patients, the clinical and immunological characteristics between anti-Ro52+/anti-Ro60– and anti-Ro52+/anti-Ro60+ SSc patients did not reveal any statistically significant differences (**Supplementary Table 2**). Similarly, clinical and immunological features were not statistically different when SSc patients were divided according to reactivity to specific Ro52 (Ro52-1, Ro52-4, Ro52-5) fragments.

DISCUSSION

This is the first comprehensive analysis of B-cell epitope mapping of anti-Ro52 Abs in patients with SSc using large polypeptidyl fragments spanning the whole Ro52 antigen. Our data show that, as in other AIRDs, such as SjS and SLE (13–19), the dominant epitopic region universally recognized by anti-Ro52 Abs in SSc is that lying within the coiled coil domain of the protein (aa 125-268) (13, 21). Lack of Ab binding of a sequence spanning the C-terminus of the antigen, reported in SjS and SLE is also confirmed in the present study (13–19, 21). However, our study revealed novel findings: patients with SSc less frequently recognize Ro52-1 compared to SLE patients. More importantly, anti-Ro60+ SSc patients showed a distinct, previously unrecognized epitopic pattern, characterized by broad recognition of Ro52 epitopes (including Ro52-1, Ro52-2, Ro52-4, and Ro52-5) compared to anti-Ro60- SSc patients where reactivity by large is restricted to Ro52-2.

In particular, SSc sera were less frequently reactive to Ro52-1 -the N-terminus fragment spanning aa 1-127—than combined SjS/SLE sera (11.6 vs. 41.7%). In a similar vein, Abs against Ro52-4 (aa 57-180)—which partly overlaps with Ro52-1 were less frequently found in SSc than in SjS/SLE (27.9 vs. 50%). This led us to assume that, while the overlapping region contains an epitope (or epitopes) of anti-Ro52 in SjS and SLE such an epitope recognition is absent, at least in part in SSc. Why Ab responses against specific Ro52 fragments are different in frequency and strength among various autoimmune diseases is not an easy task to address (13, 35–38). We can only speculate that the exact mechanisms which are responsible for the induction of anti-Ro52 Ab responses in SSc somewhat differ from those operating in SLE and SjS. It should be noted that in SSc anti-Ro52 Abs less frequently co-exist with anti-Ro60 Abs compared to SLE and SjS, which usually have both autoAb specificities (8, 10, 39). A similar to SSc pattern of less frequently recognition of Ro52-1 and Ro52-4 was also seen in PBC, suggesting that a common (or similar) mechanism of autoAbs production for both diseases may be in operation (40).

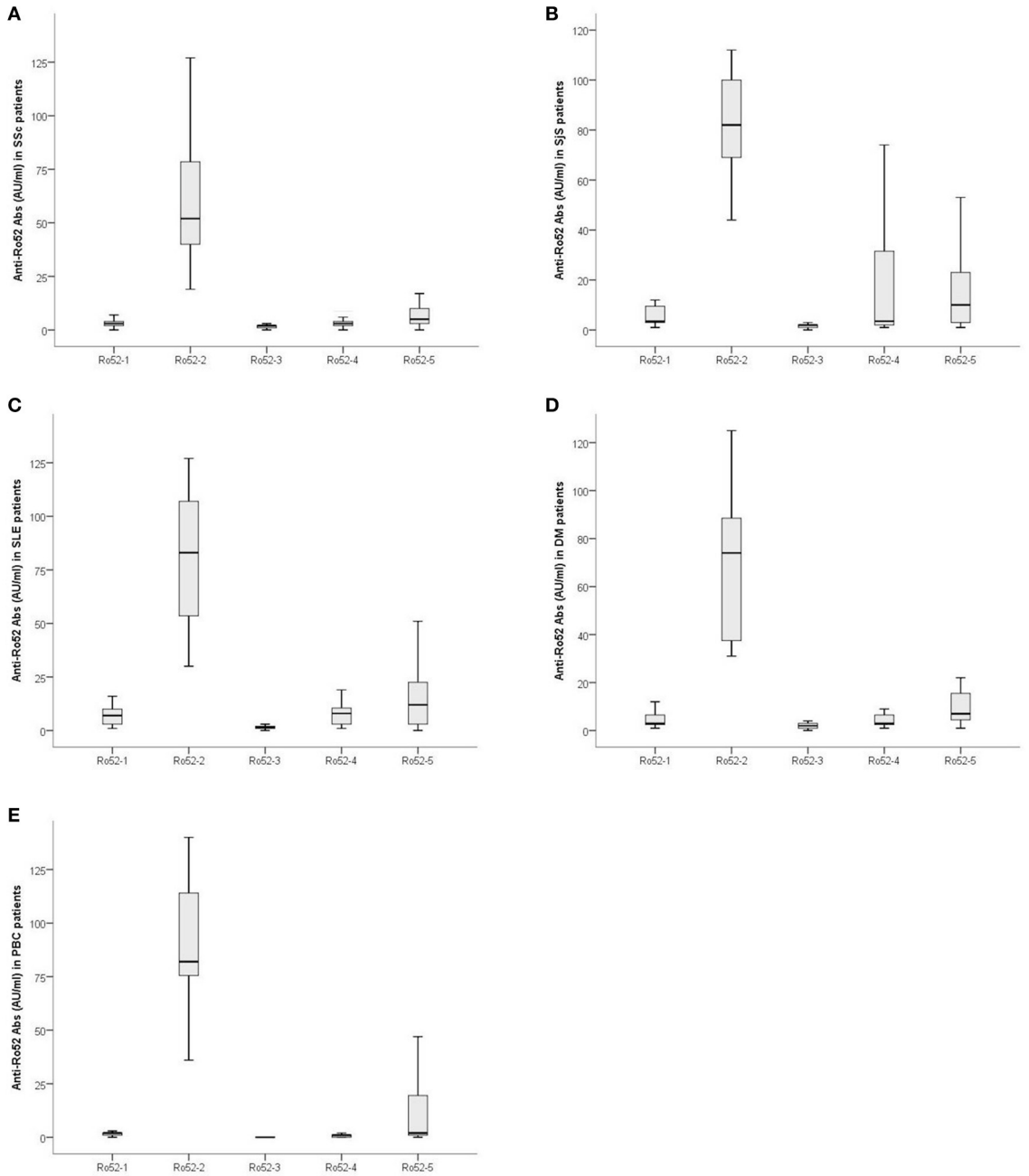

FIGURE 3 | Magnitude of Ab reactivity to individual Ro52 fragments in anti-Ro52-Ab-positive patients: **(A)** 43 patients with Systemic Sclerosis (SSc), **(B)** 20 patients with Sjögren's syndrome (SjS), **(C)** 28 patients with systemic lupus erythematosus (SLE), **(D)** 15 patients with dermatomyositis (DM) and **(E)** 15 patients with primary biliary cholangitis (PBC). Values are given as box plots which represent interquartile ranges and the solid black line at the approximate center of each box is the median. The arms of each box extend with their ends corresponding to 10 and 90% of the value.

The increased frequency of reactivity against Ro52-1 and Ro52-5 in anti-Ro52/anti-Ro60 double positive patients than in anti-Ro52+/anti-Ro60– SSc patients is difficult to explain. Currently, it is not known why some patients have reactivities to Ro52 alone, Ro60 alone or both (41). The two autoantigens are structurally unrelated but—immunologically—interrelated since anti-Ro52 and anti-Ro60 immune responses tend to co-exist (42, 43). However, anti-Ro52 autoAbs can be present without ever anti-Ro60 reactivity in many autoimmune diseases. Mechanisms, such as epitope spreading and exposure to cryptic epitopes in double positive sera at very early stages of disease may account for con-current reactivity (35–37, 44, 45). The clinical significance

TABLE 4 | Ab reactivity against Ro52 fragments in patients subdivided toanti-Ro52+/anti-Ro60+ and anti-Ro52+/anti-Ro60−.

Ro52	SSc Ro52/Ro60+ (*n* = 15) (%)	SSc Ro52+/Ro60- (*n* = 28) (%)	p	SjS Ro52/Ro60+ (*n* = 14) (%)	SjS Ro52/Ro60- (*n* = 6) (%)	p	SLE Ro52+/Ro60+ (*n* = 18) (%)	SLE Ro52+/Ro60- (*n* = 10) (%)	p	DM Ro52+/Ro60+ (*n* = 3) (%)	DM Ro52+/Ro60- (*n* = 12) (%)	P	PBC Ro52+/Ro60+ (*n* = 2) (%)	PBC Ro52+/Ro60- (*n* = 13)	P
Full-length Ro52	100	100	–	100	100	–	100	100	–	100	100	–	100	100	–
Ro52-1	33.3	0	**0.003**	50.0	16.7	NS	33.3	60.0	NS	33.3	16.7	NS	0	7.8	NS
Ro52-2	100	100	–	100	100	–	100	100	–	100	100	–	100	100	–
Ro52-3	0	0	–	0	0	–	0	0	–	0	0	–	0	0	–
Ro52-4	33.3	25.0	NS	50.0	16.7	NS	55.6	60.0	NS	33.3	33.3	NS	0	7.8	NS
Ro52-5	60.0	57.1	NS	71.4	33.3	NS	55.6	60.0	NS	33.3	41.7	NS	50.0	38.5	NS

SSc, systemic sclerosis; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; PBC, primary biliary cholangitis. Significant p-values are indicated in bold.

of epitopic recognition is underlined in experimental diseases, where the clinical phenotype largely depends on the Ro52 domain, used as an immunogen. For instance, Sroka et al. (46) have recently shown that only immunization with the coiled coil Ro52 domain and its subsequent immune response against the coiled coil Ro52 domain can induce salivary gland dysfunction (46). However, we were unable to find specific associations between clinical features and epitope profiling. The relatively recent demonstration of the true nature of the Ro52 antigen and its pleiotropic key role for signal transduction, in adaptive and innate immunity as member of the TRIM family of proteins may explain (at least in part) some of these attributes (47–49). Ro52 (TRIM21) is an intra-cytoplasmic receptor of IgG and epitope spreading mechanisms involving regions corresponding to dominant or subdominant epitopes of Ro52 and Ro60 may account for the observed distinct Ab recognition against the two antigens (11, 12).

Our data suggests that epitope mapping of anti-Ro52 Abs in systemic sclerosis reveals a common denominator, the coiled-coil related epitope which, similarly to SjS, SLE, and other autoimmune diseases, is universally reactive. The N-terminus region spanned by aa 57-180 is a dominant epitope in anti-Ro52+/Ro60+ SSc patients but not in Ro52+/Ro60- SSc patients suggesting that Ro60 directly or indirectly is involved in the shaping of the epitopic repertoire of anti-Ro52 Abs, a finding which warranties further investigation(35, 36). Understanding the mechanisms responsible for the breaking of tolerance to Ro52 in SSc may shed a light not only for the understanding of the pathogenesis of this disease but also for those of SjS and SLE, positioning Ro60 as a key player. By no means our study or other studies of this kind (21) can address the key question that arises. Are these data epiphenomenal or do they really play a role in the induction of anti-Ro52 or anti-Ro60 in SSc, other AIRDs or indeed in other autoimmune diseases? Nevertheless, our data provide the impetus for subsequent studies performed in serum samples from patients on a large scale, as well as in experimental models of the disease.

AUTHOR CONTRIBUTIONS

Each authors named as an author has made substantial contributions to the conception, design of the study, or acquisition, analysis, and interpretation of data. AG, CL, MGM performed experiments; TSc, WM prepared antigenic preparations; TS, CK, DD and LIS performed clinical assessments; TS, AG, AT, and CK prepared clinical and laboratory datasets; AG and CL analyzed the data; AG, LIS and DPB drafted the manuscript; DPB and LIS supervised the project and designed the experimental work; DPB had the original idea. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02835/full#supplementary-material

Supplementary Figure 1 | Correlation of line immunobloding readings and ELISA absorbance values testing of individual fragments from patients with systemic sclerosis. The immunobloting data were confirmed in a representative set of sera from 12 anti-Ro52 Ab positive SSc patients and 20 anti-Ro52 Ab negative controls (Data not included). A statistically significant correlation is noted for all fragments between the two assays readouts.

Supplementary Table 1 | Representation of the alternative cut off calculation, by the mean plus two standard deviations of the reactivity values of anti-Ro52 negative patients with autoimmune rheumatic diseases.

Supplementary Table 2 | Main demographic, clinical and immunological parameters in anti- Ro52+/Ro60+ and Ro52+/Ro60- patients with SSc.

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REVIEW
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Mesenchymal Stem Cells in Systemic Sclerosis: Allogenic or Autologous Approaches for Therapeutic Use?

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Systemic sclerosis (SSc) is a rare autoimmune disease, which is potentially lethal. The physiopathology of the disease is still incompletely elucidated although the role of fibroblasts, endothelial cells (ECs), immune cells. and the environment (i.e., oxidative stress) has been demonstrated. This is an intractable disease with an urgent need to provide better therapeutic options to patients. Mesenchymal stem cells (MSCs) represent a promising therapeutic approach thanks to the number of trophic and pleiotropic properties they exert. Among these, MSCs display anti-fibrotic, angiogenic, and immunomodulatory capacities that might be of interest in the treatment of SSc by acting on different processes that are dysregulated in the disease. In the recent years, the therapeutic effectiveness of MSCs has been demonstrated in different preclinical animal models and is being investigated in phase I clinical trials. Both allogenic and autologous transplantation of MSCs isolated from bone marrow or adipose tissue is being evaluated. The rationale for using allogenic MSCs in SSc, as well as in other autoimmune diseases, is based on the possibility that autologous MSCs might be altered in these diseases. In SSc, reports from the literature are controversial. Nevertheless, the role of the oxidative environment and of the crosstalk with neighboring cells (fibroblasts and ECs) on the functional properties of MSCs has been reported. Here, we review the preclinical and clinical data reporting the interest of MSC-based treatment in SSc and question the use of autologous or allogeneic MSCs in perspective of clinical applications.

Keywords: mesenchymal stem cell, systemic sclerosis, allogenic, cell therapy, bleomycin, HOCl

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INTRODUCTION

Systemic sclerosis (SSc) is a rare autoimmune disease, which affects most frequently middle-age patients with a prevalence ranging from 100 to 300 per million depending on the country. The pathophysiology of SSc is still not completely understood even though three main axes of dysfunction are reported: fibrosis, vascular activation and immune abnormalities. The disease is characterized by vascular damage and diffuse fibrosis, which mainly affects skin and lung tissues but heart and digestive tract could also be involved (1). SSc is typically classified as limited or diffuse according to the extent and distribution of skin fibrosis (2). One of the earliest and most frequent symptom is the Raynaud's Phenomenon but vasculopathy is also responsible of other

clinical signs including digital ulcers, pulmonary arterial hypertension, and telangiectasia (3). All of these symptoms are responsible for increased morbidity and lead to functional disability (reduced mouth opening and loss of hand function, for example), pain, and psychological consequences. This impacts not only the patient's quality of life but also reduces his life expectancy. In at least half of the cases, patients will die from SSc-related disorders and the other half from higher incidence of malignancies and cardiovascular diseases compared to the general population (4–6).

There is no curative treatment to date. Only symptomatic treatments are commonly proposed to patients to alleviate pain and improve function. Novel therapeutic strategies are being envisaged among which mesenchymal stem cells (MSCs)-based therapy, which is currently under evaluation in the clinics. The first clinical trial has evaluated the safety of MSCs-containing autologous stromal vascular fraction (SVF) (7) but the option of using allogeneic MSCs is also under evaluation since publications have reported potential alterations of MSCs from SSc patients. Here, we review the available literature on MSC-based cell therapies in SSc from pre-clinical models to clinical applications and discuss the interest of using allogeneic or autologous MSCs for future clinical trials.

GENERALITIES ON MESENCHYMAL STEM CELLS

MSCs are adult multipotent progenitor cells, which have been first identified in the bone marrow (BM) (8). In addition to bone marrow-derived MSCs (BM-MSCs), they have been described in different niches and isolated from several tissues, including adipose tissue (ASCs), umbilical cord (UC-MSCs), placenta, or dental pulp (9). Actually, MSCs have been proposed to be present in virtually all organs as pericytes, which show identical differentiation capacities *in vitro*. However, recent lineage-tracing experiments indicate that *in vivo*, pericytes do not contribute to other cell lineages (10). These results therefore challenge the concept that MSCs, or at least part of them, are pericytes behaving as multipotent tissue progenitors.

MSCs are defined by 3 criteria, as proposed by the International Society for Cell Therapy: (1) plastic adherence, (2) expression of the cell surface markers CD73, CD90, CD105, and lack of expression of the hematopoietic markers CD11b or CD14, CD19 or CD79α, CD34, CD45, HLA-DR, and (3) capacity of differentiation into adipocytes, chondrocytes, and osteoblasts (11). Along with their potential of multilineage differentiation, MSCs exert a number of paracrine functions: they support survival and differentiation of hematopoietic stem cells, induce cell proliferation and have anti-fibrotic, anti-apoptotic, pro-angiogenic, anti-bacterial, and anti-inflammatory effects (12). These effects are mediated primarily through the secretion of soluble mediators but can be enhanced upon contact of MSCs with the target cells (13). The soluble factors are released in the extracellular milieu or within extracellular vesicles that protect them from degradation and allow their transfer throughout the organism [for review, see (12, 14)]. Although, MSCs from different tissue sources share similar properties, they may display some differences in their differentiation potential or immunomodulatory capacity (15, 16).

Thanks to this pleiotropic activity, the therapeutic efficacy of MSCs has been investigated in different pathological conditions, from inflammatory diseases to acute or degenerative diseases. So many different applications as rheumatic diseases (17), stroke (18), lupus and scleroderma (19), heart diseases (20), or bone defects (21) have been evaluated. To date, thousands of patients have been enrolled in hundreds of clinical trials and safety of MSC injection has been proved. In 2016, a meta-analysis has reported no acute toxicity, death, infection, organ systemic failure or risk malignancy after MSC implantation (22). Only transient fever was frequently observed. Efficacy of MSC-based treatments as compared to standards of care is still however under evaluation but promising results have been achieved mostly in phase II trials. For SSc, very few trials have been initiated and results from a couple of registered phase I/II clinical trials are still pending.

THERAPEUTIC EFFECT OF MSCs IN PRECLINICAL MODELS OF SSc

Before the initiation of clinical trials, the interest of using MSCs in the treatment of SSc has been evaluated in different preclinical models. Three models have been used: the model of bleomycin-induced pulmonary fibrosis, the model of hypochlorous acid (HOCl)-induced SSc and the tight skin (Tsk1/+) mouse model of SSc.

The Model of Bleomycin-Induced Fibrosis

The model of bleomycin-induced fibrosis is the most widely used model to replicate scleroderma, or dermal or pulmonary fibrosis in mice or rats. According to the mode of administration, fibrosis can be induced in skin when bleomycin is injected daily sub-dermally or in lung after a single intra-tracheal instillation. Fibrosis in lung, skin, and internal organs may be induced via the use of osmotic pumps that deliver a constant amount of bleomycin over a number of days (23, 24). It is believed that bleomycin causes breaks in DNA, resulting in overproduction of reactive oxygen species (ROS) and inflammatory response that activate fibroblasts and subsequently fibrosis formation. Importantly, the bleomycin models replicate some of the earliest patterns of SSc but do not present with the typical clinical signs and autoantibody patterns of SSc.

The therapeutic potential of MSCs has been first demonstrated in the mouse model of lung fibrosis. One intravenous injection of 500,000 allogenic BM-MSCs on the day of bleomycin instillation was shown to reduce collagen content and inflammation in the lungs (25). At day 14 after injection, the authors could detect engraftment of BM-MSCs in areas of bleomycin-induced injury where they adopted an epithelium-like phenotype. Another study confirmed that systemic injection of allogenic BM-MSCs after intra-tracheal instillation of bleomycin protected mice from injury and fibrosis through the suppression of inflammation (26). They also reported that BM-MSCs differentiated into distinct lung cell phenotypes and secreted chemokines that

might have attracted endogenous cells. Due to the relatively low numbers of transplanted MSCs and persistence of some injury in BM-suppressed animals, the authors suggest that both endogenous and exogenous MSCs likely contribute to the repair process. Another study comparing minimally cultured (2 h) to conventionally cultured (9 days) BM-MSCs indicated that both types of cell preparations ameliorated as efficiently inflammatory and progressive fibrotic lung injury (27). Minimally cultured BM-MSCs had a higher proliferative capacity, expressed higher levels of stem cell markers, and chemokine receptors but lower levels of type I procollagen, α-smooth muscle actin (α-sma) and transforming growth factor-β (TGFβ) that might be more advantageous for cell-based therapy.

The therapeutic effect of BM-MSCs has been also confirmed in rat models of bleomycin-induced lung fibrosis. One intravenous injection of five millions of BM-MSCs resulted in decreased levels of TGFβ1, platelet-derived growth factor-A (PDGF-A), PDGF-B, insulin growth factor-1 (IGF1), and in lower collagen content in lungs (28). Another report has shown that systemic injection of syngeneic BM-MSCs ameliorated fibrosis and decreased inflammatory and angiogenic markers as well as nitric oxide metabolites (29).

In a dermal fibrosis model induced by daily subcutaneous injection of bleomycin for 4 weeks, syngeneic BM-MSCs were injected subcutaneously into the lesion skin every day (30). Treatment with BM-MSCs resulted in a basket-weave organization of collagen arrangement similar to normal skin, with few inflammatory cells and α-sma-positive myofibroblasts as well as down-regulation of TGFβ, type I collagen, and heat-shock protein 47 (HSP47) expression in skin.

The impact of MSCs from different species or tissues on treatment efficacy has been evaluated in the mouse model of bleomycin-induced lung fibrosis. Human UC-MSCs injected systemically 24 h after bleomycin instillation in SCID mice were reported to reduce inflammation markers, collagen content, and inhibit expression of TGFβ, interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) (31). In this study, improvement of tissue remodeling was shown with increased levels of matrix metalloproteinase-2 (MMP-2) and decreased levels of their endogenous inhibitors, tissue inhibitor of MMP (TIMP). Of interest, another report demonstrated that both human amnion- and chorion-derived fetal MSCs displayed similar reduction in the severity of bleomycin-induced lung fibrosis as allogeneic murine amnion- and chorion-derived fetal MSCs (32). Amniotic fluid MSCs were shown to inhibit collagen deposition and to preserve pulmonary function, which could be related to transient increase of MMP-2 (33). Cells were observed to localize within fibrotic lesions with a preferential targeting to the area of fibrosis. Similarly, compared to BM-MSCs, human amnion-derived MSCs reduced collagen deposition and increased MMP-9 activity (34). Finally, ASCs attenuated lung fibrosis induced by repetitive intra-tracheal administrations of bleomycin, namely hyperplasia of Club cells (Clara cells) and cuboidal alveolar epithelial cells, infiltration of the perialveolar ducts by inflammatory cells, septal thickening, enlarged alveoli, and extensive fibrosis (35). It also led to suppression of epithelial cell apoptosis and expression of TGFβ suggesting that irrespective of the tissue or species origin, MSCs are potent inhibitors of lung fibrosis.

The time of cell injection after disease induction may be one important factor to control for better efficacy of cell therapy. Most frequently, MSCs have been implanted at the time or within the few hours after bleomycin instillation. Interestingly, early treatment (day 0) with murine or rat BM-MSCs on the day of bleomycin instillation resulted in a significant reduction of fibrotic changes that was not seen when BM-MSCs-based treatment was delayed (day 7) (25, 36). Similarly, two injections of low (2.8×10^6cells/kg) or high (5.6×10^6cells/kg) doses of human BM-MSCs at day 7 and 15 after fibrosis induction did not lead to improvement of lung function or rescue of damage tissue (37). Injection of autologous ASCs via the trachea at day 15 of the disease did not improve the severity of lung injury but prevented further aggravation of lung damage (38). In this report, the majority of ASCs did not penetrate inside the lung region at week 3 but some cells had sprouted deep into the distorted architecture of the lung at week 6 after disease induction. By contrast, injections of murine amniotic fluid-derived MSCs at either day 0 or day 14 were both efficient to inhibit fibrosis indicating improvement of the disease in both acute and chronic remodeling events (33). Injections of amniotic MSCs or BM-MSCs at day 3 or 6 were even more efficient to reduce lung inflammation than the treatment at day 1 (39). In addition, in a model of repeated injections of bleomycin at day 0 and 7, BM-MSCs injected at day 10 were beneficial in terms of collagen deposition reduction and MMP-9 down-regulation (34).

The age of the donors may be another parameter that might change the therapeutic potential of MSCs. One study compared ASCs from aged (>22 months) and young (4 months) mice (40). At day 21 after bleomycin instillation, mice receiving young ASCs exhibited decreased fibrosis, MMP-2 activity, oxidative stress, and markers of apoptosis vs. untreated controls. Improved treatment with young-donor ASCs was associated with decreased mRNA expression of MMP-2, IGF receptor, and protein kinase B (AKT) activation.

For more a decade since 2003, the skin or lung bleomycin-induced model of local fibrosis was the single model used to evaluate the therapeutic effect of MSCs. However, it does not reproduce the main characteristics of the diffuse human SSc. With respect to the systemic nature of the disease, relevant models of systemic fibrosis have to be used to better evaluate the efficacy of MSCs in models closer to human SSc.

The Model of Bleomycin-Induced Lung and Skin Fibrosis in Aged Mice

A study relied on the use of an established aged (18–22-month-old) mouse model of bleomycin-induced lung fibrosis to test the hypothesis that fibrosis may develop simultaneously in multiple organs and notably affects lung, skin and wound healing. Mice developed irreversible lung and skin fibrosis as well as delayed wound closure at day 21 (41). In this model, intravenous single injection of allogeneic ASCs attenuated lung fibrosis as evaluated by semi-quantitative Ashcroft score on Masson's-trichrome stained histological sections. ASCs also accelerated

wound healing as shown by increased total wound size and wound gaps. This effect was associated with higher levels of caveolin-1 and lower level of $\alpha_v$integrin, TNFα, and miR-199-3p in lung and skin wounds. These results therefore support the hypothesis that ASCs may prevent systemic fibrosis and enhance wound healing but rely on the use of aged mice that are expensive and difficult to get.

