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1 **Title: Bi-directional communication: conversations between fibroblasts and**
2 **immune cells in systemic sclerosis**

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18 **Highlights**

- 19 • Systemic sclerosis is an idiopathic autoimmune disease, therapies are limited
- 20 • Dysregulated T and B cell cytokines promote myofibroblast activation and
- 21 fibrosis
- 22 • Communication between fibroblasts and immune cells alters immune cell
- 23 phenotype
- 24 • Fibroblasts drive polarization, survival, and differentiation of T cells
- 25 • Targeting both immune and stromal cells may be of therapeutic benefit in SSc
- 26

Abstract

Systemic Sclerosis (SSc) is an autoimmune idiopathic connective tissue disease, characterized by aberrant fibro-proliferative and inflammatory responses, causing fibrosis of multiple organs. In recent years the interactions between innate and adaptive immune cells with resident fibroblasts have been uncovered. Cross-talk between immune and stromal cells mediates activation of stromal cells to myofibroblasts; key cells in the pathophysiology of fibrosis. These cells and their cytokines appear to mediate their effects in both a paracrine and autocrine fashion. This review examines the role of innate and adaptive immune cells in SSc, focusing on recent advances that have illuminated our understanding of ongoing bi-directional communication between immune and stromal cells. Finally, we appraise current and future therapies and how these may be useful in a disease that currently has no specific disease modifying treatment.

Keywords: Fibroblast, cytokine, systemic sclerosis, CD4+T cell, B cell, adaptive immunity, cross-talk, fibrosis.

Introduction

Systemic sclerosis (SSc) is an autoimmune multi-organ connective tissue disease, the cause of which is currently unknown. SSc is characterised by the presence of autoantibodies, vasculopathy, inflammation and fibrosis. The disease can be broadly classified into two subtypes: limited (lcSSc) and cutaneous diffuse disease (dcSSc) [1].

The extent of skin fibrosis is the differentiating feature. dcSSc involves rapidly progressive fibrosis of the skin, lungs, and other internal organs while in lcSSc, vascular manifestations are a prominent feature and are in general accompanied by mild skin and internal organ fibrosis. Lung involvement (pulmonary hypertension and pulmonary fibrosis) significantly contributes to morbidity and mortality in SSc [2]. The disease is more prevalent in women [3], in line with all autoimmune diseases. At present there are no treatments for SSc that are effective across organ systems.

Elevated levels of immune cell derived cytokines (IL-1, IL-4, IL-6, IL-13, IL-17, IL-33, TNF- α , CCL2) have been detected in the serum of SSc patients [4-7]. Infiltrating mononuclear cells detected in the dermis of SSc patients, consisting of lymphocytes, plasma cells and macrophages have all been implicated in the development of the fibrotic milieu [8]. Aberrant immune activation is thought to precede the activation and expansion of resident fibroblast populations by pro-inflammatory and pro-fibrotic cytokines [9, 10].

Cross-talk between immune cells and stromal fibroblasts has long been considered as a major driver of SSc disease pathogenesis and progression. Cellular cross-talk can occur in a paracrine manner *e.g.* through the release of cytokines or be facilitated by direct cell-cell contact. T cells play an early role in endothelial dysfunction and contribute to the activation of macrophages and fibroblasts/myofibroblasts *via* secretion of cytokines [9]. SSc fibroblasts secrete elevated levels of extracellular matrix components;

collagens, glycosaminoglycans (GAGs) and fibronectin, promoting scar formation and dermal thickening [11, 12]. Fibroblast activation and differentiation into an alpha-smooth muscle actin (α SMA) expressing myofibroblasts promotes fibrosis [13, 14]. Sustained accumulation of activated fibroblasts accompanied by dysregulated tissue repair results in pathological deposition of ECM and progressive tissue stiffening, ultimately leading to loss of organ function.