The Murine Model of HOCl-Induced SSc

In order to evaluate the therapeutic effect of MSCs in a systemic model of scleroderma, the murine model of HOCl-induced SSc is relevant, cheap and reproducible. After daily intradermal injections of HOCl for 6 weeks, mice develop a systemic disease with the main features of the human form, including skin and lung fibrosis, vascular abnormalities and the production of Anti-Scl-70 autoantibodies, which are anti-nuclear topoisomerase antibodies mainly detected in diffuse systemic scleroderma (42). Our group demonstrated the therapeutic effect of a single intravenous injection of syngeneic BM-MSCs in preventive (day 0) but also curative (day 21) approaches (43). Interestingly, the dose escalation study revealed that the best improvement of both skin and lung fibrosis was obtained with the lowest dose of 2.5×10^5 BM-MSCs. We also compared different sources of cells, i.e., allogenic, xenogeneic, and syngeneic BM-MSCs as well as xenogeneic ASCs, which seemed to be the most potent as compared to BM-MSCs (44). The therapeutic effect was observed on all features of SSc, including reduction of fibrotic, inflammatory. and oxidative markers, but also reduction of autoantibodies Scl-70 while matrix remodeling markers were increased. Finally, infused GFP-expressing allogeneic BM-MSCs were entrapped in the lungs where they were detected at days 1 and 2 but not at day 7. Interestingly, either murine allogenic or human xenogeneic BM-MSCs did not migrate to the skin although skin fibrosis was reduced, indicating a systemic effect of BM-MSCs (44, 45).

The Model of Tight Skin (Tsk1/+) Mouse

The tight skin 1 (Tsk1/+) mouse occurred as a spontaneous mutation in fibrillin-1, which controls the biologically active levels of TGFβ through binding to the latent TGFβ binding proteins (LTBP) 1 and 4 (46). It has therefore been suggested that activation of the TGFβ signaling axis is involved in the development of the phenotype. This genetic model of SSc is characterized by hyperplasia of the sub-cutaneous loose connective tissue with abnormal dermis, osteopenia and deregulation of the interleukin-4 (IL4)/IL4 receptor (IL4R) signaling pathway. Interestingly in this model, MSCs have been shown to ameliorate osteopenia by rescuing impaired lineage differentiation of the recipient BM-MSCs (47). In this interesting study, the authors further demonstrated that MSC-derived extracellular vesicles containing miR-151-5p rescued the disease phenotypes via regulation of the IL4R pathway in recipient BM-MSCs.

Altogether, the beneficial effect of MSCs-based treatment has been proved in the different models of local or systemic scleroderma (**Table 1**). Some clues of their mechanism of action have also been investigated as discussed below.

MECHANISMS OF ACTION OF MSC-BASED THERAPY IN SSc

The capacity of MSCs to differentiate into several cell types of musculoskeletal tissues was the first property that has attracted the attention of researchers and clinicians (58). Since then, several mechanisms of action have been proposed to play important roles in various disorders and diseases via the secretion of many soluble mediators as summarized in (12). Besides anti-apoptotic and anti-bacterial functions, the support of hematopoietic stem cells, the chemoattracting effect, the proliferative, and protective role of MSCs has been largely exemplified. In SSc, the main functions of MSCs are proposed to be anti-inflammatory to counteract the dysregulation of the immune system, anti-fibrotic to down-regulate the excessive production of collagen associated to thickening of skin and internal organs but also pro-angiogenic to counteract the widespread vasculopathy. A number of reviews have discussed the interest of using MSCs for the treatment of SSc (19, 59, 60). In the next paragraph, we focused our attention on the mechanisms that have been deciphered specifically in preclinical models of SSc.

Systemic administration of MSCs results in their homing in lungs and cell engraftment is increased in lungs from bleomycin-induced mice, in particular in areas of injury where they adopt an epithelial cell morphology and display anti-oxidative role (25, 48). Of interest, in the HOCl-induced systemic model of SSc, both human or green fluorescent protein (GFP)-positive murine BM-MSCs were shown to be retained in the lungs of mice after intravenous administration while no cells were detected in the skin suggesting a systemic effect of infused BM-MSCs (43, 45). In addition, tracheal instillation of BM-MSC supernatants decreased the number of apoptotic cells, collagen content, inflammation and fibrotic scores in lungs in the model of bleomycin-induced lung fibrosis (49). These results suggest that BM-MSCs preferentially act through the secretion of soluble mediators that are distributed throughout the body but they can also act directly in contact with damaged lungs where they preferentially reside.

Some secreted mediators involved in their therapeutic effect including IL1-RA, hepatocyte growth factor (HGF) have been identified in murine models of lung fibrosis (50, 51). However, novel strategies of MSC-based treatments have been tested in order to improve their efficacy by enhancing survival after *in vivo* administration or increasing their trophic action. Indeed, preconditioning of BM-MSCs before administration was shown to enhance their therapeutic efficacy. Namely, oncostatin M- or hypoxia-preconditioned BM-MSCs improved pulmonary respiratory function thanks to decreased inflammatory and fibrotic markers as compared to unconditioned BM-MSCs and this effect was mediated at least through the upregulation of HGF (52, 53). Pre-treatment of BM-MSCs with N-acetylcysteine was able to increase both treatment efficacy in a murine model of bleomycin-induced lung injury and BM-MSC survival through the production of anti-oxidant factors (54). In order to improve their therapeutic function, strategies to over-express factors have also been tested. Overexpression

TABLE 1 | Summary of studies on the role of mesenchymal stem cells in preclinical models of systemic sclerosis.

Model/species	Number and origin of MSCs	Route of injection	Injection time	Main results	Mechanisms	References
Bleomycin intratracheal/mouse	5×10^5 allogenic BM-MSCs	IV	d0 or d7	Reduction of collagen content and inflammation in lungs after treatment at d0	ND	(25)
Bleomycin intratracheal/mouse	5×10^5 syngeneic BM-MSCs	IV	H6	Reduction of inflammation and fibrosis in lungs	Differentiation into distinct lung cell phenotypes	(26)
Bleomycin intratracheal/mouse	5×10^5 syngeneic BM-MSCs	IV	d3	Reduction of collagen content and inflammation in lungs	ND	(27)
Bleomycin intratracheal/rat	5×10^6 syngeneic BM-MSCs	IV	H12	Reduction of TGFβ1, PDGF-A, PDGF-B, IGF1 and collagen content in lungs	MSC differentiation into alveolar epithelial cells	(28)
Bleomycin intratracheal/rat	1×10^6 syngeneic BM-MSCs	IV	d4	Diminution of inflammation, collagen content, angiogenic markers and nitric oxide metabolites in lungs	ND	(29)
Bleomycin subcutaneous/mouse	1×10^6 syngeneic BM-MSCs	SC	Daily injection during 4 weeks	Improvement remodeling matrix responsible for normal collagen arrangement in skin. Reduction of inflammation and α-sma-positive myofibroblasts	Down-regulation of TGFβ, type I collagen and HSP47 expression	(30)
Bleomycin intranasal/mice	1×10^6 human UC-MSCs	IV	d1	Reduction of inflammation, collagen content and TGFβ expression and improvement remodeling of matrix	ND	(31)
Bleomycin intratracheal/mouse	4×10^6 IP or 1×10^6 IV ou IT xenogeneic & allogeneic amnion- and chorion-derived fetal MSCs	IP or IV or IT	d0	Reduction of lung fibrosis	ND	(32)
Bleomycin intratracheal/mouse	1×10^6 murine amniotic fluid MSCs	IV	d0 or d14	Inhibition of collagen deposition and preservation of pulmonary function	ND	(33)
Bleomycin intranasal/mouse	Human BM-MSCs or amnion-derived MSCs	IV	d0 and d7	Reduction of inflammation and collagen content in lungs	ND	(34)
Bleomycin intratracheal/mouse (every 2 weeks, 8 doses in total)	5×10^5 human ASCs	IP	4 doses at time of bleomycin injection	Reduction of lung fibrosis and inflammation	ND	(35)
Bleomycin intravenous/rat	5×10^5 syngeneic BM-MSCs	IV	d1 or d7	Reduction of lung fibrosis only after treatment at d1	ND	(36)
Bleomycin intratracheal/rat	2.8×10^6 or 5.6×10^6 human BM-MSCs /kg	IV	d8 or d15	Safety of MSC injection No amelioration of disease	ND	(37)
Bleomycin intratracheal/rat	Autologous ASCs	IT	d15	No improvement but prevention of lung damage aggravation	ND	(38)
Bleomycin intratracheal/mouse	5×10^5 syngeneic BM-MSCs	IV	d1 or d3 or d6	Reduction of lung inflammation & fibrosis after d3 or d6 treatment	ND	(39)
Bleomycin intratracheal/mouse	5×10^5 syngeneic ASCs from old or young mice	IV	d1	Only young ASCs induced lower lung fibrosis, oxidative stress and apoptosis	Lower levels of MMP-2, IGFR and AKT activation	(40)
Bleomycin intratracheal/mouse	5×10^5 syngeneic ASCs	IV	d1	Reduction of lung & skin fibrosis Acceleration of wound healing	Decreased miR-199-3p and increased caveolin-1 in lungs and skin	(41)

(Continued)

TABLE 1 | Continued

Model/species	Number and origin of MSCs	Route of injection	Injection time	Main results	Mechanisms	References
HOCl intradermic injection/mouse (daily, 42 days)	2.5×10^5 syngeneic or allogeneic BM-MSCs, or human ASCs & BM-MSCs	IV	d0 or d21	Reduction of fibrotic, inflammatory and oxidative markers in skin & lungs. Improvement of matrix remodeling.	ND	(43) (44) (45)
Tsk1/+ mouse	1×10^5 allogenic BM-MSC /kg bodyweight	IV	8 weeks	Improvement of osteopenia	Downregulation of the IL4R pathway by miR-151-5p in MSC-EV	(47)
Bleomycin intratracheal/rat	2.5×10^6syngeneic BM-MSCs	IV	d0 or d7	Reduction of alveolitis, pulmonary fibrosis and oxidative stress	Conversion of BM-MSC into type II alveolar epithelial cells	(48)
Bleomycin intratracheal/rat	0.2mL allogenic BM-MSC supernatants	IT	H6 and d3	Reduction of collagen content, inflammation and fibrosis in lungs	ND	(49)
Bleomycin intratracheal/mouse	5×10^5 allogenic BM-MSCs	IV	d0	Reduction of IL1α lung level	IL1RN expressing MSCs antagonizing IL1α	(50)
Bleomycin intranasal/mice	5×10^4 allogenic or HGF KO BM-MSCs/g bodyweight	IV	H6 or d9	Reduction of lung fibrosis and inflammation, and increase of HGF	HGF release	(51)
Bleomycin intratracheal/mouse	2.5×10^5 syngeneic OSM-preconditioned BM-MSCs	IT	d3	Diminution of inflammation and fibrosis in lungs and improvement of respiratory function	Production of high level of HGF	(52)
Bleomycin intratracheal/mouse	2.5×10^5 syngeneic hypoxia-preconditioned BM-MSCs	IT	d3	Improvement of lung function and matrix remodeling. Decreased pro-inflammatory and fibrotic factors in lungs	Anti-apoptotic	(53)
Bleomycin intratracheal/mouse	2×10^5 NAC-pretreated human embryonic MSCs	IV	d1	Decrease of inflammation and lung fibrosis.	Increased antioxydative capacity of MSCs	(54)
Bleomycin intratracheal/mouse	5×10^5 human BM-MSCs overexpressing let7d	IV	d7	Reduction of collagen content and inflammation in lungs	Let7d over-expression	(55)
Bleomycin intratracheal/mouse	1×10^6 xenogenic UC-MSCs over-expressing ACE2/kg bodyweight	IV	d3	Decrease of collagen content, fibrotic and pro-inflammatory factors and increase of anti-oxidative mediators	ACE2 over-expression	(56)
Bleomycin subcutaneous/mice (daily, 21 days)	1×10^6 Trx-1-overexpressing BM-MSCs	SC	Daily	Reduction of skin fibrosis and apoptosis, promotion of BM-MSC survival and differentiation into endothelial cells	TRX1-mediated inhibition of oxidative stress	(57)

ACE2, angiotensin converting enzyme 2; AKT, protein kinase B; ASCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow mesenchymal stem cells; HGF, hepatocyte growth factor; HOCl, hypochlorite; IGF, insulin growth factor; IGFR, IGF receptor; IL, interleukin; IL1RN, interleukin 1 receptor antagonist; IP, intraperitoneal; IT, intratracheal; IV, intraveinous; KO, knock-down; MMP, metalloproteinase; MSC-EV, mesenchymal stem cell-derived extracellular vesicles; NAC, N-acetylcystein SC, subcutaneous; OSM, onconstatin M; PDGF, platelet-derived growth factor; TGFβ, transforming growth factor; Trx-1, thioredoxin 1; Tsk1, tight skin; UC-MSCs, umbilical cord-derived mesenchymal stem cells.

of let7d, which is a microRNA known to have anti-fibrotic effects, in BM-MSCs resulted in slight decrease of collagen content in lungs and improved mouse survival when injected at day 7 in bleomycin-induced mice while naïve BM-MSCs did not have any beneficial effect (55). Similarly, human UC-MSCs over-expressing angiotensin-converting enzyme 2 (ACE2) were more efficient than naïve UC-MSCs to decrease collagen content, fibrotic, and pro-inflammatory factors while increasing anti-oxidative and anti-inflammatory mediators (56). Thioredoxin 1 (Trx-1)-overexpressing BM-MSCs were also shown to inhibit apoptosis and fibrosis under hypoxic conditions and to promote the formation of tubular-like structures by endothelial cells in bleomycin-induced lung injury (57). Indeed, strategies for enhancing MSCs survival and/or efficiency have been demonstrated to be of interest in preclinical models of SSc. A summary of the potential (or proven) mechanisms of action of MSCs in the treatment of SSc is provided in **Figure 1**.

CHARACTERIZATION OF MSCS FROM SSc PATIENTS

Early studies evaluating the phenotypic and functional characteristics of MSCs from SSc patients reported no major difference as compared with MSCs from healthy donors. Indeed, BM-MSCs from two patients with SSc have been compared to those of healthy donors 10 years ago (61). No major differences in their proliferative rate, adipogenic or osteogenic differentiation potential, expression of surface markers and immunosuppressive properties were reported between the two sources of BM-MSCs. Some years later, similar data were obtained using BM-MSCs from 12 scleroderma patients in comparison with 13 healthy controls (62). SSc and healthy BM-MSCs displayed the same phenotype, identical capacities to proliferate and form CFU-F, similar capacities to differentiate into adipogenic and osteogenic lineages, equal capacities to support long-term hematopoiesis, and similar anti-proliferative properties. SSc ASCs were also shown to have comparable phenotypic markers and functional characteristics (proliferative and differentiation potential) as healthy donor ASCs (63). However, this cohort of six SSc patients was characterized by cutaneous local forms of scleroderma (generalized morphea, linear and/or plaque scleroderma) and not diffuse systemic sclerosis. More recently, no difference in proliferative capacities and potential to support endothelial cell tube formation was noticed between ASCs from ten patients with diffuse SSc or eight healthy donors (64).

By contrast, other studies have reported some differences. As an example, MSCs isolated from the skin of scleroderma patients expressed more PDGF-R than those from healthy donors, resulting in the production of higher levels of ROS (65). Nevertheless, SSc-MSCs were still able to improve the antioxidant defenses by counterbalancing ROS accumulation. More recently, the impact of the pro-oxidant environment exerted by SSc sera on BM-MSCs has been investigated. Exposure of BM-MSCs to SSc sera enhanced their antioxidant capacities as well as their osteogenic and adipogenic potentials while their immunosuppressive functions were reduced (66). With the objective to evaluate the consequence of TGFβ activation in SSc, analysis of several members of the TGFβ family has been undertaken in BM-MSCs from nine patients with diffuse SSc (67). A highly significant increase in mRNA and protein levels of TGFβ receptor II (TβRII) was detected in SSc BM-MSCs as compared to healthy controls. Moreover, in response to TGFβ activation, the production of type I collagen and Smad-3 phosphorylation was up-regulated in SSc BM-MSCs and also in SSc fibroblasts. An earlier study reported that nerve growth factor receptor (NGFR)-positive BM-MSCs, a subpopulation of MSCs with high proliferative and clonogenic potentials, were less numerous in BM from SSc patients than from healthy controls (68). They displayed lower clonogenic potential (10-fold less), had a reduced fold expansion rate (105-fold less), showed signs of rapid aging and stress and, never differentiated into adipocytes or osteoblasts. In line with these data, Cipriani and coauthors have described that BM-MSCs from SSc subjects expressed up-regulated α-sma and transgelin (TAGLN or SM22α) genes and displayed reduced proliferative activity and migration potential (69). An increase in senescence markers (senescence-associated β-galactosidase (SA-βGal), p21, IL6, TGFβ) was also observed while their immunosuppressive activity on lymphocyte proliferation and regulatory phenotype induction was retained (70). Interestingly, the same team showed an impaired crosstalk between endothelial cells (ECs) and BM-MSCs (71). In coculture conditions, SSc ECs induced increased levels of VEGF-A in SSc MSCs and of TGFβ, PDGF-R, α-sma, type I collagen in both SSc and healthy MSCs. Despite the production of high levels of VEFG-A by SSc MSCs, they had lower angiogenic performance. The authors also detected increased expression of TGFβ and PDGF-BB in SSc ECs when co-cultured either with MSCs from healthy or SSc individuals. This impaired crosstalk between ECs and MSCs suggested that ECs may be involved in the early steps leading to fibrosis by producing factors that induce a phenotype switch of MSCs toward myofibroblast cells. In another study, SSc BM-MSCs exhibited altered differentiation into contractile and synthetic vascular smooth muscle cells when stimulated by connective tissue growth factor (CTGF) or b-FGF. Moreover, stimulation with TGFβ1 induced a myofibroblast-like phenotype with high production of α-sma and collagens, higher migration capacity but less proliferative capacity (72). Accordingly, SSc BM-MSCs expressed up-regulated levels of proangiogenic factors including VEGF, stromal derived factor-1 (SDF-1) and C-X-C chemokine receptor (CXCR4) (73). However, they noticed that SSc BM-MSCs promoted angiogenesis and improved capillary morphogenesis as compared to healthy MSCs.

A unique study has identified altered functions in SSc ASCs (74). No alteration in their phenotype or differentiation potential was noticed but their proliferative rate, metabolic activity and migration capacity were reduced as compared to healthy matched control ASCs. However, no data on the expression of TβRII or other fibrotic markers as well as on their immunosuppressive and angiogenic functions were available. Further functional analysis is therefore required to fully decipher the therapeutic potential of ASCs isolated from SSc patients.

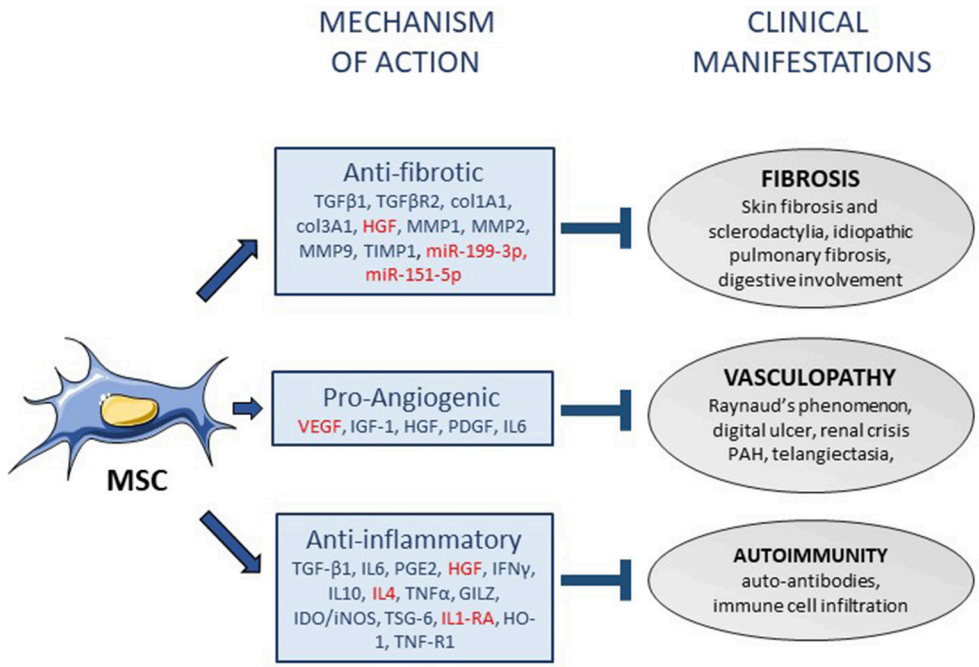

FIGURE 1 | Mechanisms of action of mesenchymal stem cells in systemic sclerosis. Mesenchymal stem cells (MSC) display various functions that might participate to their mechanisms of action in the treatment of systemic sclerosis (SSc), through the release of soluble mediators (arrows). The mediators that have been associated with anti-fibrotic, pro-angiogenic or anti-inflammatory functions of MSCs are listed in the squares (in red: mediators specifically identified in SSc). Three mechanisms of action of MSCs can be associated with the improvement of the three main clinical manifestations of SSc (blind-ended arrows). PAH, pulmonary arterial hypertension.

CLINICAL DATA ON MSC-BASED TREATMENT IN SSc

Case Reports Using Fat Injection

In recent decades, fat tissue grafting has been used to treat skin atrophy or fibrosis thanks to its biocompatibility and property of filling but also to its high content in multipotent stem or progenitor cells with regenerative potential. The first report on the implantation of fat in hands of patients who presented Raynaud's phenomenon was published in 2014 (75). Among the thirteen treated patients, nine patients suffered from scleroderma. There were no complications and the treatment showed some evidence of perfusion. In 2015, another study reported a significant improvement of digital ulcerations, hand grasping, and pain after injection of adipose tissue-derived cells into fingers of 15 SSc patients suffering from digital ulcers (76). More recently, the effect of one local injection of autologous SVF associated with platelet-rich plasma into malar and perioral areas in six patients showed improvement of skin elasticity and vascularization (77). Some indication of efficacy was suggested by these case reports but the role of ASCs contained within the SVF could not be drawn.

Case Reports Using MSCs

The first patient who received BM-MSCs in the treatment of progressive diffuse SSc has been reported in 2008 (78). A young female patient had severe disease, refractory to all immunosuppressive drugs. At time of implantation, she presented with six painful ulcerations and received 6×10^7 intravenous administration of allogeneic haploidentical-related donor BM-MSCs. No adverse events were reported and 3 months after treatment, a significant decrease in the patient's painful ulcerations was measured. Vascular improvement in the blood circulation of hands and fingers was noticed but level of Scl-70 autoantibodies was not decreased. In 2011, the same team reported four supplementary cases of allogeneic BM-MSC systemic injection (0.22 to 1.8×10^6 BM-MSCs/kg bodyweight) (79). Here again, improvement of vasculopathy and skin fibrosis was observed but the study underscored the need to set phase II clinical trials for efficacy assessment.

Local injection of autologous ASCs in combination with hyaluronic acid (HA) solution has been first tested in 2013 (63). Infiltration of 8×10^5 ASCs/mL HA was done in a single area for each patient, either the face or the arm chosen with the patient consent. The procedure improved considerably skin fibrosis for four out of six patients and moderately for one patient. All patients showed arrest of local disease progression (100%), four of them presented regression of dyschromia (67%), five patients increased skin softening (83%), four patients showed better sensitivity (67%), and one patient reported erythema reduction (17%). The study demonstrated that ASCs could be successfully implanted locally in patients with severe disease, thus representing a good, feasible, and efficient cell-based soft-tissue augmentation strategy. Of interest, the same team thereafter compared autologous fat and ASC transplantation to evaluate clinical improvement of mouth opening in two groups of five

TABLE 2 | Summary of clinical trials using mesenchymal stem cells for the treatment of systemic sclerosis.

Type of treatment	Route	Patient number	Clinical target	Clinical outcome	Adverse events	References
Autologous fat grafting (mean of 30 ml/hand)	Subcutaneous in hand	9	Raynaud's phenomenon	Improvement of perfusion and decrease of ulcer numbers	None	(75)
Autologous fat grafting (0.5–1 ml/finger)	Subcutaneous in hand	15	Digital ulcers	Improvement of digital ulcerations, hand grasping and pain	None	(76)
Combined platelet-rich plasma and lipofilling treatment	Subcutaneous in peri-oral location	6	Face skin fibrosis	Improvement of skin elasticity, labial rhyme opening and vascularization	None	(77)
Allogeneic BM-MSCs (10^6/kg bodyweight)	Intravenous	1	Systemic sclerosis	Reduction of ulceration and pain, improvement of hand vasculopathy	None	(78)
Allogeneic BM-MSCs ($0.22–1.8 \times 10^6$/kg)	Intravenous	5	Systemic sclerosis	Improvement of skin fibrosis and vasculopathy	Minor respiratory tract infection	(79)
Autologous ASCs (4 to 8×10^6)	Subcutaneous peri-oral location	6	Localized skin scleroderma	Disease stabilization for all patients and improvement of skin elasticity in 4/6 patients	None	(63)
Autologous fat (16 ml) or ASCs (3.2×10^6)	Subcutaneous peri-oral location	5	Face skin fibrosis	Improvement of skin fibrosis & mouth opening	None	(80)
Autologous SVF (5 ml/hand)	Subcutaneous in hand	12 (phase I trial)	Severe hand functional handicap	Improvement of pain, grasping capacity, finger edema, Raynaud's phenomenon, quality of life	None	(7) (81) (82)

patients (80). Both procedures obtained significant results but neither one emerged as a first-choice technique.

Clinical Trials With MSCs

The results of the first open phase I clinical trial evaluating the interest of MSCs, and more precisely of MSC-containing SVF, on 12 SSc patients with severe functional hand handicap were published in 2015 (7). Injection of autologous SVF into fingers was safe and a significant improvement in pain, grasping capacity, finger edema, Raynaud's phenomenon, and quality of life was recorded at 6 months. At 12 months follow-up, a significant improvement in skin sclerosis, motion, edema and strength of the hand was observed. SVF was therefore claimed as a promising therapy whose effect persisted at least 1 year after injection (81). Finally, at 22 and 30 months after treatment, safety, tolerability and efficacy were very encouraging (82). The same team is now including 40 patients in a randomized double blind phase II trial to evaluate efficacy of the approach.