The innate and adaptive immune system are both accepted to play a role in SSc pathogenesis [15]. It is impossible to completely un-tangle these contributions due to the complex interplay between various cell types throughout the course of disease. We summarize the evidence highlighting the importance of bi-directional communication between immune and stromal cells in the context of systemic sclerosis. Particular emphasis is placed on the contributions of cytokine producing T and B cell subsets to fibroblast phenotype and function. An improved understanding of how changes in the adaptive immune system can influence stromal cell mediated tissue remodelling and *vice versa*, will lead to improved therapeutic targeting and drug development.

Pathogenesis of SSc

The pathogenesis of systemic sclerosis in the skin can be broadly categorised into three basic pathological processes which occur in the following order, (1) vasculopathy (2) inflammation/immune activation and (3) fibrosis. The onset of these changes is characterised by initial changes in dermal microcirculation causing damage to the endothelium [9]. This is followed by the induction of progressive inflammation *via* activation of the innate and subsequently the adaptive immune system [16]. Resident fibroblasts are activated by pro-fibrotic cytokines *e.g.* transforming growth factor- β (TGF- β), platelet derived growth factor (PDGF) and connective tissue growth factor

(CTGF) released by activated immune cells. Following activation, these fibroblasts produce exuberant levels of extracellular-matrix (ECM) proteins, including type 1 collagens and inhibitors of matrix metalloproteinases. This leads to aberrant tissue remodelling, culminating in dermal thickening and tissue fibrosis.

Interstitial lung disease (ILD) is a frequent complication of SSc, this is usually progressive and has a poor prognosis [17]. SSc-ILD is characterized by a pattern of lung injury termed non-specific interstitial pneumonia (NSIP) [18]. Histologically, this presents as varying degrees of pulmonary inflammation and fibrosis, some forms consist entirely of inflammation (cellular-NSIP) and some are primarily fibrotic (fibrotic-NSIP). Patients with the fibrotic form of NSIP have worse outcomes [19]. SSc-ILD pathogenesis involves abnormal interactions between endothelial cells, lymphocytes/monocytes and fibroblasts. Ultimately this leads to excessive ECM production by fibroblasts promoting tissue hypoxia and vascular hyperreactivity [20]. Inflammatory infiltrate in the SSc lung consists predominantly of infiltrating B cells [21]. The majority of patients with SSc-associated ILD are positive for anti-nuclear antibodies (ANAs) that are frequently accompanied by anti-Scl-70, anti-topoisomerase-1 antibodies or anti-fibroblast antibodies [22-24]. Anti-centromere antibodies are more commonly found in patients with the limited form of disease [25].

Animal models of SSc: Pathogenesis and Cellular Cross-talk

Mouse models of SSc have been used to study various aspects of disease that otherwise could not be studied at length within a human population. In mice, models are either inducible *e.g.* induced *via* immunization, introduction of autoimmune factors or chemicals (Bleomycin, Topoisomerase-1) or occur as spontaneous genetic mutations, (tight-skin (Tsk/+), Fos-related antigen 2 (Fra-2-Tg) and Friend leukaemia integration

1 transcription factor (Fli1^{-/-}). The importance of cross-talk between human SSc fibroblasts and T and B cells has been previously established [6, 26]. The following disease models have been selected as examples of communication between immune and stromal cells.

Bleomycin (BLM) induced fibrosis

Subcutaneous administration of bleomycin for 4 weeks induces the development of inflammatory infiltrates and fibrosis in the affected skin and dermal sclerosis can persist for up to 6 weeks [27]. Bleomycin injections produce reactive oxygen species (ROS) causing damage to the skin [28]. The affected skin has increased infiltration of polymorphonuclear cells, T and B lymphocytes, macrophages, eosinophils, and mast cells. Inflammatory infiltrates release elevated amounts of pro-fibrotic and pro-inflammatory cytokines (TGF- β , PDGF, CCL2, IL-4, IL-6, and IL-13), driving the production of excessive amounts of ECM components and leading to cutaneous fibrosis [29-31].

Many of these cytokines are also up-regulated in BLM-induced pulmonary fibrosis. Intratracheal administration of bleomycin commonly induces an acute pneumonitis with inflammatory cells aggregating in the pulmonary interstitium, followed by aberrant collagen production [32]. The inflammatory phase of this model subsides after day 3-5 while fibrosis occurs between days 14-28, however fibrosis begins to spontaneously resolve after 28 days. T and B cells are present within the inflammatory infiltrate of lesional skin induced by bleomycin administration. Genetic inactivation of CD19 leads to a reduction in skin and lung fibrosis in the bleomycin model [33]. Depletion of T cells using anti-CD3 monoclonal antibodies reduced ECM production and fibrosis [34]. Following BLM treatment dermal fibroblasts in lesional skin

expressed CD40, interactions with CD40 and its ligand CD40L is a potential pathway for immune and inflammatory responses [35]. Anti-CD40L antibody treatment inhibited induction of skin fibrosis *via* suppression of fibroblast proliferation and CCL2 production. Indeed abatacept, which blocks the co-stimulatory pathway engagement between antigen presenting cells (APC) and T cells, reduced dermal fibrosis in the bleomycin model of SSc [36]. Interferon Regulatory factor 5 (IRF5) knockout mice are also protected from bleomycin induced skin fibrosis [37].

TSK1 mice

This is a genetic model of SSc characterised by increased hypodermal thickening due to a spontaneous tandem duplication in the fibrillin-1 (Fbn1) gene [38]. This mutation is homozygous lethal so mice are maintained as heterozygotes (Tsk1/+). The Tsk1/+ model is predominantly associated with dermal fibrosis and excessive deposition of ECM. Pathways activated by mutant fibrillin in Tsk1/+ mice resemble those that are activated in human disease *e.g.* TGF- β signalling, canonical Wnt signalling and AP-1 activation [39-41]. B cell activation and autoantibody formation is accompanied by an emphysema-like lung pathology in the Tsk1/+ mouse model [42]. There are no inflammatory infiltrates in the skin and vascular injury is absent in Tsk1/+ mice [42]. Tsk1/+ mice overexpressing CD19 have significantly elevated levels of anti-topoisomerase-1 with no evidence of dermal thickening, while B cell antigen-receptor cross-linking augmented the activation of extracellular signalling regulated kinase in Tsk1/+ cells [33]. Administration of an anti-CD20 (Rituximab) antibody, to deplete B cells, reduced dermal fibrosis and autoantibody production in new-born Tsk1/+ mice (3 days) [43]. This effect was not observed using older mice

(56 days). Taken together these findings suggest that depletion of B cells reduces fibrosis and ECM deposition as opposed to the earlier inflammatory phase of SSc. CD4⁺ and CD8⁺ T cells exert discrete pathological effects in the Tsk1/+ model. CD4⁺ T cells regulate dermal accumulation of collagen in TSK1/+ mice while in the absence of CD8⁺ T cells levels of serum anti-topoisomerase are significantly reduced [44]. Adoptive transfer of purified B and T lymphocytes from Tsk1/+ mice into normal syngeneic mice, increased collagen production and autoimmune phenotypes in recipient mice [45].

TSK2 mice

Tsk2/+ mice have a mutation in the collagen type III alpha 1 (Col3a1) gene [46]. As this mutation is homozygous lethal mice these mice are maintained as heterozygotes (Tsk2/+). In comparison to the Tsk1/+ mouse, the Tsk2/+ model displays a more generalised fibrotic phenotype. The Tsk2/+ phenotype is associated with many pathological features of human SSc, such as inflammatory changes to the skin, abnormal ECM production, fibrosis and anti-nuclear antibodies [46, 47].