There are only 4 clinical trials recorded in the United State National library of Medicine (www.clinicaltrials.gov). One trial has been completed but no result is available. Only one of those is recruiting in France since 2014. This is a phase I/II trial which assesses the effect of one intravenous injection of intrafamilial allogenic MSCs on 20 patients. The trial is still enrolling patients and data are not yet available. Another phase I/II clinical trial has started in November 2017. It evaluates the therapeutic effect of allogeneic BM-MSCs after injection in intramuscular areas of affected limbs in 20 scleroderma patients with digital ulcers and is recruiting (NCT03211793) (83).

In summary, data from case reports and clinical trials are scare and need to be taken with cautious in terms of efficacy (**Table 2**). One question still pending is whether autologous or allogeneic ASCs or BM-MSCs have to be used in the clinics since some studies have reported phenotypic or functional alterations of MSCs from SSc patients.

CONCLUSION

Many studies have now established the beneficial effect of the administration of BM-MSCs, ASCs or MSCs from other tissue sources in different preclinical models characterized by local or systemic fibrosis. Some evidence of safety and efficacy of MSC-containing SVF or culture expanded MSCs has been described from the clinics but efficacy needs to be further proved in phase II clinical trials that are ongoing. Both autologous and allogeneic MSCs from BM or adipose tissue are being assessed but the risk that the functional properties of MSCs isolated from SSc patients are altered is under debate. Contradictory results are reported in the literature but a number of reports discuss the reduction of the number of clonogenic cells, proliferative rate, differentiation, and angiogenic potentials. MSCs from SSc patients display a more mature and myofibroblast-like phenotype, probably related to microenvironmental signals dysregulated during the disease. They express higher levels of TβRII and TGFβ, which is released into the extracellular medium where it can act in an autocrine or paracrine manner. Moreover, the crosstalk between MSCs and ECs contribute to the altered expression of different molecules involved in angiogenesis, inducing a switch of perivascular MSCs toward a myofibroblast population, further supporting the fibrotic process. The finding that MSCs from SSc patients constitutively overexpress mediators involved in the fibrotic and

angiogenic processes might indicate that MSCs are altered by the environment secondary to the onset of the disease or, that they might participate to the physiopathology of the disease. With respect to the use of autologous MSCs for clinical applications, further investigation on their functional properties is likely needed.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Targeting Costimulatory Pathways in Systemic Sclerosis

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Systemic sclerosis (SSc) is an autoimmune T-cell disease that is characterized by pathological fibrosis of the skin and internal organs. SSc is considered a prototype condition for studying the links between autoimmunity and fibrosis. Costimulatory pathways such as CD28/CTLA-4, ICOS-B7RP1, CD70-CD27, CD40-CD154, or OX40-OX40L play an essential role in the modulation of T-cell and inflammatory immune responses. A growing body of evidence suggests that T-cell costimulation signals might be implicated in the pathogenesis of SSc. CD28, CTLA-4, ICOS, and OX40L are overexpressed in patients with SSc, particularly in patients with cutaneous diffuse forms. In pre-clinical models of SSc, T-cell costimulation blockade with abatacept (CTLA-4-Ig) prevented and induced the regression of inflammation-driven dermal fibrosis, improved digestive involvement, prevented lung fibrosis, and attenuated pulmonary hypertension in complementary models of SSc. Likewise, potent anti-fibrotic effects were seen with the blockade of OX40L by reducing the infiltration of inflammatory cells into lesional tissues leading to decreased fibroblast activation. Concerning clinical effects, a preliminary observational study suggested some effectiveness of abatacept on inflammatory joint involvement, whereas clinical improvement of skin fibrosis was observed in a small placebo-controlled randomized trial. Currently there is one ongoing phase II clinical trial assessing the efficacy of abatacept in SSc (ASSET trial, NCT02161406). Overall, given the lack of available effective agents and the known toxic effects of immunosuppressive agents approved for use in SSc, costimulatory pathways offer the advantage of a targeted approach to costimulatory signals and potentially a better safety profile.

Keywords: adaptive immunity, inflammation, costimulatory pathways, systemic sclerosis, fibrosis

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INTRODUCTION

Systemic sclerosis (SSc) is a rare connective tissue disease characterized by the triad of vascular damage, autoimmunity culminating in widespread fibrosis (1). It can be a devastating disease with a profound impact on life expectancy reflected by high mortality rates (2, 3). The pathogenesis of SSc involves a genetic predisposition together with some partly known environmental triggers. A growing body of evidence suggests that in early stages of the disease there is an interplay between the immune system in particular T and B cells and fibroblasts leading to the perpetuation of the fibrotic process (4).

The activation of naive T cells requires a first signal involving the recognition by the T cell receptor (TCR) of a given antigen and a second non antigen-specific costimulatory signal (5, 6). As a matter of fact, activation and proliferation of naïve T cells are unlikely in the absence of costimulatory signals (7). The CD28-CD80/CD86 pathway is considered the classical co-stimulatory pathway but other pathways such as ICOS-B7RP1, CD70-CD27, CD40-CD154, or OX40-OX40L also play an essential role (8, 9). Negative costimulatory pathways such as CTLA4-B7 or PD1-PDL1/2 play a key role in restraining adaptive immune response (10). There are numerous reports related to the implication of T cell costimulatory pathways in the pathogenesis of several different autoimmune conditions including multiple sclerosis, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (11, 12).

Positive and negative costimulatory signals might be implicated in the pathogenesis of SSc (13, 14). Although a variety of costimulatory molecules have been identified and different immunotherapeutic strategies have been tested, objective clinical responses are rare in SSc patients.

This review aims to discuss the contribution of T cell costimulatory pathways in SSc pathogenesis with a specific focus on their potential therapeutic applications.

POSITIVE COSTIMULATORY SIGNALS IN SSC

CD28, which represents one of the most relevant costimulatory pathways, is essential for naïve T cell activation. Indeed, it promotes T-cell proliferation through the induction of IL-2 secretion after binding to CD80 and CD86 (15). Increased soluble CD28 levels were detected in patients with SSc, compared to healthy subjects, but no correlation was found between soluble CD28 concentrations and extent of skin fibrosis. Furthermore, higher levels of circulating soluble CD28 were more often observed in SSc patients with associated autoimmune disorders (Sjögren's syndrome, systemic lupus, or polymyositis) (12). One of the proposed mechanisms by which CD28 could be implicated in the pathogenesis of SSc is that T cell activation through CD28 is associated with a different profile of cytokine production, with increased proinflammatory and profibrotic cytokines such as TNF, IL-2, IL-6, and IL-10 (16). Moreover, soluble CD28 was shown to inhibit T cell response *in vitro* (12).

The inducible costimulator (ICOS) is a member of the CD28 superfamily. Its structure and function are very similar to that of CD28 (15). ICOS is highly expressed in activated T cells of patients with connective tissue diseases, including RA and SLE (17, 18). ICOS has broad effects on adaptive immune system activation by promoting germinal center formation, T cell proliferation, antibody production and B cell isotype switching (19). Previous reports showed that ICOS serum levels and peripheral T cell expression were increased in patients with early diffuse cutaneous SSc (dcSSc) (20, 21). Overexpression of ICOS in activated T cells induces proinflammatory (IFN-γ, IL-17) and pro-fibrotic (IL-4) cytokine synthesis, promoting fibroblast activation and extracellular matrix synthesis (21).

OX40 and its binding partner, OX40L are members of the TNF receptor superfamily and generate a potent costimulatory signal that upregulates IL-2 production, enhances T cell survival, B cell proliferation, and differentiation and proinflammatory cytokine production (22, 23). OX40 also mediates inactivation of T-reg cell function that unleashes nearby DCs, allowing them to induce an adaptive immune response. OX40 levels were found significantly increased in SSc patients compared to controls and patients with SLE, particularly in the early-onset stage of the disease (24). Two reports confirmed the influence of OX40-ligand (OX40L) polymorphisms in SSc genetic susceptibility, highlighting its role in the disease pathogenesis (13, 25).

Serum levels of the OX40 binding partner OX40 ligand (0X40L) are increased in patients with SSc and were shown to be predictive of the worsening of dermal and lung fibrosis (26). OX40L expression is also prominent in the skin of patients with diffuse SSc. Of great interest, OX40L has been recently reported to be overexpressed in resting and activated dermal fibroblasts, in addition to lesional skin T and B cells. Thus, pathological activation of dermal fibroblasts may be directly mediated by the OX40-OX40L axis, linking directly immunity to fibrosis. The profibrotic effects of OX40L may also be related to its crosstalk with matrix metalloproteinases (MMPs), which are abnormally produced in SSc (27). OX40L has been shown to directly modulate MMP expression in the lesional skin of fibrotic mice invalidated for OX40L (26). Moreover, MMP-2 directly stimulates dendritic cells to up-regulate OX40L on the cell surface (28). MMPs also condition human naïve T cells and dendritic cells to prime TH2 phenotype via an OX40L-dependent pathway (28, 29).

CD40 is another member of the TNF receptor superfamily that plays a pivotal role in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation. The binding of CD154 (CD40L) on TH cells to CD40 activates antigen-presenting cells and induces a variety of downstream effects.

A wide array of evidence reported increased CD40 expression in activated CD4+ T cells, skin fibroblasts, and the serum of SSc patients (30–33). The upregulation of the CD40-CD40L axis in immune cells seems at least partly mediated by epigenetic modifications (Demethylation of CD40L regulatory elements) (34, 35).

Soluble CD40L serum concentrations are associated with vascular complications of the disease including pulmonary arterial hypertension (PAH), digital ulcers and destructive peripheral microangiopathy assessed by nailfold videocapillaroscopy (36, 37).

Proteomic analysis of sera from individuals with diffuse cutaneous SSc revealed a multianalyte signature, based notably on CD40L levels, associated with clinical Improvement during Imatinib Mesylate treatment. This results highlights the potential interest of CD40L to predict treatment response in SSc (38).

DNAX accessory molecule 1 (DNAM-1) is an important regulator of the adhesion and costimulation of T cells belonging to the immunoglobulin supergene family (39). Strinkingly, CD226, which encodes DNAM-1, polymorphisms have been

identified as a genetic susceptibility factor to SSc, highlighting the contribution of costimulatory pathways in the pathogenesis of this condition (40, 41). DNAM-1 is also overexpressed in the skin of patients with SSc (39) and upregulattion of DNAM-1 in CD8+ T cells is associated with disease severity, suggesting this factor to be a potential therapeutic target in SSc (42).

NEGATIVE COSTIMULATORY SIGNALS IN SSC

Since the advent of immunotherapy for the treatment of several neoplastic conditions there has been a rising interest in intrinsic immunity downregulators such as cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4) or programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1). One of the main drawbacks of immune checkpoint blockade therapy is the emergence of the so-called immune-related adverse events highlighting the role that immune checkpoint play in maintaining immunologic homeostasis (43). Recently there have been few reports of SSc and SSc-like conditions induced by immune checkpoint inhibitors (44, 45).

CTLA-4 is a T cell inhibitory molecule which binds to CD80/86 with higher affinity than CD28, resulting in a drop in IL-2 production and a decreased T cell proliferation (46). Preliminary data suggest that CTLA-4 might contribute to human SSc. Notably, serum soluble CTLA-4 levels (sCTLA4) have been shown to be increased in patients with diffuse cutaneous subset and to correlate with disease severity and activity (14). Increased sCTLA4 serum levels are also observed in several other autoimmune diseases. The biological significance of elevated sCTLA-4 serum levels is not completely clarified yet. sCTLA-4 may specifically inhibit early T-cell activation by blocking CD80/CD86—CD28 interaction. On the other hand, higher levels of sCTLA-4 could compete for the binding of the membrane form of CTLA-4 with CD80/CD86, leading to a reduction in inhibitory signaling (47). In line with this, a meta-analysis of published data showed CTLA-4 polymorphisms conferred susceptibility to SSc (48). Macrophages in particular profibrotic M2 phenotype macrophages may have an important in perpetuating the disease (49). A previous study on tumor immune escape showed that blocking CTLA-4 decreased M2 macrophages differentiation thus suggesting a close relationship between these entities (50).

PD-1 is another inhibitory molecule that regulates T cell tolerance. The expression of PD-1 and its ligands PD-L1 and PDL-2 is antagonized by their soluble forms, leading to augmented T-cell responses (51). Two previous reports showed soluble PD-1 and PD-L1 and PD-L2 to be elevated in SSc patients suggesting it to be correlated to disease development and severity (52, 53). These data seem to suggest that the elevated levels of soluble CTLA-4 and PD-1/PD-L1 and 2 observed in SSc is related to an abnormal T cell and B cell activation.

Figure 1 summarizes the putative role of costimulatory pathways in the pathogenesis of SSc.

COSTIMULATION THERAPY—DATA FROM EXPERIMENTAL MODELS OF SSC

Altogether, positive and negative costimulatory T cell signals seem to implicated in the pathogenesis of SSc. Therefore, targeting these pathways through immunotherapy might be more advantageous than current immunosuppressive therapies traditionally used in SSc. Indeed, this strategy offers the hypothetical advantage of targeting the antigen-specific T cells involved in the disease without causing generalized immunosuppression and therefore decreasing the theoretical risk of infectious events (54). Data from *in vivo* complementary animal models give us insight on the effects of the costimulatory pathways blockade as a promising strategy for the treatment of SSc.

A first set of data have shown that DNAM-1 gene invalidation through the use of DNAM-1 deficient mice or the treatment of mice with DNAM-1 neutralizing antibodies prevented the development of dermal fibrosis in the bleomycin mouse model by reducing the infiltration of lesional skin by inflammatory cells and preventing the release of proinflammatory cytokines (TNF-α and IL-6) (39).

OX40L blockade through gene invalidation or targeted therapy using monoclonal antibodies prevented and induced regression of established inflammation-driven dermal fibrosis in the bleomycin mouse model, which mimics early and inflammatory stages of SSc (26). Likewise, OX40L blockade protected against the development of interstitial lung disease and alleviated pulmonary hypertension in the Fra-2 transgenic mouse model, which is characterized by extensive inflammatory infiltrates with features of human vasculopathy, including PH, paralleled by fibrosing alveolitis similar to that in patients with SSc (26). The effects observed with OX40L blockade were mediated by a dramatic reduction of T cells, B cells, and natural killer cells as well as by reduced levels of proinflammatory cytokines such as IL-6 and TNF-α (26). Interestingly, OX40L knockout mice spontaneously develop interstitial pneumonia and severe PH thus addressing several aspects of SSc pathogenesis (24, 55).

Abatacept (CTLA4-Ig) is a recombinant fusion protein comprising the extracellular domain of human CTLA-4 and the modified Fc region of human IgG1 widely used for the treatment of rheumatoid arthritis (56). In complementary murine models of SSc abatacept prevented the development of inflammation-driven fibrosis and reversed established bleomycin-induced fibrosis. Abatacept treatment led to reduced total and activated T cell, B cell and monocyte infiltration in the lesional skin, as well as decreased release of proinflammatory and profibrotic cytokines. Abatacept demonstrated no efficacy in the treatment of late and non-inflammatory dermal fibrosis in the tight skin-1 mouse model, supporting that T cells are necessary to drive the antifibrotic effects of this molecule (57).

Abatacept also improves gastrointestinal involvement in the chronic graft-vs.-host disease (cGvHD) model by decreasing liver transaminase levels and improving colon inflammation. Abatacept alleviated interstitial lung disease and reversed PH in Fra-2 mice by improving vessel remodeling and related cardiac

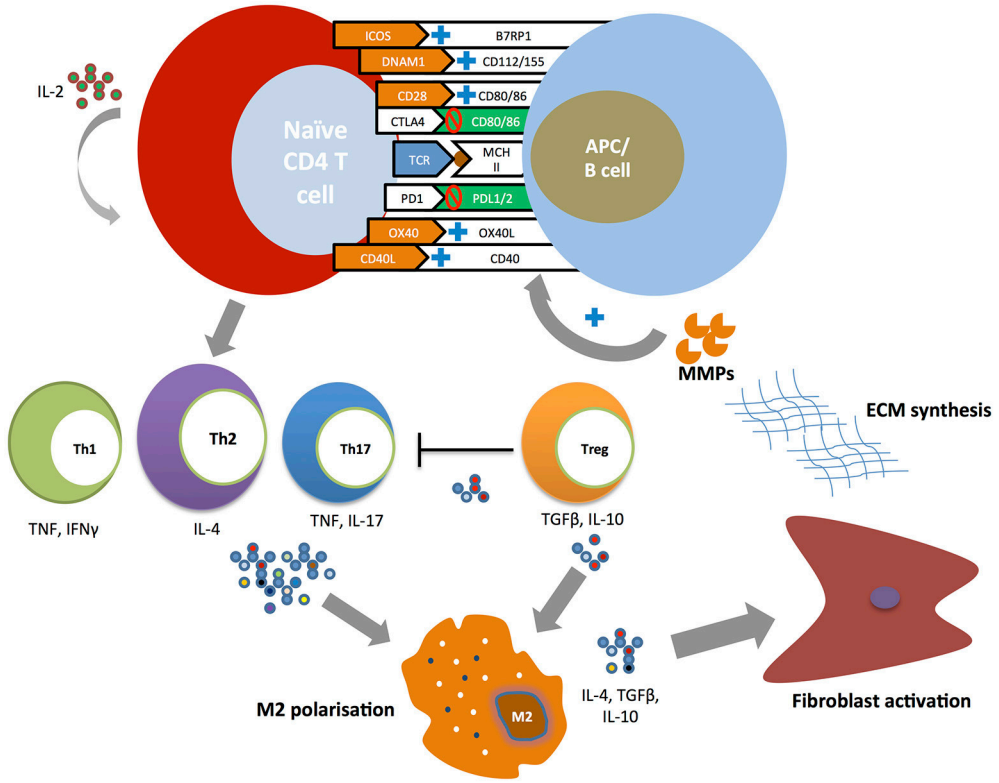

FIGURE 1 | Costimulatory pathways and T cell responses in the pathogenesis of SSc. Naïve CD4+ T cells undergo expansion and differentiation at the time of T cell receptor (TCR) binding to a major histocompatibility complex (MHC) molecule carrying a peptide antigen. The engagement of positive costimulatory pathways (ICOS-B7RP1, DNAM-CD112/115, CD28-CD80/86, CD40-CD154, and OX40-OX40L) and the inhibition of negative costimulatory pathways (CTLA4-CD80/86, PD1-PDL1/2) promote the development of T helper subsets. T helper cell cytokines (TNF, IFNγ, IL-4, IL-17) induce profibrotic type 2 macrophages (M2) polarization with T helper type 2 (Th2) playing a central role. Regulatory T cells (Treg) promote further M2 macrophage polarization and activation through the secretion of IL-10 and TGFβ. M2 activated macrophages promote fibroblast activation leading to excessive extracellular matrix production. In its turn metalloproteinases (MMPs) directly stimulate antigen-presenting cells (APCs) to upregulate positive costimulatory molecules in particular OX40L.

hemodynamic impairment. Abatacept significantly reduced, in lesional lungs of Fra-2 mice, fibrogenic markers levels, T-cell proliferation and M1/M2 macrophage infiltration (58). These findings suggest that abatacept might be an appealing therapeutic approach beyond skin fibrosis for organ involvement in SSc.

FROM BENCH TO BEDSIDE: DATA FROM OBSERVATIONAL STUDIES AND CLINICAL TRIALS

Data from an observational (59) and case control study (60) suggested beneficial effects of abatacept in patients with SSc. Indeed, in the study from de Paoli et al. (60) abatacept was added to standard therapy in four patients suffering from severe diffuse cutaneous SSc (dcSSc). In this study, abatacept induced a medically significant and pronounced improvement of the modified Rodnan skin score (mRSS) as well as in pulmonary function tests. However, these results are difficult to interpret since regression of skin fibrosis and overall disease activity over time may reflect the natural history of SSc. Data from the EUSTAR (European League Against Rheumatism Scleroderma Trials and Research group) cohort showed that abatacept induced clinical improvement particularly some effectiveness on inflammatory joint involvement on a group of 11 patients with SSc (59).

One small randomized, placebo-controlled trial assessed the efficacy of abatacept in patients with dcSSc over a period of 24 weeks (61). After randomization, 7 patients received abatacept therapy, while 3 patients in the control group received placebo. At week 24, subjects randomized to abatacept showed a trend toward improvement in mRSS (-8.6 $p = 0.0625$). After adjusting for disease duration, mRSS significantly decreased in the abatacept group as compared to the placebo group (-9.8 $p = 0.0114$). Interestingly, after differential gene expression and pathway enrichment analysis the authors showed that improvers tended to be in the inflammatory intrinsic subset at baseline. Notwithstanding, the small sample size does not allow do draw any conclusions regarding its clinical efficacy.

Conversely, pembrolizumab, a PD-1 inhibitor approved for the treatment of advanced melanoma, selected lymphomas, and advanced non-small cell lung cancer due to its robust antitumor immunity, 2 cases of treatment-induced sclerodermoid reactions resembling to SSc have been published (44). This report

TABLE 1 | Summary of the different costimulatory pathway molecules data in SSc.

	Costimulatory pathway	Tissue expression	Expression levels	Clinical manifestations	Experimental blockade in SSc animal models	Experimental activation in SSc animal models	Clinical trials	Main results
Positive costimulators	CD28-CD80/86	Serum	Increased	None	None	None		
	ICOS-B7RP1	Serum, skin	Increased	Early dcSSc	None	None		
	OX40L-OX40	Serum, skin	Increased	Early onset, worsening of dermal fibrosis	Prevented and induced regression of established inflammation-driven dermal fibrosis in the bleomycin mouse model; Protected against interstitial lung disease and pulmonary hypertension in the Fra-2 model	Spontaneous ILD Production of antiDNA antibodies		
	CD40L-CD40	Serum, skin	Increased	Digital ulcers, PH, early/active NVC pattern	None	None		
	CD112/155-DNAM-1	Skin	Increased	Correlates with more severe dermal fibrosis and ILD	None	None		
Negative costimulators	CTLA-4-CD80/86	Serum	Increased	dcSSc, correlates with disease activity and severity	None	Prevented induced dermal fibrosis; was effective in the treatment of established fibrosis	1) Pilot study evaluating the clinical and molecular effects of Abatacept in dcSSc 2) Study of Subcutaneous Abatacept to Treat Diffuse Cutaneous Systemic Sclerosis (ASSET) trial (ClinicalTrials.gov identifier: NCT02161406)	1) Trend toward improvement in mRSS 2) Estimated study completion date: September 2018
	PD1-L-PD1	Serum	Increased	Correlates with disease severity	None	None		

CD (cluster of differentiation), ICOS (inducible co-stimulatory molecule), B7RP1 (B7-related protein-1), DNAM-1 (DNAX Accessory Molecule-1), CTLA-4 (cytotoxic Tlymphocyte-associated protein 4), PD1 (programmed death 1), dcSSc (diffuse cutaneous systemic sclerosis), PH (pulmonary hypertension), NVC (nailfold vascular capillaroscopy), ILD (interstitial lung disease), mRSS (modified-Rodnan skin score)

emphasizes the role of costimulatory pathways and immune checkpoint molecules in the pathogenesis of SSc. A summary of costimulatory pathways in SSc is available on **Table 1**.

RESEARCH AGENDA

SSc is a very severe autoimmune disease that is considered a prototype for studying the pathogenesis of fibrosis in particular the links between fibrosis and immunity (2). Current therapies used in the treatment of SSc remain essentially palliative and do not reverse the natural course of the disease. Given the lack of available effective agents in SSc, and their high toxicity profiles, targeted immunotherapy in particular blocking costimulatory molecules could be a beneficial strategy for SSc and other fibrotic conditions. Hence, in this context, abatacept appears to be a promising therapy for SSc given the encouraging results presented in this review but also given its well-documented safety profile in other rheumatic diseases in particular in RA (62). To better address the issue of abatacept in the treatment SSc patients, the Study of Subcutaneous Abatacept to Treat Diffuse Cutaneous Systemic Sclerosis (ASSET) trial (ClinicalTrials.gov identifier: NCT02161406) is currently ongoing. This study is a randomized placebo-controlled double-blind phase 2 trial of patients with dcSSc comparing subcutaneous abatacept against placebo. The primary outcome of this trial is defined as the change from baseline in the mRSS to month 12. Further randomized-controlled trials assessing the efficacy of costimulation therapy against placebo and standard therapy drugs (p.e. cyclophosphamide, mycophenolate mofetil) are warranted.

CONCLUSION

There is a large body of evidence showing that T cell costimulatory pathways play a critical role in the pathogenesis of SSc. Data from *in vivo* experimental animal models and from human studies showed meaningful effects of costimulation blockade in SSc. Of most interest is abatacept a targeted immunotherapy widely used in RA for which a randomized-controlled trial is currently ongoing. Targeted innovative therapies are one of the most important issues in SSc which is a life-threatening condition free of effective therapies. Further trials are awaited enthusiastically by the medical community in order to stop the natural course of this destructive condition.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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iNOS Activity Is Required for the Therapeutic Effect of Mesenchymal Stem Cells in Experimental Systemic Sclerosis

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Objectives: Fibrosis is a hallmark of systemic sclerosis (SSc), an intractable disease where innovative strategies are still being sought. Among novel anti-fibrotic approaches, mesenchymal stromal/stem cell (MSC)-based therapy appears promising. Previously, we reported anti-fibrotic effects of MSC in an experimental model of SSc, through various mechanisms (tissue remodeling, immunomodulation, anti-oxidant defense). Since immunomodulation is a pivotal mechanism for MSC therapeutic effects, we investigated the specific role of critical molecules associated with MSC immunosuppressive properties and hypothesized that MSC defective for these molecules would be less effective in reducing fibrosis in SSc.

Methods: SSc was induced by 6-week daily intradermal injections of hypochlorite (HOCl) in mice. MSC were isolated from the bone marrow of wild type mice (WT) or mice knockout for IL1RA, IL6, or iNOS (IL1RA$^{-/-}$, IL6$^{-/-}$, or iNOS$^{-/-}$ MSC, respectively). Treated-mice received 2.5 $\times$ 10^5 MSC intravenous infusion at d21. Skin thickness, histological and biological parameters were evaluated in skin and blood at d42.

Results: IL1RA$^{-/-}$ and IL6$^{-/-}$ MSC exerted similar anti-fibrotic properties as WT MSC, with a reduction of skin thickness together with less collagen deposition. Conversely, iNOS$^{-/-}$ MSC did not exert anti-fibrotic functions as shown by a similar skin thickness progression as non-treated HOCl-SSc mice. Compared with WT MSC, iNOS$^{-/-}$ MSC kept some immunosuppressive and tissue remodeling properties, but lost their capacity to reduce oxidative stress in HOCl-SSc mice.

Conclusion: Our study highlights the crucial role of iNOS, whose activity is required for the anti-fibrotic properties of MSC in experimental SSc, with a special emphasis on NO-related anti-oxidant functions.

Keywords: systemic sclerosis, HOCl, mesenchymal stem cells, inducible NO synthase, oxidative stress

INTRODUCTION

Skin fibrosis is the hallmark of systemic sclerosis (SSc), a rare and intractable autoimmune disease characterized by multi-organ fibrosis where innovative therapeutic strategies are still being sought. Among novel anti-fibrotic approaches in development, mesenchymal stromal/stem cell (MSC)-based therapy appears promising (1). Our group previously reported dramatic anti-fibrotic and anti-inflammatory effects of MSC in an experimental mouse model of SSc (2, 3). This inducible model, based on daily exposure to hypochlorite (HOCl-SSc), mimics the main features of human SSc in its diffuse and rapidly progressive form. MSC immunosuppressive properties are pivotal for their therapeutic effects, and mainly rely on paracrine mechanisms depending on soluble factors secretion (4–6).

We previously demonstrated that MSC efficacy in HOCl-SSc was associated with huge decrease in tissue inflammation characterized by less T-lymphocytes and macrophages infiltrates, and lower levels of inflammatory cytokines (7). In the present study, we further investigated the specific role of some critical molecules associated with MSC immunosuppressive properties and hypothesized that MSC defective for interleukine-1 receptor-antagonist (IL1RA), interleukine-6 (IL6) or inducible nitric-oxide (NO)-synthase (iNOS) would be less effective in reducing fibrosis in SSc. Herein, through a concise report, we present preliminary results giving evidence of the crucial role of iNOS for the anti-fibrotic properties of MSC.

MATERIALS AND METHODS

Isolation and Culture of MSC

MSC from C57BL/6 wild-type-(WT)-mice or from IL6-, iNOS-knock-out C57BL/6J-mice, or IL1RA-knock-out BALB/c-mice ($IL6^{-/-}$, $IL1RA^{-/-}$, $iNOS^{-/-}$-MSC, respectively) were isolated from bone-marrow (BM), as reported earlier (4, 5). BM was flushed out from long bones and the cell suspension was plated in DMEM supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria), 2 mM glutamine, 100 U/ml penicillin, 100 mg/mL streptomycin (Lonza, France), and 2 ng/ml human bFGF (R&D Systems, France). Cells were passaged till obtaining homogeneity for mesenchymal marker expression and lack of hematopoietic markers as analyzed by flow cytometry. They were used between passages 10 and 15.

HOCl Preparation

HOCl was generated extemporaneously by adding NaClO (9.6% as active chlorine) to KH_2PO_4 solution (100 mM, pH: 6.2), usually using a 1:100 ratio. The right amount of NaClO was adjusted so as to obtain the desired HOCl concentration, defined by the absorbance of the mixture at 292 nm (optical density between 0.7 and 0.9 read on a Nanodrop spectrophotometer, Thermoscientific). Stock solutions were stored at 4°C in the dark and NaClO was replaced every 3 weeks.

Experimental Design and Animals

Six-week-old female BALB/c mice purchased from Janvier were housed and cared for according to the Laboratory Animal Care guidelines. Approval was obtained from the Regional Ethics Committee on Animal Experimentation (approval APAFIS#5351-2016050919079187) and the French Ministry for Education, Higher Education and Research. The mice had their backs shaved the day before the disease induction. Skin thickness was assessed with a caliper before disease induction and every week during the whole experiment by a blinded experimenter. As previously described, a total amount of 300 µL of freshly prepared HOCl was injected in two sites into the backs of the mice with a 29 G needle, 5 days a week for 6 weeks (8). Control mice received PBS in the same conditions. Treated-mice received an infusion of MSC ($2.5x10^5$ cells conditioned in 100 µL PBS), in the tail vein at day 21. Groups of 7 to 10 mice were made for each condition (PBS-, HOCl-, and MSC-treated HOCl-mice). After 6 weeks and a 2-day recovery time without HOCl injections, all animals were sacrificed. Blood samples were collected and serum was recovered after centrifugation (1,500 g, 10 min) and stored at −20°C for ELISA. Skin biopsies (6 mm punches) were performed on the backs of the mice and lungs were removed and washed in PBS. Samples were stored at −80°C for RT-qPCR and collagen content determination or fixed in 4% formaldehyde for histopathological analysis.

Histopathology

Skin samples were embedded in paraffin and 5µm thick sections were stained with Masson-trichrome. Histological slides were scanned using Nanozoomer (Hamamatsu).

RT-qPCR Analysis

Skin samples were crushed in RLT-buffer and total RNA was extracted using the RNeasy mini-kit and Qiacube robotic workstation (Qiagen, France). One microgram of RNA was reverse-transcribed (M-MLV RT, Invitrogen, France). qPCR was performed on 20 ng cDNA using LightCycler480 SYBRGreenI Master-mix and real-time PCR instrument (Roche, France). Primers were designed using the applications Primer3 and BLAST as already described (2). Samples were normalized to mRNA expression of TATA binding protein *(Tbp)* housekeeping gene, and results provided either as relative expression to *tbp* using the formula $2^{-\Delta Ct}$ or as fold-change vs. PBS-mice using the formula $2^{-\Delta\Delta Ct}$.

Collagen Content in Skin

Collagen content assay was based on the quantitative dye-binding Sircol method (Biocolor, Ireland). Skin biopsies taken from the site of injection were suspended in 2 mL of a 0.5 M acetic acid—pepsin (2.5 mg/mL) solution and dissociated using UltraTurrax (vWR, France). Collagen extraction was performed overnight at 4°C under stirring. The solution was then centrifuged at 12,000 g for 10 min and 20 µL of each sample were added to 1 mL of Syrius red reagent. Tubes were rocked at room temperature for 30 min and centrifuged at 12,000 g for 10 min. The supernatants were discarded and the tubes washed with 750 µL of ice-cold salt acid wash. After another 12,000 g centrifugation of 10 min, the

collagen-dye pellets were resuspended in 1 ml of 0.5 M NaOH Alkali solution. Optical density (OD) was then read at 555 nm on a microplate reader (Varioskan Flash, Thermo scientific) vs. a standard range of bovine collagen type I concentrations (supplied as a sterile solution in 0.5 M acetic acid). Results were expressed as the collagen content in $\mu g/mm^2$ of skin.

Determination of Advanced Oxidation Protein Product (AOPP) Concentrations in Sera

AOPP concentration was measured by spectrophotometry as previously described (3). Twenty microliters of acetic acid was added to 200 µL of serum diluted 1:20 in PBS. In standard wells, 20 µL of acetic acid was added to 200 µL of chloramine-T solution (range from 0 to 1,000 µM) followed by 10 µL of 1.16 M potassium iodide. Absorbance was read at 340 nm on a microplate reader (Varioskan Flash) before and immediately after adding acetic acid and potassium iodide. AOPP concentration was expressed as chloramine-T equivalents (µM).

Total Anti-oxidant Capacity of Serum

The total antioxidant capacity was determined on sera diluted 1:10, measuring the formation of the radical cation 2,29-azino-bis (3- ethylbenzthiazoline-6-sulfonic acid) using the Antioxidant Assay Kit (Cayman Chemical, Interchim, France). The absorbance was read at 750 nm on a microplate reader (Varioskan Flash) vs. a standard range of Trolox, and was expressed as mM Trolox equivalents.

Determination of Glutathione (GSH) and Glutathione Disulfide (GSSG) Concentrations in Serum

At sacrifice, 100 µL of blood was collected with heparinized syringe, immediately mixed with 100-µL trichloro-acetic acid (10% in EDTA) and centrifuged at 10,000 g, at 4°C for 10 min allowing plasma recovery. Concentrations of GSH and its oxidized form GSSG were determined using ultraperformance liquid-chromatography–tandem-mass spectrometry (UPLC, Waters Acquity, Milford, USA).

Statistical Analysis

Quantitative data were expressed as mean ± SEM. Data were compared using Mann–Whitney's test for non-parametric values or Student's *t*-test for parametric values as evaluated using the Shapiro–Wilk normality test. When analysis included more than two groups, one-way ANOVA was used. All statistical analyses were performed using Prism 6 GraphPad software for Mac OS (California, USA). A $P < 0.05$ was considered significant.

RESULTS

iNOS Activity Is Required for MSC-Based Therapy of SSc

In a first series of experiments, we compared the effects of MSC defective for IL1RA, IL6, and iNOS production with those of WT-MSC when injected during the course (d21) of HOCl-SSc. In this setting, we observed that disease progression was hampered in mice treated with WT, IL1RA$^{-/-}$, or IL6$^{-/-}$-MSC, indicating that neither IL1RA nor IL6 were involved in the therapeutic effect of MSC in this model (**Figure 1A**). Conversely, iNOS$^{-/-}$-MSC did not affect the course of skin thickness, which followed the progression of non-treated HOCl-SSc mice. Concordantly, at d42, skin thickness was significantly lower in mice treated with WT, IL1RA$^{-/-}$, or IL6$^{-/-}$-MSC, compared with non-treated mice or iNOS$^{-/-}$-MSC-treated mice; and no difference in skin thickness was found between the two latter groups (**Figure 1B**).

These clinical data were corroborated by the measurement of collagen content in skin, significantly lower in mice treated with WT, IL1RA$^{-/-}$, or IL6$^{-/-}$-MSC compared with HOCl-SSc mice, while no significant effect was noted for iNOS$^{-/-}$-MSC-treated mice (**Figure 1C**). On histology, treatment with WT-MSC and to the same extent with IL1RA$^{-/-}$ or IL6$^{-/-}$-MSC reduced dermal collagen infiltration, while no reduction in collagen deposition was observed in iNOS$^{-/-}$-MSC-treated mice (**Figure 1D**).

iNOS$^{-/-}$ MSC Show Preserved Anti-inflammatory and Remodeling Capacities

Since IL1RA$^{-/-}$ or IL6$^{-/-}$-MSC exerted similar anti-fibrotic effects as WT_MSC, we next focused on iNOS$^{-/-}$-MSC in a second series of experiments. We confirmed that iNOS$^{-/-}$-MSC were unable to reduce skin thickening (**Figure 2A**) or collagen deposition (**Figure 2B**) during the induction of HOCl-SSc, compared with WT-MSC.

In order to decipher the underlying mechanisms, we then investigated the effect of MSC treatment on skin inflammation and tissue remodeling. We first noted that iNOS$^{-/-}$-MSC were almost as efficient as WT-MSC in reducing the expression of IL1β and IL6, two main inflammatory cytokines that are found at high levels within the skin of HOCl-SSc mice (**Figure 2C**). Concerning their ability to improve ECM remodeling, we noted that mice treated with iNOS$^{-/-}$-MSC disclosed reduced expression of tissue inhibitor of metalloprotease-1 (TIMP-1), and higher matrix metalloproteinase (MMP)1/TIMP1 ratio compared with HOCl-SSc mice, indicating enhanced remodeling capacity, similar to what is observed using WT-MSC (**Figure 2D**). Of note, concerning systemic involvement in HOCl-SSc, iNOS$^{-/-}$-MSC were not able to reduce fibrotic markers such as collagen 1 and α-SMA and inflammatory cytokines (IL-1β and IL-6) in lung tissue, while WT-MSC had a positive impact on pulmonary fibrosis in this model (data not shown).

iNOS$^{-/-}$ MSCs Fail to Dampen HOCl-Induced Oxidative Stress

Since immunomodulatory and remodeling capacities of iNOS$^{-/-}$-MSC seemed relatively preserved, we next looked at their effects on oxidative parameters in HOCl-SSc mice. Interestingly, we observed that iNOS$^{-/-}$-MSC failed to reduce the levels of AOPP in serum compared with WT-MSC,

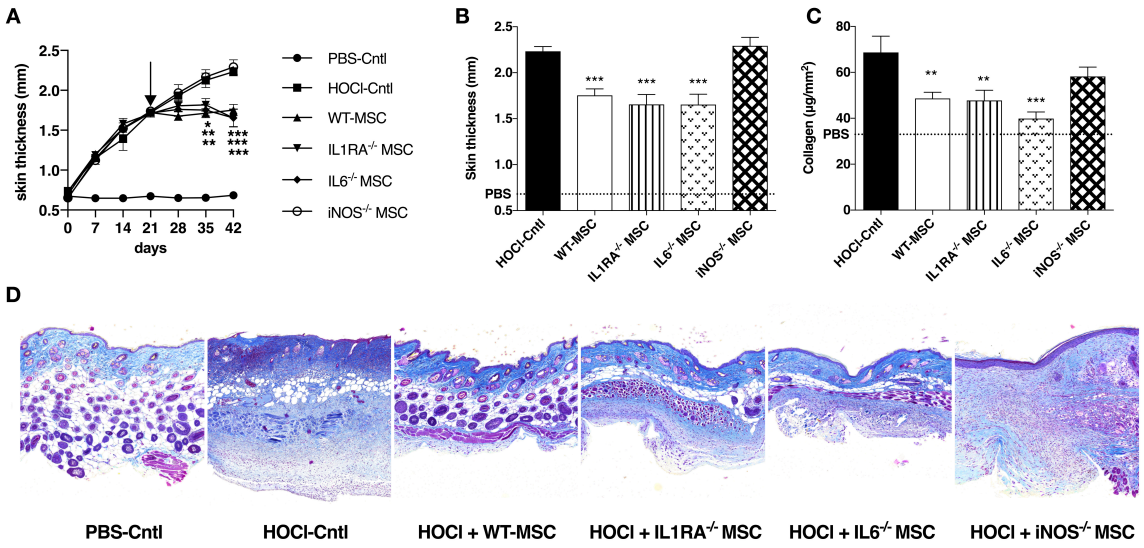

FIGURE 1 | Comparative effects of WT MSC, IL1RA$^{-/-}$, IL6$^{-/-}$, and iNOS$^{-/-}$ MSC in HOCl-SSc. **(A)** Skin thickness evolution (d0 to d42) from control PBS-mice, HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, IL1RA$^{-/-}$, IL6$^{-/-}$, or iNOS$^{-/-}$ MSC at d21. **(B)** Skin thickness at d42 in previously mentioned groups of mice (control PBS-mice are represented by a discontinued line). **(C)** Collagen content in skin samples from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, IL1RA$^{-/-}$, IL6$^{-/-}$, or iNOS$^{-/-}$ MSC (mean level for control PBS-mice is represented by a discontinued line). **(D)** Representative skin sections at d42 (original magnification 10x; Masson Trichrome staining). $N = 8$ for PBS-mice, HOCl-mice, and IL6$^{-/-}$ MSC-treated mice, $n = 7$ for IL1RA$^{-/-}$ and iNOS$^{-/-}$ MSC-treated mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, data are presented as mean ± SEM.

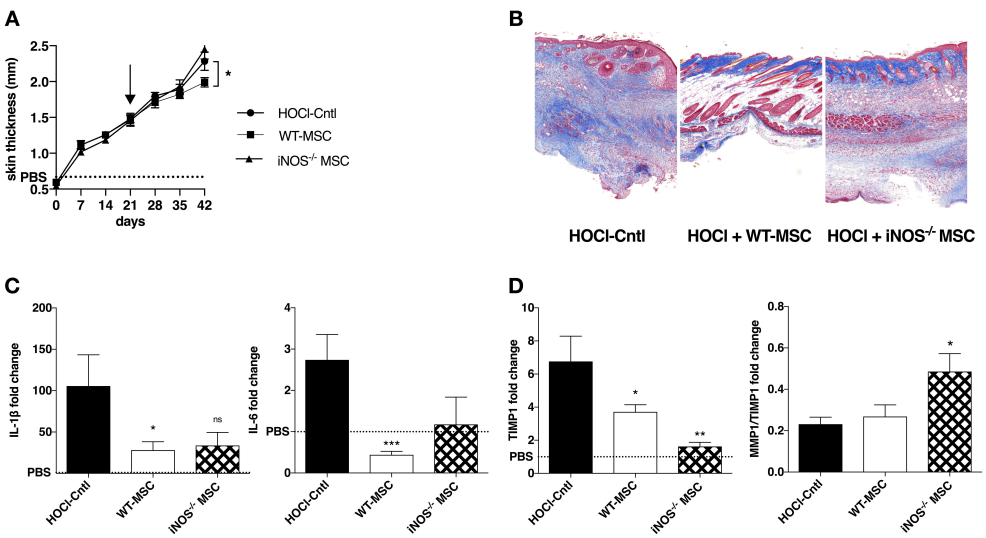

FIGURE 2 | Effects of iNOS$^{-/-}$ MSC on fibrosis, inflammation, and tissue remodeling in HOCl-SSc. **(A)** Skin thickness evolution (d0 to d42) from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSCs at d21 (mean levels for control PBS-mice are represented by a discontinued line). **(B)** Representative skin sections at d42 (original magnification 10x; Masson Trichrome staining). **(C,D)** mRNA expression of *IL1β, IL6, MMP1,* and *MMP/TIMP1* at d42 in skin sections from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC. Results are given as fold-change vs. control PBS-mice normalized at 1.

but induced higher levels of gluthatione and enhanced anti-oxidant capacity (AOC) (**Figures 3A–C**). In the end, the overall oxidative balance represented by AOPP/AOC ratio remained high under iNOS$^{-/-}$-MSC treatment whereas it significantly decreased under WT-MSC treatment (**Figure 3D**).

DISCUSSION

We previously demonstrated pleiotropic mechanism of MSC, acting through the abrogation of myofibroblastic activation and the reduction of tissue inflammation through potent immunosuppression, but also inducing tissue remodeling via

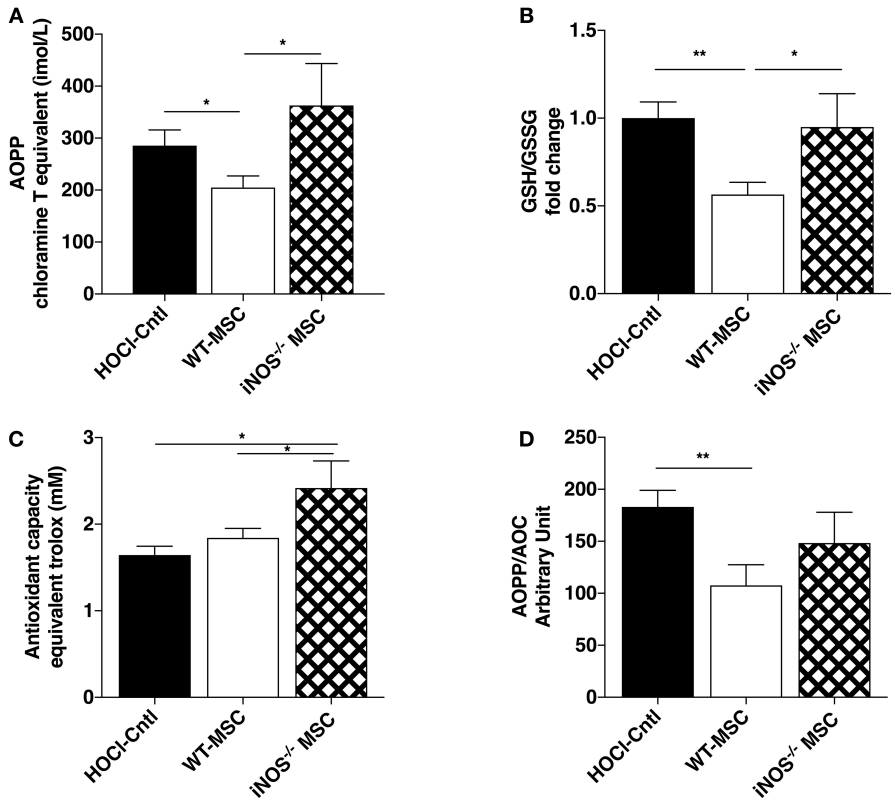

FIGURE 3 | Effects of iNOS$^{-/-}$ MSC on oxidative balance in HOCl-SSc. **(A)** Advanced Oxidation Protein Product (AOPP) concentrations in sera from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC. **(B)** Glutathione levels in sera from HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC (GSH/GSSG ratio). **(C)** AOC and **(D)** AOPP/AOC serum levels in HOCl-mice and HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSCs. $N = 7$ HOCl-mice, $n = 6$ for HOCl-mice treated with 2.5 × 10^5 WT-, or iNOS$^{-/-}$ MSC. *$P < 0.05$, **$P < 0.01$, data are presented as mean ± SEM.

metalloproteinase activation, and improving oxidative imbalance (2, 6, 7). These different mechanisms may depend on the pathological environment at the time of MSC infusion and argue for MSC adaptive capacities. This seems even more relevant in the setting of SSc, a heterogeneous and multifaceted disease.

In the present study, we aimed at deciphering possible mechanisms of MSC efficacy in HOCl-SSc and focused on their paracrine functions through molecules that were known to support their immunosuppressive capacities. Using MSC defective for these molecules, we observed that only iNOS seemed to be mandatory for the anti-fibrotic effects of MSC in HOCl-SSc. By contrast, IL1RA and IL6 were not involved in this function while they were required in previous *in vitro* and *in vivo* studies on other inflammatory models, such as collagen-induced-arthritis, a preclinical model for rheumatoid arthritis, another pathological condition where inflammatory cytokines such as IL6 play a critical role (4, 5).

Focusing on inflammation in mice treated with iNOS$^{-/-}$-MSC, we noticed that there were slightly less capable of reducing cytokine production than WT-MSC. This seems consistent with the importance played by iNOS for the immunosuppressive function of MSC in literature (9). Another explanation for these results may lie in the fact that inflammation in HOCl-SSc mainly pertains to the onset of the fibrogenic process (before d21), a period of time where the role of MSC might be more devoted to immunosuppression (Frontiers Immunology, in revision).

Since we did not demonstrate a dramatic reduction of anti-inflammatory function using iNOS$^{-/-}$-MSC, we looked for other mechanisms involved in the lack of therapeutic effect in murine SSc. We first turned to tissue remodeling, because this process seems of particular importance in the last 3 weeks of HOCl-SSc model (from d21 to d42), a phase characterized by less inflammation but strong collagen deposition in tissue. Interestingly, iNOS$^{-/-}$-MSC improved some remodeling parameters, with higher levels of the MMP1/TIMP1 ratio in skin, as compared with those found in WT MSC treated-mice. The strong decrease of TIMP-1, one main inhibitor of metalloproteinases, under iNOS$^{-/-}$ MSC treatment, suggested that iNOS is poorly involved in tissue remodeling.

Still, iNOS$^{-/-}$ MSC failed to prevent collagen deposition in tissue, which led us to consider the impact on oxidative balance. Indeed, in HOCl-SSc as well as in human disease, the role of oxidative stress seems pivotal (10, 11). Notably, AOPP were reported to play a critical role in fibrosis and autoantibody formation both in human and mice (8, 12). Herein, we showed that iNOS$^{-/-}$-MSC, unlike WT-MSC, were not able

to reduce the levels of AOPP in serum. Even though anti-oxidant defenses (i.e., serum AOC and glutathione levels) were upregulated in iNOS$^{-/-}$-MSC-treated mice, to even higher levels than in WT MSC-treated mice, the overall oxidative balance seemed unfavorable in these mice. In that sense, the strong upregulation in some parameters (i.e., glutathione or tissue remodeling enzymes MMP1/TIMP1), even overpassing what is observed using WT-MSC, might be a compensatory mechanism to counteract the persistent oxidative stress in these mice treated with defective iNOS$^{-/-}$-MSC.

On the whole, through this concise report, we demonstrate the crucial role of iNOS in the therapeutic effects of MSC in the murine HOCl-SSc model resulting in a global anti-fibrotic impact. This is supported by another study where iNOS$^{-/-}$-MSC failed to prevent tissue fibrosis in a model of liver cirrhosis (13). Conversely, another study reported that NO increased the anti-fibrotic properties of MSC in the same disease model (14).

Actually, NO plays a complex role in tissue remodeling and in oxidative stress regulation. On the one hand, the short-term production of NO by iNOS induces reactive oxygen species (ROS) formation such as peroxynitrites (15). On the other hand, NO is required for wound healing (16–18) and is also considered as an antioxidant (19, 20). Moreover, while prolonged iNOS blockade induces renal, heart or liver fibrosis in rodents (21–23), NO has shown proper antifibrotic roles, through the inhibition of myofibroblast activation, the abrogation of TGFβ pathway and the activation of MMP and hepatocyte growth factor (HGF), leading to less collagen deposition in other models (13, 20, 24). Interestingly, in the specific context of SSc, molecules up-regulating the NO pathway have been developed to treat pulmonary arterial hypertension in the clinics (25), and demonstrated anti-fibrotic effects in various preclinical models (26). This strengthens the interest of these preliminary results. Even if iNOS might not be the only mediator of importance in MSC therapeutic effects, this work underlines the role played by oxidative stress in SSc, and brings the perspective of enhancing MSC anti-oxidant activity to ameliorate their anti-fibrotic properties for future applications.

AUTHOR CONTRIBUTIONS

AM participated in the design of the study, acquisition, analysis and interpretation of data, manuscript redaction and final approval. PR, GF, TS, MM, and KT participated in acquisition and analysis of data, manuscript proofreading and final approval. J-PC and CJ participated in the design of the study, interpretation of data, manuscript preparation and final approval. DN and PG carried out the conception and design of the study, participated in analysis and interpretation of data, manuscript redaction and final approval.

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REVIEW
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Intriguing Relationships Between Cancer and Systemic Sclerosis: Role of the Immune System and Other Contributors

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Systemic sclerosis (SSc) is an autoimmune connective tissue disorder, characterized by multisystem involvement, vasculopathy, and fibrosis. An increased risk of malignancy is observed in SSc (including breast and lung cancers), and in a subgroup of patients with specific autoantibodies (i.e., anti-RNA polymerase III and related autoantibodies), SSc could be a paraneoplastic syndrome and might be directly related to an immune response against cancer. Herein, we reviewed the literature, focusing on the most recent articles, and shed light onto the potential relationship between cancer and scleroderma regarding temporal and immunological dimensions.