Fra-2 Transgenic (TG) mouse

Ectopic overexpression of Fra-2 (Fra-2 Tg model) causes fibrosis in multiple organs, resulting in systemic inflammation and inflammatory infiltrates in the skin and the lung [48]. The precise mechanism by which fibrosis arises in this model has been disputed, arising either from vascular remodelling mediated by hyperproliferative myofibroblasts and smooth muscle cells (SMCs) or *via* repeated alveolar epithelial injury/insult. Fra-2 expression is elevated in SSc patients and is crucial for the release of collagen by dermal fibroblasts [49]. Fra-2 activates pulmonary remodelling and inflammatory pathways *via*

induction of chemokines from the C-C motif chemokine ligand (CCL) and the C-X-C motif chemokine ligand (CXCL) families. These chemokines then drive the recruitment of inflammatory cells to the lungs of Fra-2 Tg mice. Elevated numbers of cytokine producing CD4⁺ and CD8⁺ lymphocytes and B cells are present in the lungs Fra-2 overexpressing mice [50]. The differentiation and proliferation of B cells is mediated *via* induction of Foxo1 and Irf4 [51]. Declining lung function in these mice strongly correlates with the influx of inflammatory cells in bronchoalveolar lavage fluid (BALF) [52]. Elevated levels of Th2 cytokines IL-4, IL-13, IL-5 and eotaxin are also present. Fra-2 mice treated with Abatacept displayed decreased T cell proliferation, reduced expression of fibrogenic markers (CCL2 and TGF β) and decreased lung vascular remodelling [53].

Fli-1 Models

Fli-1 is a member of the ETS family of transcription factors, expression of Fli-1 is downregulated in human dermal SSc fibroblasts, endothelial cells and perivascular inflammatory cells [54]. Fli-1 acts as a suppressor of the Col1 gene [55]. Fli1^{-/-} deficient mice develop skin fibrosis characterised by increased fibrillar collagen content and dysregulated fibril size and organisation. Haploinsufficiency of Fli-1 (Fli1^{+/+}) potentiates the response to bleomycin, with increased numbers of α -SMA⁺ myofibroblasts accompanied by elevated levels of IL-1 β , IL-6 and TGF- β [56]. Mice that are heterozygous for both Kruppel-like factor-5 (Klf5) and Fli-1 develop spontaneous dermal fibrosis with characteristic dermal thickening and increased collagen content [57]. These mice display fibrosis and vasculopathy of the skin and lungs in addition to B cell activation and autoantibody production. Perivascular B-cell accumulation eventually progresses to prominent B-cell lymphoid aggregates [57].

Fli1/+ dermal fibroblasts amplify aberrant immune responses and promote the trans-differentiation of Tregs into Th2 like cells [56]. These changes are mediated by IL-33 expression in lesional skin. Taken together these findings point to Fli-1 a crucial regulator of the interactions between dermal fibroblasts and immune cells in pathological skin fibrosis.

All the models discussed above involve aberrant ECM production and progressive fibrosis, with substantial contributions from fibroblasts, T and B cells. Despite being initiated by different stimuli *e.g.* spontaneous genetic mutations in ECM proteins and transcription factors, immunizations or chemical induction of ROS, these models reinforce the importance of cellular cross-talk and cytokine production in driving SSc pathogenesis.

Innate immunity in SSc

Innate immune cells are essential for host defence and tissue homeostasis. Myeloid cells (granulocytes, dendritic cells, monocytes and macrophages) promote an inflammatory phenotype through increased Toll-like receptor (TLR) signalling and interferon (IFN) pathways (type-1 interferons, IFN α /IFN β) [58, 59]. Newer players in innate immunity include platelet-derived danger-associated molecular patterns (DAMP), innate lymphoid cells, plasmacytoid dendritic cells (pDCs) and pathogen-recognition receptors (PRR). Activation of TLRs on dendritic cells, monocyte/macrophages and B cells stimulate inflammatory cytokines, antigen presentation and development of the adaptive immune response. Aberrant activation of numerous members of the TLR signalling family of receptors has been well documented in SSc [59]. TLRs themselves are expressed on fibroblasts, albeit to a lower degree of expression, and can themselves

promotes fibrosis [16]. Recent findings involving innate immune cells in SSc are summarised in **Table 1**.