Keywords: Systemic sclerosis (scleroderma), cancer, immunosurveillance, paraneopastic syndrome, immunoediting of cancer, autoimmunity

INTRODUCTION

Systemic sclerosis (SSc, or scleroderma) is a rare connective tissue disorder affecting middle-aged women and characterized by tissue fibrosis, vascular dysfunction (microangiopathy) and autoimmunity with the production of specific autoantibodies (1). Skin fibrosis (or scleroderma) is the hallmark of SSc and a leading cause of disability. According to the extent of skin thickening, two main phenotypes are described: limited or diffuse SSc. In diffuse SSc, multi-organ involvement (i.e., pulmonary fibrosis) is associated with poorer prognosis and shorter life expectancy. Unfortunately, there remains an unmet medical need in SSc, a peculiar autoimmune condition where usual immunosuppressant drugs lack efficacy (2).

With population aging, cancer incidence is continuously increasing and represents a major public health problem. In France for instance, in 2017, 400,000 new cases were estimated, including 214,000 men and 186,000 women (3). The most common cancers were prostate cancer followed by lung cancer and colorectal cancer in men. In women, breast cancer, followed by colorectal cancer and lung cancer were the most common cancers, with an increase in lung cancer incidence that tends to become the second cancer in frequency. On the whole, cancer caused about 150,000 deaths that year.

Many studies have shown an increased risk for malignancy in SSc Similar to the general population, an increase in the incidence of malignant tumors has also been noted in the last decades. Together with a better management of scleroderma-related complications due to medical progress in SSc, cancer has thus become a leading cause of mortality in this disease, resulting in about 11% of deaths (third cause) according to the study on death certificates EUSTAR database (4). As a consequence, overall mortality has not improved in SSc in the last four decades, and the management of cancer including early diagnosis and adapted treatment becomes a challenging issue in this disease (2, 5). If no consensual recommendation has been made to date, some authors have suggested screening strategies, based on the most recent data on identified risk factors in this disease (6, 7).

The association of SSc and cancer is not fortuitous and the temporal clustering observed in some patients raises the possibility of SSc as a paraneoplastic syndrome in some patients, as described in other autoimmune conditions such as dermatomyositis (6, 8). However, the connections between the two are more complex than once imagined, and may result from many and diverse mechanisms (9). While immunosuppressants used to treat this autoimmune condition may lead to cancer development (10, 11), cytotoxic anti-cancer therapies have been associated with the development of scleroderma-like features such as Raynaud phenomenon, digital ischemia and fibrosis (12–15). Moreover, common mechanisms and pathways may be involved both in fibrogenesis and oncogenesis, and recent data have suggested that autoimmunity in SSc may be triggered by antigen mutation in tumor cells (6, 16, 17). In that sense, the immune response observed in these patients could be considered as protective, enhancing anti-tumor defenses. More recently, the advent of immune checkpoint inhibitors to treat numerous cancer types, sometimes triggering autoimmune responses, underscores the importance of immunity in cancer emergence and spread. Altogether, a growing body of data points out the intriguing bilateral relationships between SSc and cancer. Herein, we reviewed the most recent literature on this subject, and shed light onto their relationships through epidemiological, biological, and immunological dimensions.

CANCER IN SYSTEMIC SCLEROSIS: EPIDEMIOLOGY

General Aspects

Published studies demonstrated an increased risk of all cancers in SSc, but the associated factors and the increased risk according to cancer subtypes widely differs. This heterogeneity is probably related to the number of patients included, to different follow-up durations, and to the different methods of analysis used in each study. In the study by Wooten and coll. published in 2008, 3.6 to 10.7% of SSc patients had developed at least one cancer. Lung cancer was the most common, followed by breast cancer. The risk factors included female gender, older age, and cutaneous diffuse form of SSc (18).

In a Danish cohort of 2,205 SSc patients, 222 had cancer after the onset of SSc, a higher incidence than in the general population with a standardized incidence ratio (SIR) of 1.5 (95% CI 1.3–1.7) (19). In this cohort, male patients had a higher risk than female and lung cancer and lymphoma exhibited the highest incidence rates. Of note, there was no association between SSc and breast cancer.

Two meta-analyzes performed in 2013 provided additional information. The first one by Bonifazi et al. included 16 studies involving more than 7,000 patients (20). The relative risk of cancer in SSc patients was 1.75 (95% CI 1.41–2.18). There was a strong association with lung cancer with a relative risk (RR) of 4.35 (95% CI 2.08–9.09), and hematological disorders with a RR of 2.24 (95% CI 1.53–3.29). Again, the association with breast cancer was not confirmed.

The second meta-analysis by Onishi et al. involved more than 6,000 patients through six cohort studies and observed a SIR of 1.41 (95% CI 1.18–1.69) and a higher SIR in male than in female patients (1.85 vs. 1.33) (21). An increased SIR was observed for the following cancers: lung cancer (SIR = 3.18, 95% CI 2.09–4.85), malignant hematological disorder (2.57, 1.79–3.68), hepatocellular carcinoma (SIR = 4.36, 2.00–9.51), and bladder cancer (SIR = 2.00, 1.06–3.77). There was no significant difference between cutaneous limited and diffuse SSc.

Recently, Igusa et al. analyzed the cancer risk in subgroups according to SSc phenotype, autoantibodies, and temporal clustering (22). In this study involving 2,383 SSc patients, 205 patients also had cancer. Surprisingly, the overall risk of cancer was not found increased compared to the general population. However, an increased risk for malignancy was found in anti-RNA-Pol-III-positive patients (SIR = 2.84, 1.89–4.10) and sero-negative patients (SIR = 1.83, 1.10–2.86). In contrast, anti-centromere-positive patients had a lower risk of cancer (SIR = 0.59, 0.44–0.76). Interestingly, among anti-RNA-Pol-III-positive patients, diffuse SSc phenotype was associated with breast cancer, while limited SSc phenotype was associated with lung cancer. These data would allow stratification of cancer risk by clinical and serological phenotype and thus allow targeted screening in this population.

DEMONSTRATED ASSOCIATIONS WITH SPECIFIC CANCERS

Lung Cancer

The association of lung cancer and SSc is widely documented in the literature with a SIR ranging from 4.2 to 5.9 (23–25). In a retrospective Italian study of 318 patients, 16 patients had lung cancer (5%) (26). There was an association with the male gender, a longer duration of the disease, a younger age at the diagnosis of SSc. Pulmonary fibrosis was a risk factor for lung cancer with an OR of 6.7 (95% CI 2.2.−20.7). Anti-Scl70 antibodies were also a risk factor (95% CI 1.7–24.1). Over the sixteen SSc patients with lung cancer, thirteen died because of lung cancer. Another study did not identify any increase in lung cancer incidence compared to the general population, but in this single-center study there was a significant number of lung cancer in their general population (27). Notably, both the existence and the duration of interstitial lung disease (pulmonary fibrosis)

in SSc have been confirmed in numerous other studies as an independent risk factor for cancer (5, 28, 29). Various common mechanisms involving chronic inflammation, tissue remodeling, cell cycle, and the sequestration of carcinogens by fibrosis have been suggested as a possible explanation for this association (5, 6).

Breast Cancer

The incidence of breast cancer in scleroderma patients is extremely variable and discordant according to the studies. This variability remains unexplained to date but is potentially related to study methods and inter-country heterogeneity (19, 30). In an Italian study on 318 scleroderma patients, a significant increase in breast cancer incidence compared with the general population was observed. The SIR was evaluated at 2.1 (95% CI 1.13–3.90) (26). An increased risk of breast cancer has been described mainly in other small cohorts of patients but one of the largest cancer studies (Danish cohort of 2,205 patients with systemic scleroderma, 222 with cancer) and two recent meta-analyses did not confirm these results (19–21). However, a close temporal relationship may be observed between SSc and breast cancer in a subgroup of patients. Indeed, there appears to be a short delay between breast cancer and SSc diagnosis, according to several case series or retrospective cohort studies. In a literature review, a majority of patients (61.4%) were diagnosed with cancer within a period from 1 year before to 1 year after SSc diagnosis (31). In the Italian study by Colaci, the median time between these two conditions was 2.5 years (26). These findings might suggest common pathogenesis pathways between these two diseases (6, 8, 15, 29).

Additionally, the group of SSc patients with breast cancer would exhibit some characteristics, such as the presence of lung fibrosis and the absence of anti-nuclear antibodies (32). Patients who developed breast cancer after the diagnosis of SSc had distinct characteristics from others: older age at the time of SSc diagnosis, more frequent interstitial pneumonia with lung fibrosis, and less frequent familial history of breast cancer. However, more recent studies have suggested a link between specific autoantibodies such as RNA Polymerase 3 (RNA-PolIII), PM/Scl or RNA binding Protein Containing 3 (RNPC) antibodies and breast cancer (7, 17, 33–35).

Concerning the association between SSc and breast cancer, two points should be underlined. First, the female susceptibility observed in SSc suggests influence of the same hormonal factors that are found involved in breast cancer. For instance, elevated prolactin levels and decreased levels of DHEA (dehydroepiandrosterone sulfate) were found in patients with SSc and in those breast cancer patients (36, 37). Secondly, calcium channel blockers (CCB), a cornerstone treatment for SSc vasculopathy, have been suspected as a risk factor for breast cancer in the general population in a case-control study (38). The long-term use of CCB (over 10 years) was associated with an OR of 2.3 (95% CI 1.2–4.9) for ductal carcinomas and an OR of 2.6 (95% CI 1.3–5.3) for lobular carcinomas. Another study showed that patients with CCB had an increased risk of aggressive breast tumors with an OR of 1.96 (95% CI 1.09–3.53) (39). Some authors therefore suggested that CCB are potentially a confounding factor that might explain the increased incidence of breast cancer in SSc observed in some studies (40). However, all these results have recently been questioned by an epidemiological study taking into account confounding factors and involving 28,000 patients (41). The possible pathophysiological relationship between breast cancer and CCB remains unclear, but a hypothetical mechanism might be an impaired functionality of intracellular calcium associated with CCB, particularly in the initiation of the pro-apoptotic signal.

Esophageal Cancer

The standardized ratio of esophageal cancer incidence in scleroderma patients is estimated at 15.9 (95% CI 4.2–27.6) (42). This increased risk of esophageal cancer is undoubtedly related to the high frequency of chronic gastro-esophageal reflux disease (GERD), increasing the risk of Barrett's esophagus, and thus the risks of dysplasia and adenocarcinoma of the esophagus. Several studies focused on this risk, which was estimated at 3% per year for patients with Barrett's esophagus over a three-year prospective study from EULAR network centers (43, 44). Among 46 SSc patients who had Barrett's esophagus at baseline and completed the follow-up, four developed esophageal adenocarcinoma. Dysplasia at baseline was a major factor risk of cancer. These data prompt endoscopic follow-up of Barrett's esophagus in SSc.

Hematological Malignancies

The association between autoimmune diseases and lymphoma has been described in several studies (45). An epidemiological study in Denmark and Sweden of 25,000 patients with non-Hodgkin's lymphoma found an association with autoimmune diseases (including SSc) with an OR ranging from 1.6 to 5.4 (46). In another Swedish study, the risk of lymphoma in SSc patients was increased with an SIR of 2.1 (47). In these studies, associated Sjogren's syndrome was not always reported, although it remains an independent risk factor for the emergence of lymphoma.

Bladder Cancers

The use of high cumulative dose of cyclophosphamide may lead to the development of bladder cancer in SSc, as reported in various studies (5, 48).

Suspected Associations With Other Cancers

Concerning gynecological malignancies, a Canadian study found a higher prevalence of cervical dysplasia in the scleroderma population than in the general population (25.4 vs. 13.8%), particularly in patients with diffuse SSc (49). In another study, overall frequency of human papilloma virus (HPV) was not higher in SSc patients than in general population (50). However, the high risk HPV52 was the most frequent genotype and a greater multi-HPV infection rate was observed in SSc, particularly in diffuse SSc. Interestingly, cyclophosphamide was reported as a risk factor for cervical intraepithelial neoplasia with a dose-dependent cumulative risk (plus 13% increased risk of cervical dysplasia per each increase of one gram according to a series of patients with systemic lupus erythematosus)

(51). These data prompt to screen carefully patients for whom immunosuppressive therapy is indicated.

Autoimmune thyroiditis is a condition commonly associated with SSc. The percentage of patients with hypothyroidism varies from 2.4 to 26% and the percentage of anti-TPO antibodies varies from 12 to 52% according to the studies (52). Some cases of scleroderma patients with thyroid cancer have been reported. In a study of 769 patients with SSc, no significant association was found and only 2 cases of thyroid cancer were observed (42). Another recent study suggested an increase in thyroid papillary carcinomas in SSc patients but all those patients with cancer had autoimmune thyroiditis (53). Once again, chronic inflammation might promote cancer development in this condition.

Rare cases of cutaneous squamous cell carcinoma have been reported in scleroderma patients, mostly associated with localized scleroderma (morphea, including pansclerotic morphea), (5, 54). Intriguingly, some patients with melanoma treated with interferon or immune checkpoint inhibitors have developed SSc (55–57).

Interestingly, a few cases of soft tissue malignant tumors (i.e., sarcomas) have been described in association with SSc, although no statistical link can be established, mainly because of the rarity of both conditions (58–64). This association between connective tissue disease and malignancy highlights the possibility of common mechanisms between the loss of connective tissue homeostasis in SSc and oncogenesis in sarcoma, including angiogenesis defects.

We summarized the demonstrated and putative associations between SSc and cancer in a table (see **Table 1**).

CHRONIC INFLAMMATION, TISSUE HOMEOSTASIS, FIBROSIS AND CANCER DEVELOPMENT

SSc cannot be considered as a systemic inflammatory disease such as systemic vasculitides or inflammatory bowel diseases. However, some locations of the disease (in particular, esophagus and lungs) are exposed to chronic tissue inflammation, which is considered nowadays as a major factor leading to the development of cancer. At least 25% of cancers might be related to persistent inflammation or chronic infection (65). In fact, inflammatory mediators, such as pro-inflammatory cytokines and free radicals, could induce genetic and epigenetic modifications, leading to alterations in the cell signaling pathways and thus breaking normal cell homeostasis. For example, free radicals can randomly induce point mutations in tumor suppressor genes and contribute to the progression of cancer (66–68).

According to this concept, some authors proposed that chronic tissue damage with recurrent tissue repair mechanisms may be one of the mechanisms for the development of lung and esophageal cancer in SSc patients with pulmonary fibrosis and GERD, respectively (15). This phenomenon is well documented for SSc patients with chronic GERD, since these patients exhibit an increased risk of Barrett's esophagus, high-grade dysplasia and esophageal cancer. Similarly, chronic inflammation within fibrosis could contribute to cancer development in SSc patients with lung fibrosis (5, 15).

It is noteworthy that since SSc may be associated with other connective tissue disorders or organ-specific autoimmune diseases, patients may be exposed to an increased specific risk of cancer through these additional autoimmune conditions and their related-chronic inflammation. Notably, primary biliary cirrhosis (whose association with SSc represents "Reynolds syndrome") and autoimmune thyroiditis (Hashimoto's) are associated with increased risk of cholangiocarcinoma (69) and papillary thyroid carcinoma respectively (53).

Another point to consider is fibrosis itself, as a condition potentially associated with cancer. Indeed, the association between pulmonary fibrosis and cancer is well known, especially in patients with idiopathic pulmonary fibrosis (IPF), which represents the key condition of lung fibrosis. About one in ten patients with IPF will develop lung cancer during follow-up (70). Apart from the role of chronic inflammation in pulmonary carcinogenesis, carcinogenic molecules may be sequestrated by fibrosis, secondary to an altered lymphatic drainage (71, 72).

Moreover, mesenchymal cells are crucial for the development of cancer (73). This is well documented in lung cancer, in which tumor-associated fibroblasts contribute to a "growing" loop with malignant epithelial cells, through the production of specific growth factors such as epidermal growth factor (EGF), fibroblastic growth factor (FGF), or transforming growth factor (TGF). In this context, epithelial–mesenchymal transition (EMT) contributes to the development and spread of tumor cells, and is also a source of fibroblasts. This complex process including numerous phenotypic transitions toward mesenchymal cells (i.e., fibroblasts) may thus be potentially implicated both in cancer and fibrosis. On the one hand, EMT has been clearly observed during the development of lung cancer, when epithelial cells transform into malignant cells under the activation of oncoproteins such as mutant Kras (Kirsten rat sarcoma viral oncogene) (74, 75). Thus, EMT may participate to epithelial cell plasticity and modify their properties in the context of lung injury. On the other hand, EMT also represents a potential mechanism in SSc, where epidermal cells may acquire mesenchymal and fibroblastic features under the activation of TGF-beta signaling pathway within lesional skin, further increasing the fibrotic burden (76).

SHARED MECHANISMS IN CANCER AND SYSTEMIC SCLEROSIS

Genetics and Epigenetics

Common features may be observed in cancer and scleroderma, with common actors promoting disease development. Strikingly, a recent gene profiling study revealed oncogenic gene patterns in SSc (77). As abovementioned, the implication of EMT in both diseases is particularly interesting and may originate from common genetic and epigenetic alterations, involving telomere shortening, chromosomal instability, senescence, increased

TABLE 1 | Associations between systemic sclerosis and cancer: evidence from epidemiological data and suspected risk factors.

Cancer	Evidence for association	Mean SIR	Scenario	Comments
Lung	YES	4–6	A,B	There is a strong association between lung cancer and SSc in the presence of interstitial lung disease and anti-scl-70 Ab. Chronic inflammation in PF may lead to tumor development.
Breast	YES	2–3	B,C	Breast cancer is the most frequent cancer found in SSc patients. The risk of developing breast cancer in SSc is associated with the presence of anti-RNA-PolIII Ab through a pathophysiological process leading to paraneoplastic autoimmunity. CCB could also contribute to the development of breast cancer.
Esophageal	YES	15	A	GERD, a hallmark of SSc, is responsible for chronic inflammation in esophagus, and can lead to Barrett's esophagus, dysplasia, and adenocarcinoma.
Bladder	YES	2	C	The use of cyclophosphamide in SSc patients can lead to the development of hemorrhagic cystitis and bladder cancer through a cumulative dose-effect.
Hematological	YES	2	A	An increased risk of lymphoma has been described in numerous autoimmune diseases, including SSc, and may be more specifically associated with the presence of secondary SjS.
Cervix	NO	N/A	C	An increased frequency of high-risk HPV infection has been reported in SSc. The use of cyc may lead to the development of cervical neoplasia in these patients.
Thyroid	NO	N/A	A	Thyroiditis, an autoimmune condition frequently associated with SSc may lead to chronic inflammation of the thyroid and the development of papillary carcinoma.
Skin	NO	N/A	A	Cases of squamous cell carcinoma have been reported in association with morphea, (i.e., localized scleroderma), a condition where chronic tissue inflammation may lead to cancer development.
Sarcoma	NO	N/A	A,B	Few cases of soft tissue malignant tumors (i.e., sarcomas) have been described in association with SSc. No statistical link can be established because of the rarity of both conditions.

Scenario A: SSc leads to cancer development (via chronic tissue inflammation); Scenario B: suspected paraneoplastic phenomenon between SSc and cancer onset; Scenario C: SSc treatments favor cancer development.

SIR, standardized incidence ratio; PF, pulmonary fibrosis; Ab, antibody; CCB, calcium channel blockers; GERD, gastro-esophageal reflux disease; cyc, cyclophosphamide; SjS, Sjogren's syndrome; HPV, human papilloma virus.

proliferation rates, immune deregulation, and impaired cell metabolism.

Telomere Shortening

While telomere maintenance is complex as well as essential for cancer progression (78), deficient telomerase activity and telomere shortening have been both reported in SSc (79, 80). In another fibrotic condition, IPF, telomere shortening is also a potential contributor for the pathological process and reduction in telomere length of circulating leukocytes could even have an impact on overall mortality (81).

Epigenetic Alterations

Epigenetic mechanisms could contribute to the pathogenesis of SSc, as a consequence of the exposure to environmental factors such as silica (see below paragraph 2.4.1). This would result in cytokine network modulation toward the development of autoimmunity. Similarly, an important body of data argues for the essential contribution of epigenetics to the development of cancer. Three main mechanisms should be considered with striking similarities between both diseases: changes in DNA methylation, histone modifications, and microRNAs.

First, a reduced expression of several genes regulating the process of methylation (such as DNA methyltransferase-1, methyl-CpG-binding domain 3, and MBD4) could induce DNA hypomethylation in CD4+ T cells in SSc (82, 83). Recently, Lian et al. observed an overexpression of CD40L in CD4+ T cells from female SSc patients (but not from male patients), which could be explained by a demethylation of specific CpG–DNA within regulating regions of CD40L (84). This overexpression of CD40L may be of great importance in SSc, since the interaction CD40/CD40L play key roles in autoimmune diseases, in particular in SSc. The demethylation within CD40L gene could result in the reactivation of the inactive X chromosome, maybe contributing to the female susceptibility of the disease. Other alterations in the methylation patterns have been observed in SSc. Endothelial cells are concerned by such epigenetic changes, since CpG hypermethylation of the bone morphogenetic protein receptor factor type 2 (BMPR2) was recently reported. Hypermethylation resulted in downregulation of the protein, inducing an increased sensitivity of endothelial cells to apoptosis and oxidative stress (85). Another example is represented by the hypomethylation of TNFSF7, the promoter of CD70, observed in SSc, which results in the overexpression of CD70 by CD4+ T cells (86). In fact, CD70 is a B cell co-stimulatory molecule, whose overexpression may contribute to autoimmunity, as observed in other autoimmune diseases like lupus and Sjögren's syndrome. So, CD70 hypomethylation could contribute to autoimmune diseases whereas hypermethylation would be rather associated with the development of malignancies, in particular breast cancers (87). Last but not least, SSc fibroblasts also appear to

be affected by methylation changes. Indeed, hypermethylation of the CpG rich regions in the Fli-1 (Friend leukemia virus-induced erythroleukemia-1) promoter region was demonstrated in fibroblasts and skin from SSc patients (88). Interestingly, the latter also plays an important role in cancer development and appears as a possible link between autoimmunity and malignancy (89). Furthermore, in an experimental animal model of bleomycin-induced lung fibrosis, the regulatory effects of the methyl CpG binding protein 2 (MeCP2) and its implication in tissue homeostasis have been shown. The authors demonstrated that MeCP2 binds to the methylated region of α-SMA gene promoter and activates its expression (90). They then studied the effects of a loss of expression of MeCP2, using siRNA or knockout mice. Resulting in the methylation of α-SMA promoter, they observed less activation of this gene. Hence, MeCP2 deficient mice were less sensitive to bleomycin, highlighting the pivotal role of MeCP2 in myofibroblast transformation and fibrosis development.

Second, impaired transcription of genes and deregulated gene expression induced by histone modifications may also contribute to the development of both cancer (91) and SSc (83). Notably, novel therapies targeting epigenetic contributors such as histone demethylase and histone deacetylase inhibitors have been developed in the field of oncology and may emerge in the field of pulmonary fibrosis (92). In SSc, a recent study highlights the role of the histone acetyltransferase p300 in the development of fibrosis (93). In this study, increased p300 levels within SSc skin under TGF-beta activation resulted in collagen transcription in a Smad-independent manner involving Egr-1 (early growth response 1). Actually, p300-mediated histone H4 hyperacetylation appears to be a pivotal epigenetic modification in SSc, whose deleterious profibrotic effects could be counteracted by the blocking effects of antiaging deacetylase enzyme sirtuin 1 (SIRT-1) on Smad-dependent transcription (94). SIRT-1 belongs to the group of histone deacetylases called sirtuins (SIRTs) that includes 7 proteins (from SIRT1 to SIRT7) implicated in the regulation of aging process, clock control, and cell metabolism. Interestingly, in this study, the expression of SIRT-1 was found reduced in SSc skin and its activation by resveratrol reversed the fibrotic response of fibroblasts (94). Emerging data corroborate this observation in SSc (95) and also suggests the role of SIRTs proteins in the development of cancer (96). Altogether, SIRTs proteins could contribute to SSc and cancer through different mechanisms: TGF-β signaling, mTOR pathway, oxidative stress and cellular senescence.

Third, numerous microRNAs, including miR-21, miR-29 family and let-7d, have been reported to play key roles in the pathogenesis of cancer and fibrosis, and could even represent potential therapeutic targets in both diseases (77). In SSc, their effects concern collagen gene expression in fibroblasts, collagen degradation, thus extracellular matrix remodeling, apoptosis, and epithelial-mesenchymal transition. Firstly, miR-21 may function as an amplifying factor, enhancing TGF-β signaling events in SSc fibrosis (97). Exosomal miR-21 might be used as a cancer biomarker, and its combination with other miRNAs within a specific panel may become a relevant diagnosis tool for cancer (98). Notably, it has been associated with breast cancer. Secondly, the miRNA-29 family, consisting of miR-29a, miR-29b, and miR-29c, could also be involved in cancer development and fibrosis. miR-29b functions either as a tumor suppressor or an oncogene under specific conditions and could mediate cancer chemo-sensitivity or resistance. The miR-29 family members also appear to be antifibrotic mediators, modulating collagen expression and degradation. Indeed, the expression of miR-29a is reduced both in fibroblasts and skin in SSc (99, 100), and its overexpression induced a reduction of fibroblast proliferation and collagen synthesis *in vitro* (101). A similar reduced expression of miR-29a has also been found both in broncho-alveolar cells from IPF and lung cancer patients, suggesting a common link between the two conditions (102). Altogether, miR-21 and miR-29 family members exhibit synergistic functions to modulate fibroblast fate both in healthy and fibrotic conditions. Thus, an imbalance between these two mediators might contribute to fibrosis. Other miRNAs strongly involved both in cancer and fibrosis are miR-16 (77) and let-7d. The latter is considered as a key regulator of cell proliferation and can act as a tumor suppressor (103). It is also involved in the regulation of EMT and prevention of lung fibrosis (104). Its expression is reduced in SSc skin (105). The role of the miRNAs in cancer and scleroderma also underscores the importance of exosomes in both diseases, since miRNAs are contained and conveyed by such vesicles (106). Interestingly, anti-PM/Scl autoantibodies found in SSc and associated with malignancy recognize a complex that is the human homolog of *saccharomyces cerevisiae* exosome (35, 107). This further strengthens a pathological link between autoimmune response in SSc and cancer.

Common Signal Transduction Pathways

Signal transduction pathways may also be shared by cancer and fibrosis, as highlighted by the recent gene profiling study by Dolcino et al. revealing oncogenic signature in SSc patients, involving numerous well-known oncogenic proteins such as Ras, janus kinase (jak), Avian Myelocytomatosis Viral Oncogene Homolog (c-myc), B-cell lymphoma (bcl-2), Myeloid differentiation primary response 88 (myd88), poly(ADP-ribose) polymérase (PARP), and the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway (77). Interestingly, the transcription factor Fra (Fos-related antigen) is also involved both in breast cancer (108) and in scleroderma (109).

The PI3K/Akt pathway is implicated in lung fibrosis, and has been documented in experimental *in vitro* and *in vivo* models and may be a target for therapy (110). The antagonist of this pathway, Protein Phosphatase and Tensin homolog (PTEN), is also implicated in SSc: its expression is reduced in skin fibroblasts from patients with cutaneous diffuse SSc. Furthermore, the gene deletion of PTEN in adult mouse fibroblasts induced skin thickening, activation of PI3K/Akt pathway and increased expression of connective tissue growth factor (CTGF/CCN2) (111), the latter mediator representing an essential actor in this model (112). The Wnt/beta-catenin pathway, strongly implicated in cancer development, has also been reported playing a role in lung fibrosis (113). An aberrant activation of this pathway would result in accumulation of beta-catenin within pulmonary

tissue, promoting EMT. In the field of cancer, the dysfunction in Wnt pathway could favor cancer emergence and spread, but also resistance to anti-tumor treatments (including immunotherapy such as immune checkpoint inhibitors). Aberrant Wnt pathway could in fact induce deficient immunosurveillance toward cancer, leading to immune-evasion (114). The activation of the Wnt pathway was also observed in lesional skin from SSc patients. It stimulated a Smad-dependent fibrotic process in mesenchymal cells. It repressed adipogenesis in subcutaneous pre-adipocytes, while inducing myofibroblast differentiation (115, 116).

Some authors hypothesize that dysfunction of regulation of the TGFβ / SMAD pathway by caveolin-1 (Cav-1) is involved in both the development of fibrosis and breast cancer (117). In SSc, there is a decrease in Cav-1 expression in the lung and skin of SSc patients. KO mice for Cav-1 develop pulmonary and cutaneous fibrosis. *In vitro*, when Cav-1 function is restored in cultured SSc fibroblasts, their phenotype was normalized and stimulation of the TGFβ pathway was stopped by the inhibition of SMAD3 phosphorylation. Thus Cav-1 would allow the inhibition of SMAD3 phosphorylation and regulate the fibrosis process (118). In breast cancer, Cav-1 gene is a tumor suppressor gene, and a dominant negative mutation is present in 16% of breast cancers. Using a transgenic mouse model of breast cancer, the authors have demonstrated that cav-/- mice have a significant increase in tumor volume and rapidly develop pulmonary metastases, unlike cav+/+ or cav+/- mice (119).

On the whole, the oncogenic pathways found activated in SSc might contribute to fibrosis development while predisposing to malignancy in this disease.

Environmental Factors and Treatments as Inducers of Both Diseases

Environmental and Occupational Exposures in SSc and Cancer

Crystalline silica is a known carcinogen for bronchopulmonary cancers and also an environmental factor involved in the development of SSc (120). Many other environmental components have been suspected or confirmed as playing a role in the emergence of SSc, such as organic solvents, pollutants, welding fumes, pesticides, etc (121–124). Some of them are recognized as carcinogens.

Immunosuppressant-Induced Cancers

Several immunosuppressive drugs can be used in SSc, as recommended by EULAR, but may contribute to cancer (125). Among them, cyclophosphamide is a well-known agent, which is able to promote bladder cancer with a dose-dependent relation (10, 11). In a retrospective study with control group, there was 4 times more cancer in the SSc population treated with cyclophosphamide, with a consequent higher number of hematological malignancies (126). Recent studies have suggested that mycophenolate mofetil (MMF) is an alternative to cyclophosphamide for the treatment of interstitial lung lesions, including improved tolerance (127, 128). However, few case series have suggested a possible relationship between squamous cell carcinoma and mycophenolate mofetil in scleroderma, with cancer regression after MMF withdrawal (129).

Anti-cancer Therapy-Induced SSc

Several scleroderma-like syndromes have been described after anti-mitotic treatment. Docetaxel, a molecule used in the therapeutic arsenal of many cancers, including breast cancer, is known to have skin toxicity, with possible scleroderma-like lesions. More than a dozen cases of patients with authentic limited or diffuse SSc have also been reported secondary to docetaxel. The mechanisms leading to docetaxel cutaneous fibrosis are not fully understood. Some authors suggest that the deposition of an extracellular matrix glycoprotein (i.e., versican) after docetaxel or paclitaxel treatment may play a role in the pathogenesis of docetaxel-induced scleroderma (130). Recently, the first case of scleroderma secondary to docetaxel with organ involvement (PAH and renal scleroderma crisis) has been published (131). One of the explanations would be the possibility of endothelial cell damage induced by oxidative stress secondary to docetaxel (132).

Other chemotherapies have been associated with tissue fibrosis and/or scleroderma, such as bleomycin and gemcitabine (12, 14).

Ionizing radiation may be responsible for morphea (133) and may exacerbate pre-existing systemic scleroderma, according to some authors (13, 134, 135).

Strikingly, graft versus host disease (GVHD), a common complication of allogeneic hematopoietic stem cell transplantation (HSCT), somehow mimics SSc (136). Indeed, is skin fibrosis is a hallmark of chronic GVHD, resembling scleroderma. Beyond common clinical features, shared pathways are involved as observed in preclinical models for the two conditions (137–139).

More recently, anticancer immunotherapy using immune checkpoint inhibitors has been shown to trigger autoimmunity, and a few cases of scleroderma have been reported in the literature (55–57, 140). In this context, the complexity of the interplay between anti-cancer pre-existing autoimmunity, genuine paraneoplastic syndromes and the effects of immune system stimulation by biologics is striking, and strengthens the relationships between SSc and malignancy.

AUTOANTIBODIES AS BIOMARKERS PREDICTING MALIGNANCY IN SYSTEMIC SCLEROSIS?

Anti-RNA Polymerase III

In a monocentric retrospective study in England, out of 2,177 patients with SSc, 7.1% had a history of cancer (33). The frequency of cancers was significantly increased in patients with anti-RNA-PolIII (14.2%) compared to patients with anti-Scl70 (6.3%) and anti-centromere (6.8%) autoantibodies ($p < 0.0001$ and $p < 0.001$). In patients diagnosed with cancer within 3 years of SSc onset, 55.3% had anti-RNA-PolIII. In addition,

SSc patients with anti-RNA-PolIII had twice as much risk of developing cancer as anti-centromere patients ($p < 0.0001$).

These data were confirmed by a study of EULAR, where anti-RNA-PolIII antibodies were associated with synchronous malignancy (-6 months + 12 months) with an OR estimated at 7.38 (95% CI 1.61–33.8) (7). The association with breast cancers was even stronger, with an OR of 20.2 (95% CI 1.45–355). Epidemiology and the shared role of sex hormones predisposing to both conditions may explain this specific association. In multivariate analysis anti-RNA-PolIII was also associated with older age, acute renal crisis and diffuse skin involvement. These results led to specific cancer-screening recommendations in anti-RNA-PolIII positive patients, over a period of 2 to 5 years, including routine mammography (repeated every year) and non-invasive investigations, such as prostatic specific antigen (PSA) testing, blood stool test, and gynecological examination. Of note, in an Australian prospective study, patients with anti-RNA-PolIII positive and anti-RNA-PolIII negative antibodies had the same percentage of cancer (13%) but the diagnosis of cancer within 5 years after the diagnosis of scleroderma was more frequent in anti-RNA-PolIII positive patients (13 vs. 3.9%) (141). A direct pathophysiological link between RNA-PolIII antigen modification in tumor and autoimmunity is even suggested in this condition, as developed below (paragraph Anti-RNPC-3).

Anti-RNPC-3

Recent studies have identified in "triple negative" scleroderma patients with cancer (patients without anti-centromere, anti-scl70 and anti-RNA-PolIII antibodies), a new autoantibody that targets RNPC-3. RNPC-3 is a protein member of the minor spliceosome, ribonucleoprotein complex that participates in the splicing of pre-messenger RNAs (17). Like the anti-RNA-PolIII positive patients, these patients have 4 times more risk of developing cancer within 2 years after the onset of scleroderma than the anti-centromere positive patients (95% CI 1.1–16.9) (142). The authors also noted an association between anti-RNPC-3 and severe interstitial lung disease, as well as more frequent muscle involvement (142). These anti-RNPC3 autoantibodies would possibly be indicative of cancer-induced autoimmunity in this subgroup of patients.

Anti-PM/Scl

A recent Spanish retrospective study involving 432 patients including 53 cancers (12.2%), found no association between anti-RNA-PolIII antibodiesand cancer (35). In contrast, the anti-PM/Scl autoantibody was associated with cancer with an OR of 3.90 (95% CI 1.31–11.61), while aspirin treatment was protective with an OR of 0.33 (95% CI 0.12–0.90) (35). As above mentioned, the link between miRNAs regulation and PM/Scl, a complex homolog to yeast exosome is intriguing. However, these results have not been confirmed by a Dutch study (143).

Anti-Scl70

Rosen's team has shown that patients with anti-scl70 antibodies were associated with short cancer-scleroderma interval (34). Advanced age was an independent risk factor for cancer in this study. An Italian team has demonstrated that anti-Scl70 antibodies were associated with lung cancer (26).

PARANEOPLASTIC SCLERODERMA: FROM EPIDEMIOLOGICAL OBSERVATION TO PATHOPHYSIOLOGICAL DEMONSTRATION

Overview of the Concept of Paraneoplastic Autoimmune Disease

As said earlier, the observation of an elevated risk of cancer in patients with numerous systemic autoimmune conditions compared with the general population argues for close and bidirectional relationships between malignancy and autoimmunity. Even more striking is the close temporal clustering in some patients, which leads to consider autoimmune disease as a genuine paraneoplastic entity. The exact definition of a paraneoplastic syndrome may vary greatly among authors and remains debatable. First, one prerequisite for paraneoplastic phenomenon lies in this "temporal clustering," viz. a short interval between the onset of cancer and the onset of autoimmune disease. Of note, cancer could precede SSc onset, and vice-versa. This "short" interval is however vague, but 3 to 5 years before and after SSc diagnosis could be acceptable since it corresponds to a "peak" of frequency in cancer diagnosis in previous studies (33). Second, there should be a parallel evolution between cancer and autoimmunity, with autoimmune flares accompanying cancer relapses. Conversely, cancer resection should lead to the remission of the associated autoimmune condition. However, such a theoretical conception of paraneoplastic phenomena may be far away from the reality observed concerning SSc, where no resolution of autoimmunity has been described after cancer healing.

Conceptually, autoimmunity may be associated with oncogenesis and anti-tumor defense. This is also consistent with the observation of cancer developing some time after autoimmunity, since autoimmune response in this context may be triggered in the very early stages of cancer development, even in premalignant disease. For instance, autoantibody production has been shown to appear years before cancer diagnosis in such context (144). Long ago considered as non-specific, the presence of some antibodies could have some clinical relevance for patients developing autoimmune disorders and cancer, and become novel biomarkers for both conditions, in terms of early diagnosis, but also prognosis and response to therapy. Interestingly, specific anti-tumor activity of antinuclear antibody (ANA) *via* antibody-dependent cell-mediated cytotoxicity (ADCC) has been described (145). For some authors, antibody clustering could even correspond to distinct underlying malignancies, beyond autoimmune disease classification (145). For instance, in dermatomyositis, anti-NXP2 and anti-TIF1γ– but not anti-MDA-5 antibodies- are strongly associated with

cancer development (more than 80% of patients presenting with cancer and dermatomyositis) (146).

Interestingly, and consistent with what is usually reported with paraneoplastic syndromes, autoimmunity in the context of cancer is most often associated with a better outcome. For instance, the presence of a paraneoplastic syndrome is associated with smaller tumors and less metastatic disease. Moreover, tumor-infiltrating lymphocytes, the presence of ANA

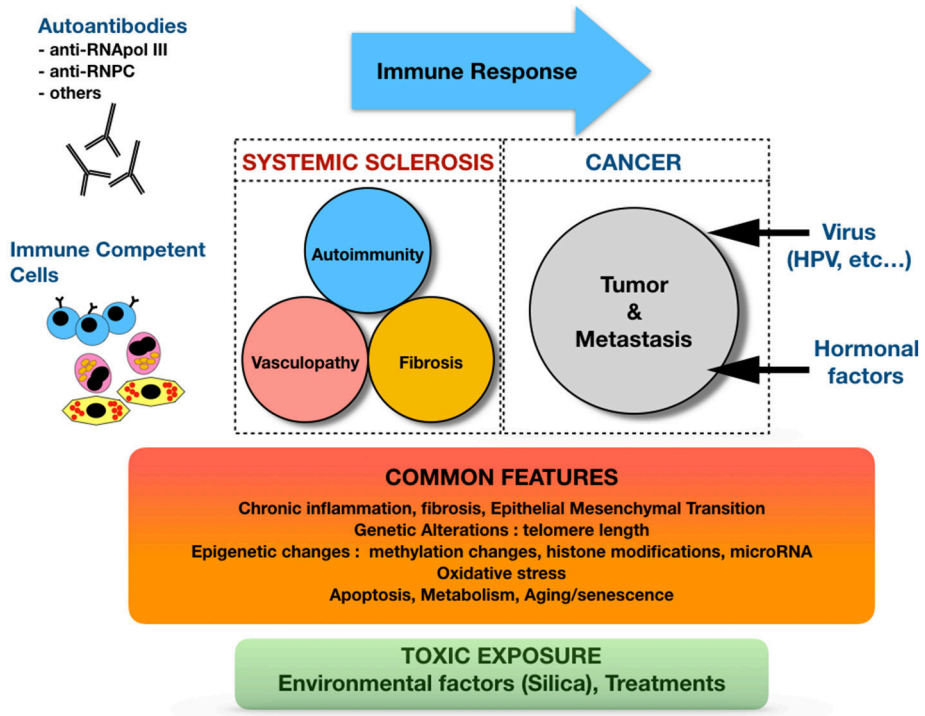

FIGURE 1 | Complex and bilateral relationships between systemic sclerosis and cancer.

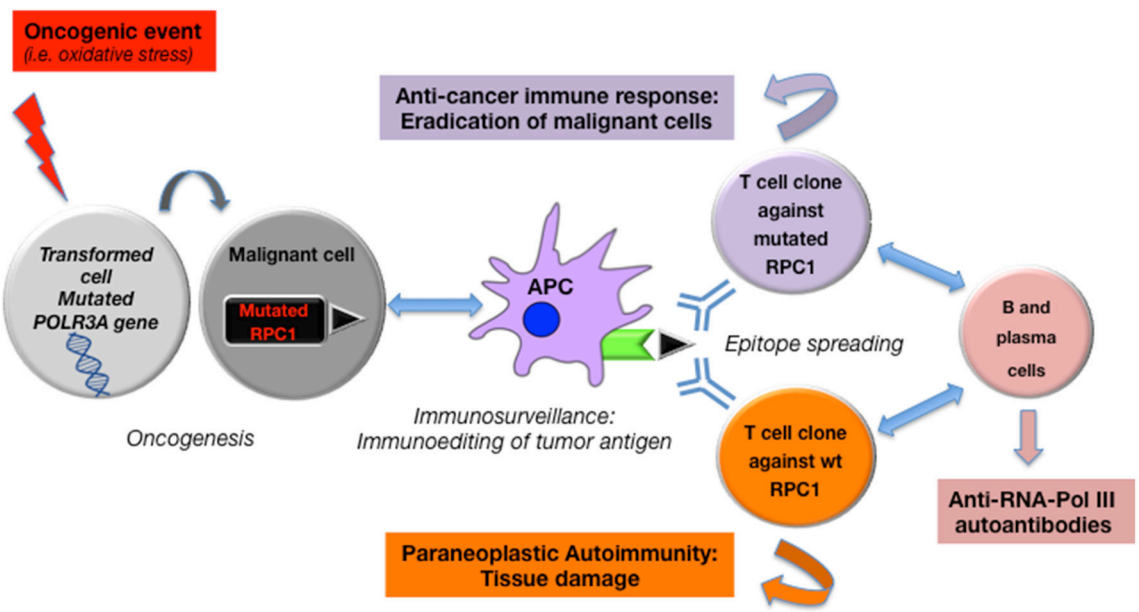

FIGURE 2 | Anti-tumor driven auto-immunity: from mutated RNA polymerase III in cancer to paraneoplastic systemic sclerosis.

and vitiligo are clearly demonstrated as positive prognostic factors (8, 145). In addition, in the context of cancer immunotherapy using immune checkpoint inhibitors (i.e., anti-PD1, -PDL1 and CTLA-4 agents), the presence of tumor infiltrating lymphocytes before treatment and the observation of autoimmunity (i.e., immune related adverse events) under treatment are usually predictive of a better response to therapy (147).

All these observations support the hypothesis of a common primary event in oncogenesis that could make a self-antigen become immunogenic, and next trigger an autoimmune response against tumor cells. This specific autoimmune response would contribute to anti-tumor defense, but in some extent, in the case of a shared antigen, this response could spread to non-mutated antigens and be responsible for healthy tissue damage in relation with autoimmunity. In SSc, this concept has recently been thoroughly demonstrated in patients with anti-RNA-PolIII antibodies and contributes to the possibility of scleroderma being a paraneoplastic disease.

Cancer-Induced Scleroderma: The Role of Mutated RNA-PolIII in Autoimmunity

Several recent studies conducted by the team of Rosen, Casciola-Rosen and Shah (Johns Hopkins University School of Medicine, Baltimore, USA) among others, have shown that the anti-RNA-PolIII antibodies were associated with cancer in SSc, with a temporal clustering between the two conditions suggestive of a paraneoplastic disease and a common underlying mechanism (7, 16, 33, 141). Patients with anti-RNA-PolIII antibodies are indeed 5.08 (95% CI 1.60–16.1) times more likely to develop cancer within 2 years of SSc onset (34). Furthermore, a specific nucleolar expression of RNA-PolIII was observed in the malignant cells from these patients, suggestive of a link between cancer-related auto-antigen and autoimmune response (16).

In order to decipher the potential common pathophysiological process underlying this association, Rosen et al. comparatively studied eight tumors from anti-RPC1 positive patients (RNA polymerase III subunit) and eight tumors from anti-Scl70 or anti-cm positive patients. Genetic alterations (somatic mutation or loss of heterozygosity) of the POLR3A gene coding for RNA polymerase III were identified in the tumors of six out of eight anti-RCP1 positive patients, while no mutation of this gene was found in the tumors of anti-cm or anti-Scl70 positive patients. Hence, the presence of this mutated auto-antigen in malignant cells could be the *primum movens* triggering autoimmunity in these patients, inducing cellular and humoral responses, with the production of anti-RNA-PolIII autoantibodies (148). Of particular interest, anti-RNA-PolIII antibodies in these patients were found to recognize both mutated and non-mutated (wild-type, wt) RNA-PolIII. This important finding, observed in other situations (149), is related to an "epitope-spreading" mechanism, that would be responsible for healthy tissue damage in SSc. In other terms, their demonstration argues for the role of a shared antigen (i.e., RNA polymerase III) that could undergo genetic alterations (for instance under DNA oxidative damage), and lead to the emergence of a mutated and immunogenic antigen in transformed cells. These alterations would next trigger a mutant-specific clonal immune response that could subsequently spread to wt antigens in healthy cells, contributing to autoimmune-mediated tissue damage in SSc (**Figure 2**). According to this hypothesis, the appearance of scleroderma in the context of malignancy would be the "price to pay" for eliminating the cancer (6). This could also explain why 80% of RNA-PolIII patients never develop cancer: the latter ones may have benefited from anti-tumor immunosurveillance with efficient eradication of malignant cells. However, as summarized by Schreiber et al., immunosurveillance in cancer is a dynamic process, better-called immunoediting, that evolves in three stages, from tumor elimination, equilibrium, and finally to escape under tumor high mutational rate with loss of expression of immunogenic antigens(150).

CONCLUSION AND PERSPECTIVES

Beyond simple epidemiological observations, intriguing and complex bilateral relationships exist between SSc and malignancy, supported by a growing body of data involving the immune system and other contributors such as genetic and epigenetic changes, environmental factors, including oxidative stress. These relationships are summarized in **Figure 1**.

On the whole, based on these observations, a novel concept of autoimmunity as a response to underlying malignancy is coming up in scleroderma, and may contribute to new strategies in patients' care. Indeed, autoantibodies could be useful biomarkers for screening strategies, as proposed by some authors who postulate that early diagnosis of malignancy may ameliorate cancer but also SSc outcome (6, 7, 35). A detailed screening algorithm has been proposed by Shah et al., based on gender, specific risk factors and "red flags," leading to specific non-invasive and invasive investigative procedures (6). Even if the beneficial effects in terms of overall survival in the scleroderma population remain to be demonstrated, a strategy based on repeated and more aggressive screening in patients with specific autoantibodies subsets (i.e., anti-RNA-PolIII, Pm/Scl or RNPC3) or in seronegative patients may be tantalizing.

Deciphering the mechanisms of autoimmunity through the prism of cancer immunosurveillance is even more fascinating in the era of anticancer immunotherapy, and will undoubtedly lead to new breakthroughs both in the field of autoimmunity and cancer. One could hope this may 1 day contribute to a better prognosis of scleroderma.

AUTHOR CONTRIBUTIONS

AM, LP, RG, and PG participated in the review of literature, and in the manuscript redaction. SR, PR, CB, AL, JM, and DN participated in the manuscript redaction and final approval.

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OTUD6B-AS1 Might Be a Novel Regulator of Apoptosis in Systemic Sclerosis

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Antisense long non-coding RNAs (AS lncRNAs) have increasingly been recognized as important regulators of gene expression and they have been found to play key roles in several diseases. However, very little is known about the role of AS lncRNAs in fibrotic diseases such as systemic sclerosis (SSc). Our recent screening experiments by RNA sequencing showed that ovarian tumor domain containing 6B antisense RNA1 (OTUD6B-AS1) and its sense gene OTUD6B were significantly downregulated in SSc skin biopsies. Therefore, we aimed to identify key regulators of OTUD6B-AS1 and to analyze the functional relevance of OTUD6B-AS1 in SSc. OTUD6B-AS1 and OTUD6B expression in SSc and healthy control (HC) dermal fibroblasts (Fb) after stimulation with transforming growth factor-β (TGFβ), Interleukin (IL)-4, IL-13, and platelet-derived growth factor (PDGF) was analyzed by qPCR. To identify the functional role of OTUD6B-AS1, dermal Fb or human pulmonary artery smooth muscle cells (HPASMC) were transfected with a locked nucleic acid antisense oligonucleotide (ASO) targeting OTUD6B-AS1. Proliferation was measured by BrdU and real-time proliferation assay. Apoptosis was measured by Caspase 3/7 assay and Western blot for cleaved caspase 3. While no difference was recorded at the basal level between HC and SSc dermal Fb, the expression of OTUD6B-AS1 and OTUD6B was significantly downregulated in both SSc and HC dermal Fb after PDGF stimulation in a time-dependent manner. Only mild and inconsistent effects were observed with TGFβ, IL-4, and IL-13. OTUD6B-AS1 knockdown in Fb and HPASMC did not affect extracellular matrix or pro-fibrotic/proinflammatory cytokine production. However, OTUD6B-AS1 knockdown significantly increased Cyclin D1 expression at the mRNA and protein level. Moreover, silencing of OTUD6B-AS1 significantly reduced proliferation and suppressed apoptosis in both dermal Fb and HPASMC. OTUD6B-AS1 knockdown did not affect OTUD6B expression at the mRNA level and protein level. Our data suggest that OTUD6B-AS1 regulates proliferation and apoptosis via

cyclin D1 expression in a sense gene independent manner. This is the first report investigating the function of OTUD6B-AS1. Our data shed light on a novel apoptosis resistance mechanism in Fb and vascular smooth muscle cells that might be relevant for pathogenesis of SSc.

Keywords: systemic sclerosis, ovarian tumor domain containing 6B-antisense RNA1 (OTUD6B-AS1), antisense long non-coding RNA (AS lncRNA), cyclin D1, proliferation, apoptosis, dermal fibroblasts, human pulmonary artery smooth muscle cells

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease characterized by immune abnormalities, microvascular dysfunction, and fibrosis in the skin and multiple internal organs. Patients are sub-classified into limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc), based on the extent of skin involvement. Fibrosis in multiple internal organs is an important contributor to the high mortality in SSc. Upregulation of profibrotic cytokines such as PDGF and TGFβ and their respective signaling pathways is a key feature of SSc (1, 2). Microvascular manifestations include loss of capillaries and vessel wall thickening of small arteries leading to reduced perfusion and hypoxia. Major clinical manifestations of the microvasculopathy are digital ulcers and pulmonary arterial hypertension (PAH). PAH is another major contributor to death in SSc patients. However, the molecular pathogenesis of SSc leading to these manifestations is still not fully understood and there is no registered approved anti-fibrotic therapy available to date (3–5).

Long non-coding RNAs (lncRNAs) represent a class of transcripts longer than 200 nucleotides that are not translated into proteins. LncRNAs have been classified by their genomic location into intergenic lncRNA, intronic lncRNA, bidirectional lncRNA, enhancer lncRNA, sense lncRNA, and antisense (AS) lncRNA (6). AS lncRNAs are transcribed from the opposite strand of protein-coding genes and overlap one or several exons and introns with the sense gene. High-throughput RNA sequencing analysis showed that for most AS lncRNAs the expression is about 10-fold lower than for their coding gene (7, 8). Moreover, the expression of AS lncRNAs is more tissue specific than those of protein coding genes (8–10). In recent years, AS lncRNAs have increasingly been recognized as important regulators of their sense gene expression. However, some AS lncRNAs can exert their biologic effects independently of the sense gene. For example, NKX2-1-AS1 is an AS lncRNA that has been linked to human lung carcinoma. NKX2-1-AS1 regulates CD274, the gene encoding the Programmed Death-Ligand 1 (PD-L1), and cell-cell interaction genes, but not the adjacent protein-coding gene NKX2-1 (11). In general, AS lncRNAs can function either in *cis* or in *trans*. In *cis*, lncRNAs regulate the expression of transcription sites on the same chromosome. In *trans*, lncRNAs regulate the gene expression on other chromosomes (9, 12). AS lncRNAs affect almost all stages of gene expression processes via pre-transcriptional, co-transcriptional, or post-transcriptional mechanisms (12–14).

Recently, a variety of studies have reported that AS lncRNAs play key roles in the pathogenesis of different diseases including cancers (15–17), cardiac vascular diseases (6, 18), kidney diseases (18), and central nervous system diseases (19). However, only very few AS lncRNAs have been linked to SSc and fibrosis (20, 21). Previous RNA sequencing analysis of SSc and healthy control (HC) skin biopsies performed by our groups revealed 676 differentially expressed non-coding genes, 38% of these non-coding genes were classified as antisense genes. The differentially expressed antisense genes included ovarian tumor domain containing 6B (OTUD6B)-AS1 (22).

The sense gene, OTUD6B, encodes for a deubiquitinating enzyme. Deubiquitinating enzymes are classified into five families based on the architecture of their catalytic domains. OTUD6B belongs to the ovarian tumor proteases (OTUs) family. Additional families are ubiquitin specific proteases (USPs), ubiquitin COOH-terminal hydrolases (UCHs), Josephins, and the JAB1/MPN/MOV34 family (JAMMs) (23). It has been shown that deubiquitinating enzymes have crucial roles in many biological processes, including cell cycle regulation, apoptosis, and DNA repair. In particular, they can regulate specific molecular pathways such as Wnt/β-catenin and NFκB signaling (24).

Little is known about the function of OTUD6B. OTUD6B has been suggested to be involved in cell cycle regulation in B lymphocytes after prolonged cytokine stimulation and in DNA synthesis in non-small cell lung cancer cells (25, 26). Moreover, defective OTUD6B function has been associated with cognitive dysfunction and dysmorphic features in development (27). There is no information available about the function of the antisense lncRNA, OTUD6B-AS1. OTUD6B-AS1 is transcribed from the opposite strand of the OTUD6B gene, which is located on chromosome 8 in head-to-head orientation to OTUD6B-AS1 (**Figure 1A**).

Here, we analyzed the differential expression of OTUD6B-AS1 in SSc skin biopsies, identified pro-inflammatory and pro-fibrotic regulators of OTUD6B-AS1 expression, and defined the functional roles of OTUD6B-AS1 in the pathophysiology of SSc. In particular, we focused on OTUD6B-AS1 role in proliferation and apoptosis of human dermal fibroblasts and human pulmonary artery smooth muscle cells (HPASMC), which represent the two major cellular players in fibrosis and vasculopathy of SSc.

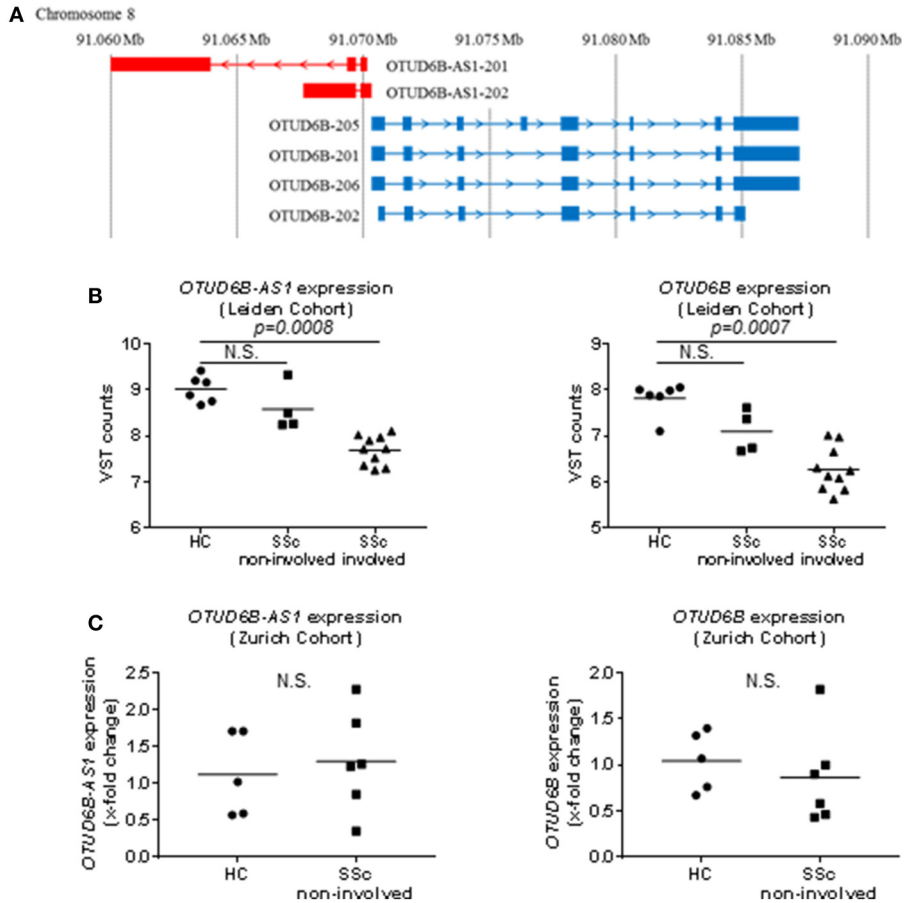

FIGURE 1 | Genomic localization and basal expression in skin of OTUD6B-AS1 and OTUD6B. **(A)** OTUD6B-AS1 and OTUD6B are located on chromosome 8 and oriented head-to-head (GENCODE v27). **(B)** In the Leiden cohort, OTUD6B-AS1, and OTUD6B expression was analyzed in healthy control (HC), non-involved and involved skin of systemic sclerosis patients (SSc) using next generation sequencing [HC: $n = 6$, SSc (non-involved): $n = 4$, SSc (involved): $n = 10$, (22)]. **(C)** In the Zurich cohort, OTUD6B-AS1, and OTUD6B expression was measured in HC and non-involved SSc patients skin by qPCR (HC: $n = 5$, SSc (non-involved): $n = 6$). Expression level was normalized using *RPLP0*. **(C)**. Data are shown as single values and mean. Statistical analysis was performed by unpaired *t*-test. N.S, not significant.

METHODS

Cell Culture

Skin biopsies were obtained from the forearm of SSc patients at the Department of Rheumatology, University Hospital Zurich, Switzerland or at the Department of Internal Medicine, Division of Rheumatology, University of Texas Health Science Center, Houston, USA. All patients fulfilled the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2013 criteria for SSc. Healthy donors were obtained from the Department of Plastic Surgery and Hand Surgery of the University Hospital Zurich. Donors characteristics are listed in **Table 1**. Patients and healthy subjects from the Leiden and Dartmouth cohort have been described elsewhere (22). In general, skin areas with clinically detectable fibrotic changes are referred to as "involved skin," and skin areas without clinically detectable fibrotic changes are referred to as "non-involved skin." This study was carried out in accordance with the recommendations of World Medical Association Declaration of Helsinki, ICH-GCP guidelines or ISO 14155. The study was approved by the Ethics Committee of the Canton of Zurich (approved ethical applications KEK-ZH 515, PB-2016-02014, and KEK-Nr. 2018-01873). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Human dermal fibroblasts (Fb) were isolated from SSc and site-matched HC skin biopsies by outgrowth culture. Dermal Fb were maintained in Dulbecco's modified eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 50 units/ml of penicillin, 50 µg/ml of streptomycin and 2 µl/ml 2-mercaptoethanol. Fb from passages 4 to 11 were used for all experiments.

Human pulmonary artery smooth muscle cells (HPASMC) were purchased from ScienCell and maintained in smooth muscle cell medium (SMCM, ScienCell) supplemented with 1% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and

TABLE 1 | Characteristics of systemic sclerosis (SSc) patients and healthy control (HC) donors in the Zurich cohort.

Donor	Gender	Site of biopsy	Disease subtype	Disease duration (years)	mRSS	Fibrosis at the site of biopsy	Skin biopsy expression analysis	PDGF stimulation/ functional assays
SSc 1	Female	Forearm	Diffuse	10.16	4	No	x	x
SSc 2	Male	Forearm	Diffuse	1.32	16	Yes	x	x
SSc 3	Female	Forearm	Diffuse	9.16	6	No	x	
SSc 4	Male	Forearm	Diffuse	0.72	n.a.	n.a.		x
SSc 5	Male	Forearm	Limited	n.a.	0	No	x	
SSc 6	Female	Forearm	Limited	6.0	0	No	x	
SSc 7	Female	Forearm	Limited	n.a.	0	No	x	x
SSc 8	Female	Forearm	Limited	n.a.	6	No		x
SSc 9	Female	Forearm	Limited	n.a.	3	No		x
HC 1	Female	Abdomen					x	
HC 2	Female	Breast					x	
HC 3	Female	Hernia surgery					x	
HC 4	Male	Forearm						x
HC 5	Male	Forearm					x	
HC 6	Male	Forearm					x	x
HC 7	Male	Forearm					x	x
HC 8	Female	Breast						x
HC 9	Male	Forearm						x
HC 10	Male	Forearm						x
HC 11	Female	Forearm						x
HC 12	Female	Forearm						x
HC 13	Female	Forearm						x
HC 14	Male	Forearm						x

Skin biopsies were obtained from patients fulfilling the American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) 2013 criteria. The disease subset was determined according to the criteria proposed by LeRoy et al. (28). Disease duration was measured from the onset of the first non-Raynaud symptoms attributable to SSc. n.a, not available.

smooth muscle cell growth supplement (all, ScienCell). HPASMC from passages 4 to 9 were used in all experiments.

Dermal Fb and HPASMC were cultured at 37°C in a humidified 5% CO_2-containing atmosphere. Dermal Fb and HPASMC were stimulated with transforming growth factor-β (TGFβ; 10 ng/ml, PeproTech), Interleukin (IL)-4 (10 ng/ml, ImmunoTools), IL-13 (10 ng/ml, ImmunoTools), and platelet-derived growth factor (PDGF; 20 ng/ml, PeproTech) for 6, 24, 48, and 72 h.

Cell Fractionation

To detect the localization of lncRNA OTUD6B-AS1, dermal Fb were fractionated as previously described (29). Cells were trypsinized, lysed with hypotonic lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.3% NP-40 and 10% glycerol) supplemented with RNase inhibitor (SUPERase-In, Thermo Fisher Scientific) and incubated on ice for 20 min. Cells were then centrifuged at 10,00 g at 4°C for 3 min. Supernatant (cytoplasmic fraction) was taken and RNA precipitated in 150 mM ethanol sodium acetate at −20°C for 1 h. Cell pellets (nuclear fraction) were washed with hypotonic lysis buffer and centrifuged at 200 g at 4°C for 2 min. Precipitated cytoplasmic RNA was pelleted, washed in ice-cold 70% ethanol and centrifuged at 17,000 g at 4°C for 5 min. Finally, Trizol was added to both fractions and RNA was extracted.

Transient Transfection of Dermal Fb and HPASMC

To identify the function of the lncRNA OTUD6B-AS1, HC Fb, or HPASMC were transfected with locked nucleic acid antisense oligonucleotide (ASO, QIAGEN) targeting OTUD6B-AS1 (k.d.: 5′- GAATGAAATAGACGTT−3′, k.d._2: 5′-TTCAGTAATTCGATT-3′, k.d._3: 5′-GGACCAAAATCAAAGA-3′, final concentration of 25 nM for dermal Fb and 50 nM for HPASMC) using a concentration of 2 μl/ml Lipofectamine 2,000 transfection reagent according to the manufacturer's protocol (Thermo Fisher Scientific). Cells were harvested for gene and protein expression analysis 24, 48, 72 h after transfection or prepared for functional assays.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from cultured cells using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. RNA concentration of isolated RNA was assessed using spectrophotometer (NanoDrop). For complementary DNA (cDNA) synthesis, 200 ng of total RNA was reverse-transcribed

using random hexamers and reverse transcriptase using the Transcriptor First Strand cDNA Synthesis Kit (Roche).

RNA Sequencing and Quantitative Real-Time PCR (qPCR) Analysis

RNA sequencing performed on the skin biopsies from Leiden has been described recently (22). Total RNA extracted from skin biopsies from Zurich as well as functional *in vitro* experiments were analyzed by qPCR (Stratagene Mx3005P QPCR system, Agilent Technologies) using SYBR Green Master Mix (Promega). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein lateral stalk subunit P0 (RPLP0) was used to normalize the obtained expression levels for the gene of interest. Primer sequences are listed in the (**Supplementary Table 1**). Specific amplification was verified measuring dissociation curves. Differential gene expression was calculated using the comparative threshold cycle method (ΔΔCt method) (30).

Western Blotting

Cells were lysed using ice-cold RIPA buffer (Sigma) supplemented with phosphatase inhibitor cocktail (PhosSTOP, Roche) and protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche). Insoluble material was removed by centrifugation at 12,000 rpm, 20 min at 4°C. Whole cell lysates were separated on 10 or 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred by electroblotting onto nitrocellulose membranes (Amersham Protran, GE Healthcare). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween20 (TBS-T) and incubated for 1 h. Western blots were performed using rabbit anti OTUD6B antibody (Abcam, #ab127714, 1:3,000), rabbit anti Cyclin D1 antibody (Cell signaling, #2978, 1:1,000), rabbit anti Cyclin D2 antibody (Cell signaling, #3741, 1:1,000), rabbit anti c-MYC antibody (Cell signaling, #13987, 1:1,000), rabbit anti Caspase 3 antibody (Cell signaling, #9662, 1:1,000) which detects both cleaved and uncleaved forms of caspase 3, rabbit anti E2F1 antibody (Abcam, #ab179445, 1:1,000), and rabbit anti GAPDH antibody (Cell signaling, #2118, 1:10,000). Bands were detected using goat anti rabbit secondary antibodies conjugated to horseradish peroxidase (Abcam, #ab6721, 1:5000). Cyclin D1, Cyclin D2, c-MYC, E2F1 expression was normalized to the expression of GAPDH, and cleaved caspase 3 expression was normalized to uncleaved caspase 3 expression. Calculation of the relative expression of protein was performed using ImageJ software.

BrdU Cell Proliferation ELISA

To quantify cell proliferation, dermal Fb, and HPASMC were seeded in 96-well plates at a density of 2,000 cells per well and transfected with the ASO negative control or ASO targeting OTUD6B-AS1. Seventy-two hours after transfection, 5-bromo-2′-deoxyuridine (BrdU) was added to each well and incubated for four additional hours. Incorporated BrdU was detected using the BrdU (colorimetric) cell proliferation ELISA kit (Roche/Sigma-Aldrich) according to the manufacturer's instructions.

Real-Time Monitoring of Proliferation

Real-time monitoring of cell proliferation was performed using the xCelligence RTCA DP system (ACEA Biosciences). Background impedance was measured 30 min after adding 100 μl of culture medium to E-plates 16 PET (ACEA Biosciences). Next, either 2,500 dermal Fb or 3,000 HPASMC per 100 μl of culture medium per well were seeded. After 30 min incubation at room temperature, plates were placed into the xCelligence system. The relative change in electrical impedance termed the Cell Index (CI) was measured. Fb and HPASMC were transfected with negative control or ASO targeting OTUD6B-AS1 26 h or 50 h after seeding, respectively. CI was monitored every 5 min from 0 h to 13 h, every 15 min from 13 h to 25 h and every 30 min from 25 h until CI reached plateau. CI was normalized at the time of transfection for every experiment. The RTCA software 2.0 (ACEA Biosciences) was used to calculate the slope of the CI curve as measure of cell proliferation.

Caspase 3/7 Assay

To measure caspase-3 and caspase-7 activities, dermal Fb, and HPASMC were seeded in 96-well plates at a density of 2,000 cells per well and transfected with either ASO negative control or ASO targeting OTUD6B-AS1 (final concentration of 25 nM for dermal Fb and 50 nM for HPASMC) as described above. To induce caspase 3/7 activation, cells were treated with 1 μM staurosporin (STP) 16 h before starting the assay (31, 32). Forty-eight or seventy-two hours after transfection, equal volumes of Caspase-Glo® 3/7 Reagent (Promega) were added into the cell culture medium and incubated for additional 1.5 h at room temperature. Luminescence as a measure of caspase 3/7 activity was recorded by plate-reading luminometer (Synergy HT, BioTek).

Statistics

All data are presented as mean ± standard deviation (SD). Paired samples were analyzed by two-tailed paired *t*-test, and unpaired samples were analyzed by unpaired *t*-test. Comparison of multiple groups was performed by one-way ANOVA with Dunnett's multiple comparisons test. $P < 0.05$ were considered statistically significant. All statistic tests were performed with GraphPad Prism version 7.0.

RESULTS

OTUD6B-AS1 and OTUD6B Expression Is Downregulated in SSc Skin

RNA sequence analysis of 6 HC and 14 SSc patients skin biopsies performed at the Leiden University Medical Center showed that OTUD6B-AS1 expression was significantly downregulated in SSc skin and this was the most differentially expressed AS lncRNA. Moreover, the sense gene OTUD6B expression was also significantly downregulated in SSc skin (22). Interestingly, additional subanalysis showed that this downregulation in SSc skin was mostly seen in skin biopsies from involved skin, while non-involved skin did not show statistically significant changes as compared to healthy controls (**Figure 1B**). In addition, recently performed RNA sequencing analysis of additional 6 HC and 14 SSc biopsies performed at Geisel School of

Medicine at Dartmouth, confirmed OTUD6B-AS1 as one of the top downregulated AS lncRNAs (22). The expression of OTUD6B-AS1 and OTUD6B was not changed in biopsies from the Zurich cohort, which only consisted of non-involved SSc skin biopsies as revealed by qPCR analysis (**Figure 1C**). Together, these data from different cohorts indicate that the expression of OTUD6B-AS1 and OTUD6B is downregulated in skin biopsies from SSc patients, mostly in clinically involved, fibrotic areas.

OTUD6B-AS1 and OTUD6B Expression in Dermal Fb Is Tightly Regulated by PDGF

OTUD6B-AS1 and OTUD6B are strongly expressed in dermal Fb as well as in other cells types present in the skin (22). Therefore, we compared the expression levels of OTUD6B-AS1 and OTUD6B in dermal Fb. Under basal conditions, there was no statistically significant difference between HC and SSc Fb (**Supplementary Figure 1**).

Based on the expression in fibrotic areas in skin biopsies, we hypothesized that immunomodulatory cytokines involved in the pathophysiology of fibrosis might be important for the downregulation of OTUD6B-AS1 and OTUD6B. We therefore analyzed their time course response after stimulation with PDGF, TGFβ, IL-4 and IL-13.

OTUD6B-AS1 expression in dermal Fb from SSc patients was significantly downregulated after 24, 48, and 72 h of PDGF stimulation. OTUD6B expression was significantly upregulated after 6 h of PDGF stimulation, while after longer stimulation, the expression levels decreased to become significantly downregulated at 72 h (**Figure 2A**).

We also analyzed the time course response to PDGF of OTUD6B-AS1 and OTUD6B in HC dermal Fb. In general, the effects of PDGF on the expression of OTUD6B-AS1 and OTUD6B in HC dermal Fb were similar to that observed in SSc dermal Fb (**Figure 2B**). These data suggest that PDGF strongly suppresses OTUD6B-AS1 and OTUD6B expression in dermal Fb, reflecting the expression pattern observed in SSc skin samples.

The effects after stimulation with other pro-fibrotic and pro-inflammatory cytokines were overall less consistent. OTUD6B-AS1 expression was slightly, but significantly upregulated after 72 h of TGFβ stimulation and significantly downregulated after 72 h of IL-4 stimulation. IL-4 slightly increased OTUD6B expression after 48 h of stimulation. However, we could not detect any strong and consistent effect after TGFβ, IL-4, and IL-13 stimulation (**Supplementary Figures 2A,B**) as observed for PDGF.

OTUD6B-AS1 Knockdown Reduces Proliferation and Suppresses Apoptosis of Dermal Fb

In order to further understand its function in the pathogenesis of SSc, we knocked down OTUD6B-AS1 using ASO (33). We used HC dermal Fb to mimic the downregulation seen in the SSc patients and to recapitulate the extent of OTUD6B-AS1 dysregulation effects in healthy cells. Successful knockdown of OTUD6B-AS1 was confirmed using qPCR at 48 and 72 h after transfection in HC dermal Fb (**Figure 3A**). First, we looked at effects of OTUD6B-AS1 knockdown on the expression of genes relevant for key processes of fibrosis. However, we could not detect significant differences in the gene expression of collagen 1α1 (COL1A1), fibronectin-1 (FN1) and alpha smooth muscle actin (α-SMA) (**Supplementary Figure 3**). These data indicate that OTUD6B-AS1 does not directly affect the expression of fibrotic genes.

Proliferation and apoptosis are also important processes in SSc pathogenesis (3). Thus, we assessed OTUD6B-AS1 function on dermal Fb proliferation using the BrdU assay and real-time monitoring of cell proliferation. Seventy-two hours after OTUD6B-AS1 knockdown, we observed a minor reduction in cell proliferation as detected by BrdU assay, however there was no statistically significant difference (**Figure 3B**). Real-time monitoring of cell proliferation showed that after more than 75 h of OTUD6B-AS1 knockdown, dermal Fb proliferated significantly slower than scrambled control treated Fb (**Figure 3C**).

We also assess apoptosis after OTUD6B-AS1 knockdown using Caspase 3/7 Glo assay® and cleaved caspase 3 WB analysis. Caspase 3/7 activity was slightly, but significantly downregulated 72 h after OTUD6B-AS1 knockdown (**Figure 3D**). Moreover, the ratio between cleaved and uncleaved caspase 3 was significantly reduced 72 h after OTUD6B-AS1 knockdown in dermal Fb (**Figure 3E**, **Supplementary Figure 4A,B**).

OTUD6B-AS1 Knockdown Reduces Proliferation and Suppresses Apoptosis in HPASMC

In the pathophysiology of SSc, cell cycle regulation, proliferation, and resistance to apoptosis is particularly important for the development of microvascular lesions, where vascular smooth muscle cells strongly proliferate resulting in vessel wall thickening and an occlusion of small arteries. The main clinical manifestation is PAH (3–5, 34). Therefore, we also performed OTUD6B-AS1 knockdown in HPASMC. OTUD6B-AS1 expression was significantly reduced in HPASMC at 48 h after transfection (**Figure 4A**, **Supplementary Figure 4C–E**). We also assessed proliferation and apoptosis after knockdown of OTUD6B-AS1 in HPASMC. HPASMC proliferation was significantly reduced already 48 h after transfection as revealed by BrdU assay (**Figure 4B**). Real-time monitoring of cell proliferation confirmed that HPASMC showed reduced proliferation earlier than dermal Fb and continued to proliferate significantly slower than scrambled control treated cells (**Figure 4C**). The apoptosis assay revealed that caspase 3/7 activity was significantly lower 48 h after OTUD6B-AS1 knockdown (**Figure 4D**), and the ratio of cleaved and uncleaved caspase 3 was significantly reduced 48 h after knockdown (**Figure 4E**) similar as for dermal Fb. These data suggest that OTUD6B-AS1 might be a regulator of proliferation and apoptosis not only for dermal Fb, but also for HPASMC.

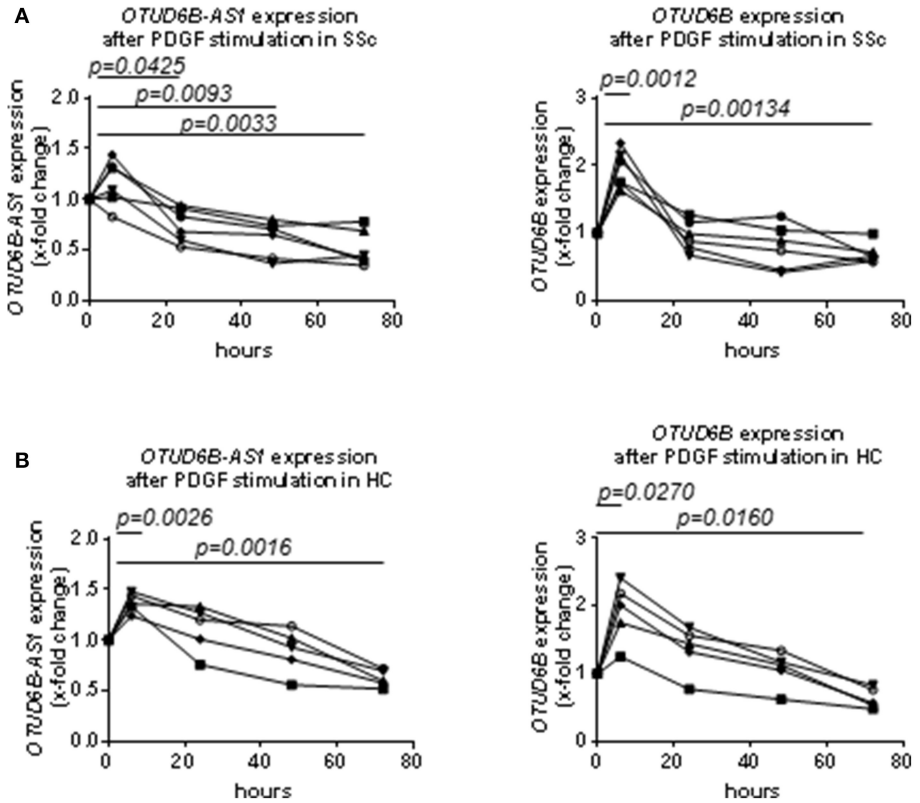

FIGURE 2 | Time course analysis of the expression of OTUD6B-AS1 and OTUD6B in SSc and HC dermal Fb after platelet-derived growth factor (PDGF) stimulation. Dermal Fb were stimulated with PDGF (20 ng/ml) for 6, 24, 48, and 72 h. **(A)** Time course analysis of the expression of OTUD6B-AS1 and OTUD6B in SSc dermal Fb after platelet-derived growth factor (PDGF) stimulation ($n = 6$). **(B)** Time course analysis of the expression of OTUD6B-AS1 and OTUD6B in HC dermal Fb after platelet-derived growth factor (PDGF) stimulation ($n = 5$). Expression levels were measured by qPCR, normalized by GAPDH and RPLP0 and compared with non-stimulated dermal Fb. Data are shown as single values. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparisons test.

OTUD6B-AS1 Knockdown Increases CyclinD1 Expression in Dermal Fb and HPASMC

Cyclins are among the most important cell proliferation associated genes. They form active complexes with cyclin-dependent kinases and regulates cell cycle progression (35, 36).Thus, we analyzed the expression of the cell cycle regulators Cyclin D1, Cyclin D2, and pro-proliferative transcription factor MYC in dermal Fb after OTUD6B-AS1 knockdown. Cyclin D1 expression was significantly upregulated at mRNA level and protein level, respectively, 48 and 72 h after transfection (**Figure 5A**, **Supplementary Figure 5**). Cyclin D2 and MYC expression was unchanged both at the mRNA and protein level (**Supplementary Figures 6A–D**). We also analyzed additional pro-fibrotic and/or proinflammatory cytokines such as IL-6 and IGFBP3, and other cytokines that act on Fb to promote fibrotic responses, such as TGFB1 (37). However, after OTUD6B-AS1 knockdown IL-6, TGFB1, and IGFBP3 expression was unchanged (**Supplementary Figure 7**). Upregulation of Cyclin D1 was confirmed in an independent set of experiments using different two different anti-sense oligonucleotides targeting OTUD6B-AS1 (**Supplementary Figure 8A**).

As observed in dermal Fb, Cyclin D1 expression was significantly upregulated 48 h after OTUD6B-AS1 knockdown at the mRNA and at the protein level in HPASMC (**Figure 5B**, **Supplementary Figure 8B**). Cyclin D2 expression at the mRNA level was slightly upregulated, while MYC expression was unchanged (**Supplementary Figure 9**).

In addition, we analyzed the expression of the transcription factor E2F1, which is located downstream of Cyclin D1 and has been suggested as an apoptosis repressor in this context (38). However, we could not detect a consistent upregulation of the expression of E2F1, neither on the mRNA nor on the protein level in dermal Fb (**Supplementary Figures 10A,B**). These data suggest that OTUD6B-AS1 knockdown reduces cell proliferation and suppresses apoptosis in dermal Fb likely independent from E2F1.

Similarly, we did not detect any difference of E2F1 expression at the mRNA level and protein level 48 h after transfection in HPASMC (**Supplementary Figures 10C,D**).

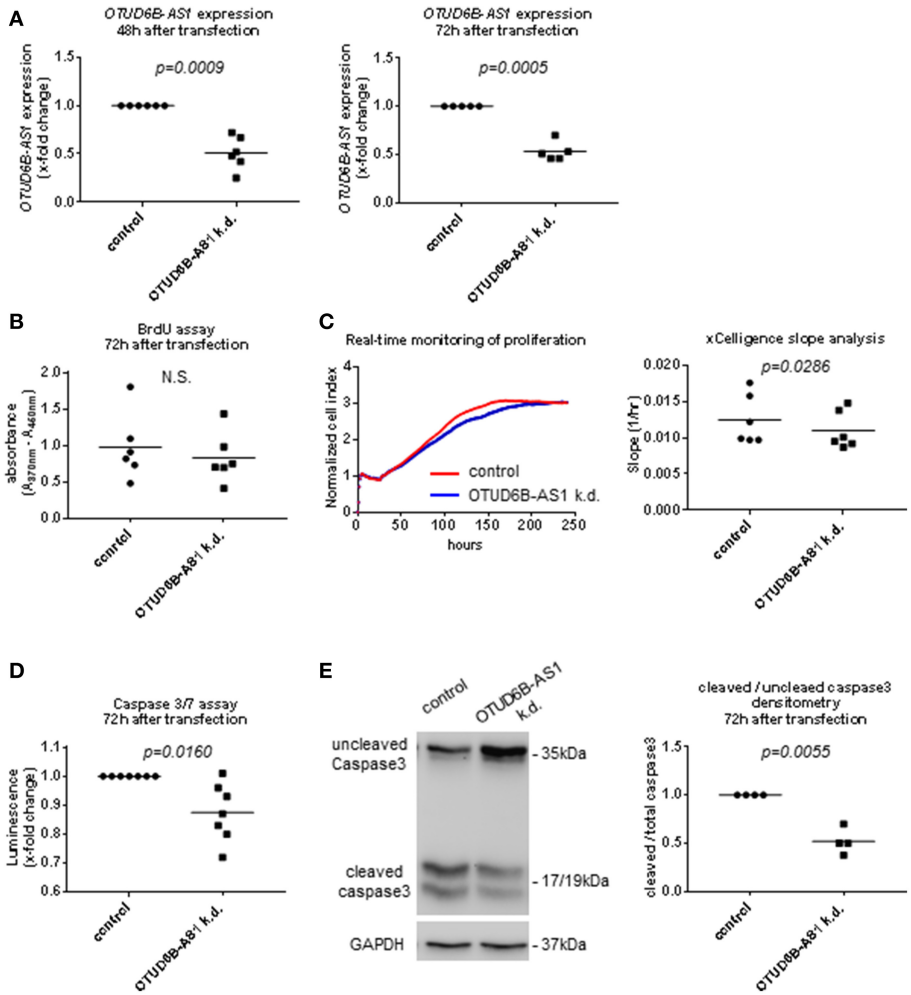

FIGURE 3 | OTUD6B-AS1 knockdown in dermal fibroblasts (Fb): effects on proliferation and apoptosis. HC dermal Fb were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting OTUD6B-AS1. **(A)** Efficacy of OTUD6B-AS1 knockdown 48 and 72 h after transfection in dermal Fb was confirmed by qPCR ($n = 5$–6). **(B)** BrdU cell proliferation assay performed 72 h after transfection ($n = 6$). **(C)** Real-time monitoring of cell proliferation analysis. Cell index (CI) was monitored every 5 min from 0 to 13 h, every 15 min from 13 to 25 h and every 30 min from 25 h until it reached plateau. CI was normalized at the time of transfection. Slope analysis was performed by RTCA software 2.0 (ACEA Biosciences) ($n = 6$, representative picture is shown). **(D)** Caspase 3/7 assay performed 72 h after transfection. Cells were treated by 1 μM staurosporin (STP, Sigma) for a period of 16 h to induce apoptosis ($n = 7$). **(E)** Western blot analysis of uncleaved and cleaved caspase 3 was performed 72 h after transfection. Cells were treated with 1 μM staurosporin (STP, Sigma) for a period of 16 h to induce apoptosis. GAPDH was used as a loading control. Densitometry analysis of cleaved caspase 3 was normalized to uncleaved caspase 3 ($n = 4$). Data are shown as single values and mean. Statistical analysis was performed by paired *t*-test. k.d, knockdown.

All together, these results demonstrate that OTUD6B-AS1 targets Cyclin D1 and therefore it might be involved in cell proliferation and cell cycle regulation.

OTUD6B-AS1 Knockdown Does Not Affect OTUD6B Expression

Regulation of corresponding sense gene expression is the most common mechanism of action of antisense transcripts (12–14, 39). Therefore, we analyzed mRNA expression and protein expression of OTUD6B after OTUD6B-AS1 knockdown. Total OTUD6B expression at the mRNA and protein level was unchanged after OTUD6B-AS1 knockdown in dermal Fb (**Figure 6A**, **Supplementary Figure 8A** and **Supplementary Figure 11A,B**). Similarly, in HPASMC, OTUD6B expression after OTUD6B-AS1 knockdown was not changed (**Figure 6B**, **Supplementary Figure 8B** and **Supplementary Figure 11C,D**).

During processing, lncRNAs are transported into the cytoplasm or remain in the nucleus (8, 40) to exert their function. Thus, the location of OTUD6B-AS1 can further give hints about its mechanism of action. By using cell fractionation experiments, we found that OTUD6B-AS1 was mainly localized in the nucleus of dermal SSc Fb (**Figure 6C**).

Overall, these data indicate that OTUD6B-AS1 knockdown does not affect expression of its sense gene OTUD6B in dermal Fb and HPASMC. The effects on target gene Cyclin D1, apoptosis, and proliferation are likely to occur in the nucleus and might be due to OTUD6B-AS1

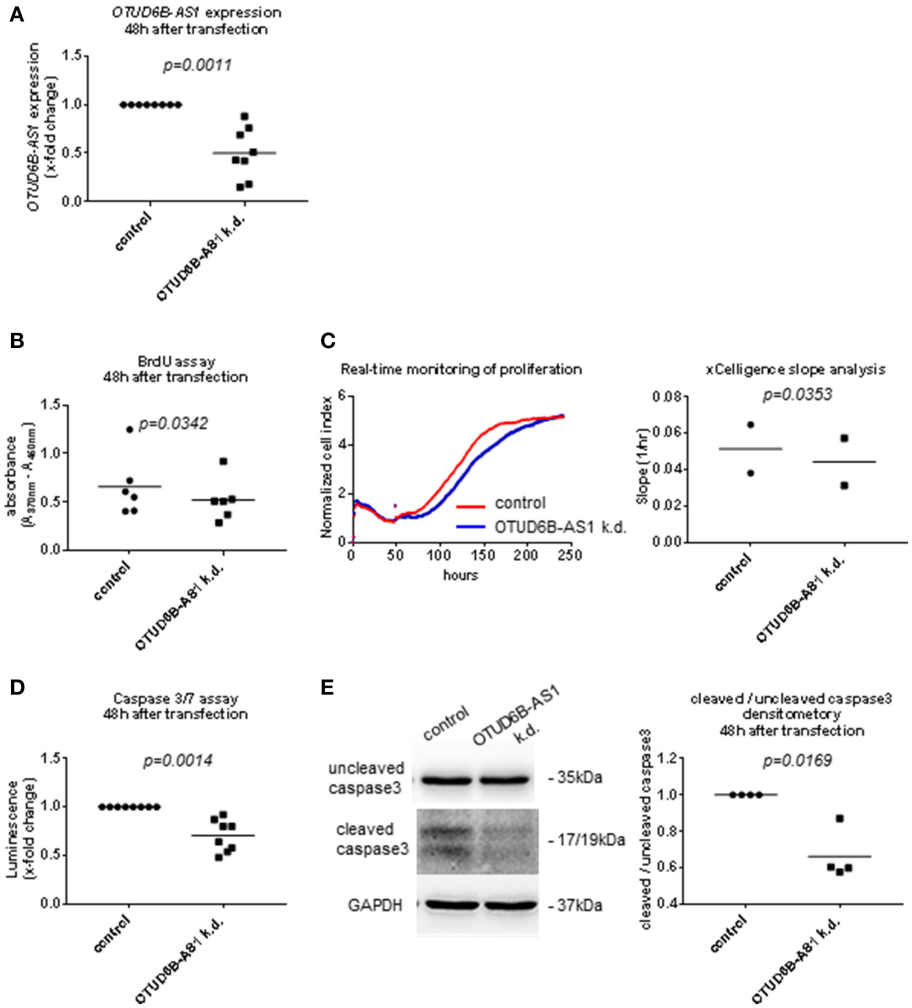

FIGURE 4 | OTUD6B-AS1 knockdown in human pulmonary artery smooth muscle cells (HPASMC): effects on proliferation and apoptosis. HPASMC were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting OTUD6B-AS1. **(A)** Efficacy of OTUD6B-AS1 knockdown 48 h after transfection in HPASMC was confirmed by qPCR. **(B)** BrdU cell proliferation assay performed 48 h after transfection ($n = 6$, biological replicates). **(C)** Real-time monitoring of cell proliferation. Cell index (CI) was monitored every 5 min from 0 to 13 h, every 15 min from 13 to 25 h and every 30 min from 25 h until it reached plateau. CI was normalized at the time of transfection. Slope analysis was performed by RTCA software 2.0 (ACEA Biosciences, $n = 2$, biological replicates, a representative picture is shown). **(D)** Caspase 3/7 assay performed 48 h after transfection. Cells were treated by 1 μM staurosporin for a period of 16 h to induce apoptosis ($n = 8$, technical replicates). **(E)** Western blot analysis of uncleaved and cleaved caspase 3 was performed 48 h after transfection. Cells were treated with 1 μM staurosporin (STP, Sigma) for a period of 16 h to induce apoptosis before cell collection. The same nitrocellulose membrane was used to detect uncleaved caspase 3 and cleaved caspase 3 with different exposure time for detection. GAPDH was used as a loading control. Densitometry analysis of cleaved caspase 3 was normalized to uncleaved caspase 3 (n=4, biological replicates). Data are shown as single values and mean. Statistical analysis was performed by paired t-test. k.d, knockdown.

trans function rather than influencing OTUD6B sense gene expression.

DISCUSSION

AS lncRNAs have been identified as crucial players in the pathogenesis of different diseases such as cancer, however little is known in fibrotic disorders including SSc. In this study, we investigated the role of OTUD6B-AS1 in the pathogenesis of SSc.

First, our additional analyses on the expression pattern showed that the downregulation of OTUD6B-AS1 and OTUD6B mRNA was seen particularly in clinically involved, fibrotic skin biopsies of SSc patients. This finding might give hints on their pathophysiological role. However, the cohorts studied in these experiments were rather limited in patient numbers and thus, our findings should be confirmed in additional, larger cohorts. In addition, due to the limited availability of protein from skin biopsies, the downregulation of OTUD6B should be confirmed by Western blots from biopsies of involved skin in additional studies. We then analyzed the effects of pro-fibrotic and pro-inflammatory cytokines on the expression of OTUD6B-AS1 and OTUD6B. Pro-inflammatory cytokines are playing a major role

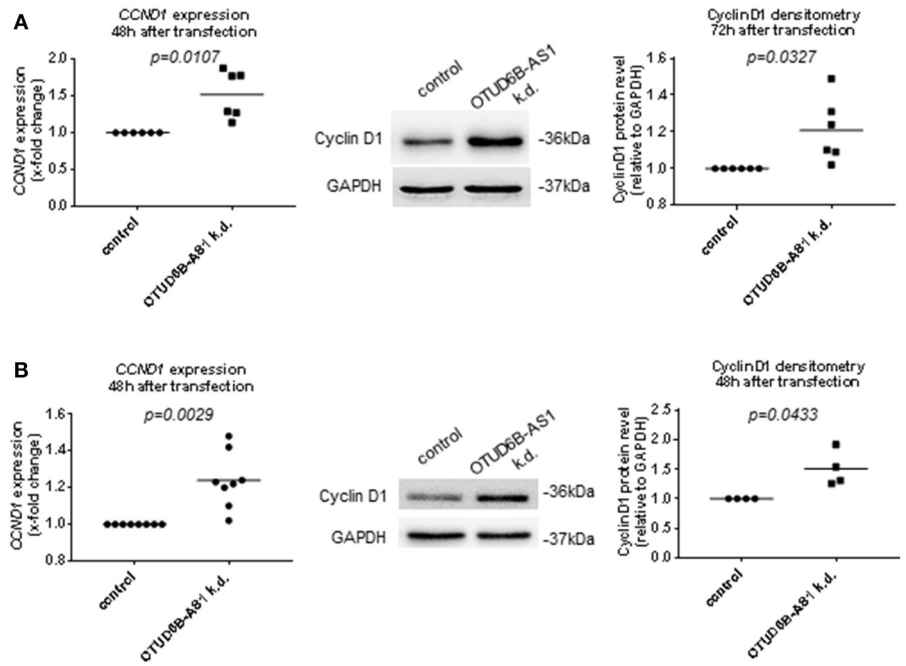

FIGURE 5 | Cyclin D1 expression after OTUD6B-AS1 knockdown in dermal fibroblasts (Fb) or human pulmonary artery smooth muscle cells (HPASMC). HC dermal Fb or HPASMC were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or ASO targeting OTUD6B-AS1. **(A)** mRNA level of cyclin D1 (CCND1) expression was measured 48 h after transfection ($n = 6$) and Cyclin D1 protein was measured 72 h after transfection ($n = 6$) in dermal Fb by Western blot. **(B)** mRNA level of cyclin D1 (CCND1) expression ($n = 8$, biological replicates) and Cyclin D1 protein ($n = 4$, biological replicates) was measured 48 h after transfection in HPASMC by Western blot. mRNA expression was normalized by GAPDH and RPLP0 and protein expression was normalized by GAPDH. Data are shown as single values and mean. Statistical analysis was performed by paired *t*-test. k.d, knockdown.

in the pathogenesis of SSc, in particular in early, but also in later stages of the disease (41). We could show that the expression of OTUD6B-AS1 and OTUD6B was significantly downregulated after PDGF simulation in dermal Fb. PDGF plays an important role in fibrosis and regulation of inflammation. PDGFs are primary mitogens and chemo-attractants for mesenchymal cells and they are secreted from platelets, macrophages, fibroblasts, and endothelial cells (1). PDGF and PDGF receptor β are highly expressed in SSc skin (42). Activated microvascular pericytes express more PDGFβ receptors in early SSc patients than normal control or late-stage SSc patients (43). Transgenic mice with constitutively activated PDGF receptor α develop multiple organ fibrosis, including skin fibrosis (44). Our data indicated that PDGF is an important regulator of OTUD6B-AS1 and OTUD6B expression in dermal Fb.

In order to identify the functional role of OTUD6B-AS1, we performed knockdown experiments of OTUD6B-AS1 using ASO in HC dermal Fb and HPASMC. Dermal Fb from SSc patients display increased proliferation in culture (45), and it has been demonstrated that proliferation of vascular smooth muscle cells is a key feature of the proliferative microangiopathy characteristic for the vascular manifestations of SSc (46). Cyclin D1 was identified as an OTUD6B-AS1 target in both dermal Fb and HPASMC. Cyclin D1 is a well-characterized cell cycle regulator and oncogene (36, 47, 48). Surprisingly, after OTUD6B-AS1 knockdown, we observed increased cyclin D1 expression, but reduced cell proliferation. Cyclin D1 expression levels are subjected to change throughout the cell cycle. Specifically, a high level of cyclin D1 expression is required for G1 phase. However, cyclin D1 decreases during the S phase for efficient DNA synthesis and increase again in G2 phase to continue proliferation (49, 50). This phenomenon was observed in different cell types including fibroblasts (50). Moreover, Cyclin D1 also has an inhibitory function on DNA synthesis via binding with proliferation cell nuclear antigen (PCNA) (51). Overexpression of cyclin D1 in human Fb prevents DNA repair and prevents cells from entering in S phase (52). This mechanism could explain the reduction in proliferation that we observed in dermal Fb and HPASMC after OTUD6B-AS1 knockdown. Thus, while the OTUD6B-AS1 knockdown favored apoptosis resistance (see below), which is supporting the pathogenesis of SSc, an inhibition of proliferation would have to be interpreted as a compensatory mechanisms. We did use HC cells for these experiments to better mimic the downregulation found in SSc skin. In further studies, these effects should be confirmed in primary SSc cells, to exclude influence of SSc specific pathway alterations on our results.

We showed that OTUD6B-AS1 knockdown suppresses apoptosis in dermal Fb and HPASMC. Fibrosis is a predominant phenomenon in SSc. Fibrotic tissue is characterized by apoptosis resistant myofibroblasts that arise from resident fibroblasts and other cellular sources (3–5). The mechanisms of the resistance to apoptosis are largely unknown. We have identified regulation of cyclin D1 by OTUD6B-AS1 in dermal Fb as a possible novel contributor to apoptosis resistance in SSc. Accordingly, it has

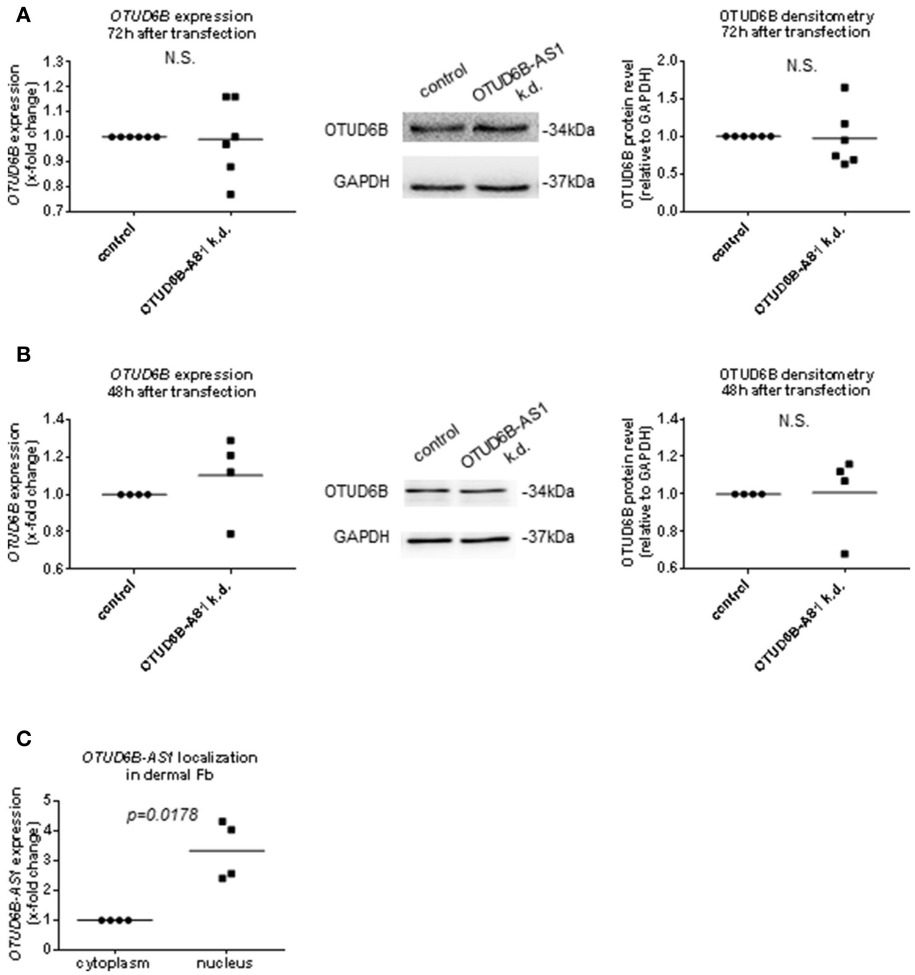

FIGURE 6 | OTUD6B expression after OTUD6B-AS1 knockdown in dermal fibroblasts (Fb, **A**) or human pulmonary artery smooth muscle cells (HPASMC, **B**). HC dermal Fb or HPASMC were transfected with locked nucleic acid antisense oligonucleotide (ASO) negative control or with ASO targeting OTUD6B-AS1. **(A)** mRNA of total OTUD6B was measured 72 h after transfection ($n = 5$). OTUD6B protein was measured 72 h after transfection ($n = 6$) in dermal Fb by Western blot. **(B)** mRNA of total OTUD6B was measured 48 h after transfection ($n = 4$, biological replicates). OTUD6B protein expression was measured 48 h after transfection by Western blot ($n = 4$, biological replicates) in HPASMC. mRNA expression was normalized by GAPDH and RPLP0 and protein expression was normalized by GAPDH. **(C)** The subcellular localization of OTUD6B-AS1 in SSc dermal Fb was analyzed by qPCR ($n = 4$). Expression levels were normalized by GAPDH and RPLP0. Data are shown as single values and mean. Statistical analysis was performed by paired *t*-test. k.d, knockdown.

been reported that D-type cyclins (D1, D2, and D3) can repress apoptosis in hematopoietic cells. Previous studies showed that this effect of cyclins could be mediated via the transcription factor E2F1, which in turn regulates the expression of Fas and Fas ligand (38). However, we could not detect consistent changes in the expression of E2F1 after knockdown of OTUD6B-AS1. With our experimental set-up, we cannot completely exclude that there are subtle changes of E2F1 and it downstream targets Fas and Fas ligand at other time points, but other, E2F1 independent mechanisms are much more likely. Knockdown of cyclin D1 could provide further insight into downstream targets mediating the apoptosis resistance in SSc fibroblasts.

We also investigated the relationship between the expression of OTUD6B-AS1 and sense gene OTUD6B. Our data showed that PDGF stimulation downregulated the expression of both OTUD6B-AS1 and OTUD6B, initially pointing to linked mechanisms of action. However, while the majority of anti-sense genes mediate their effects via direct interaction with the sense gene, we did not detect any change of the expression of OTUD6B on the RNA and protein level after OTUD6B-AS1 knockdown. Recent investigations showed that in some circumstances, the mature AS transcript does not influence sense gene expression and the AS transcription itself is required for sense gene expression (14, 53). This means, silencing AS transcript using ASO does not necessarily affect sense gene expression. In our experiments, we used ASO to reduce mature OTUD6B-AS1 levels and this might explain why OTUD6B expression was not affected. On the other hand, our data suggest that OTUD6B-AS1 has an independent mechanism from its sense gene.

Taken together, we provide the first evidence for a functional role of OTUD6B-AS1 in two cell types that play a major role in SSc. We showed that the expression of OTUD6B-AS1 and OTUD6B was significantly downregulated in dermal Fb after PDGF stimulation. OTUD6B-AS1 knockdown experiments

revealed that OTUD6B-AS1 controls proliferation and apoptosis of dermal Fb and HPASMC through the regulation of cyclin D1 expression. The OTUD6B-AS1 mode of action was independent of its sense gene OTUD6B. These results suggest that OTUD6B-AS1 downregulation might promote apoptosis resistance of Fb and HPASMC contributing to cell dysregulation on the pathophysiology of SSc.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of World Medical Association Declaration of Helsinki, ICH-GCP guidelines, or ISO 14155. The study was approved by the Ethics Committee of the Canton of Zurich (approved ethical applications KEK-ZH 515, PB-2016-02014, and KEK-Nr. 2018-01873). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MT, EP, and OD: study conception and design. MT, EP, MF-B, AK, MW, SA, MC, TM, JdV-B, and FK: acquisition of data. MT, EP, MF-B, AJ, TM, JdV-B, TH, FK, GK, and OD: analysis and interpretation of data. MT, EP, and OD: drafting and revising the article. All authors have seen and approved the manuscript and its content and are aware of the responsibilities connected to authorship.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01100/full#supplementary-material

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