274 **Table 1: Recent studies highlighting the contributions of selected innate immune**
275 **cells to SSc pathogenesis**
276

Cell type	Role in Systemic Sclerosis Pathogenesis	References
Platelets	High mobility box-group protein-1 positive microparticle products trigger fibrosis in SSc <i>via</i> damage to the endothelium	[60]
Neutrophils	Autophagic neutrophils detected in the blood of SSc patients are accompanied by neutrophil extracellular trap (NET) by-products Higher neutrophil count predicts more extensive skin/lung involvement and higher mortality in SSc	[60] [61]
Innate lymphoid cells (ILCs)	Elevated ILC2 numbers correlated with increased mRSS score and decline of lung function in SSc ILC3s release elevated cytokine levels in the fibrotic skin of SSc patients	[62] [63]
Dendritic cells (DCs)	cDC2s spontaneously produce more CXCL10 <i>ex vivo</i> compared to healthy individuals while dcSSc cDC2s produce more CCL4 A population of inflammatory dendritic cells (InfDC, CD14+CD163+) was identified in SSc patients	[64] [65]
Plasmacytoid DCs (pDCs)	pDCs can infiltrate the skin of SSc patients and exist in a chronically activated state due to dysregulated TLR8 signalling RUNX3 is downregulated in SSc pDCs and deletion of RUNX3 in DCs leads to spontaneous induction of skin fibrosis and worsens the response to BLM	[66] [67]
Monocytes	Circulating monocytes in SSc have an M1/M2 phenotype that is predictive of lung involvement Monocyte-derived macrophages from SSc patients are enriched for genes involved in glycolytic metabolism linked with pro-inflammatory activation Numbers of total circulating monocytes are elevated in SSc (expansion of IL-6 producing intermediate and non-classical monocytes)	[68] [69] [70]
Macrophages	Circulating cells co-express M1 and M2 macrophage surface markers in SSc patients Adoptive transfer of bone marrow derived macrophages treated with low dose LPS slowed progression of skin thickening in mice injected with HOCl LPSlow-macrophages dampen fibro-inflammatory phenotype of diseased human SSc fibroblasts <i>in vitro</i> SSc macrophages express markers associated with both alternative (CD163, CD206 surface expression and secrete TGF- β and CCL2) and inflammatory macrophage activation (HLA-DR surface expression and secrete IL-6)	[68, 71] [72] [72] [73]

Fibroblasts: Innate responders and adaptive sentinels

Fibroblasts regulate the behaviour, retention and survival of immune cells. They can promote recruitment of circulating immune cells to sites of tissue injury *via* the release of chemokines or inhibit the recruitment of circulating immune cells. Emerging evidence indicates fibroblasts can express numerous functionally active TLRs (TLR-1--TLR-9); synthesize antimicrobial peptides/defensins and secrete multiple pro-inflammatory cytokines [74, 75]. Activation of TLR signalling in SSc fibroblasts potentiates collagen production and ECM deposition [76, 77], TLR-4-dependent fibroblast activation promotes lung and skin fibrosis *in vivo* [78]. TLR ligands are generated from matrix molecules in response to/during tissue injury. Studies by Varga and colleagues highlighted an imbalance in DAMP release and/or PRR signalling leads fibroblasts and macrophages to release sustained levels of inflammatory cytokines and fibrosis [78, 79]. Conversely, hepatocyte growth factor (HGF) and fibroblast growth factor (FGF-1 and FGF-2) secretion by fibroblasts counteracts lung fibrosis *in vivo*, with the latter controlling TGF β R1 expression and degradation [80].

A recent study by Bhandari *et al.*, showed that the activation profile of SSc macrophages is pro-fibrotic and co-culture with dermal fibroblasts drove activation and myofibroblast transition [73]. It has been proposed that myofibroblasts can impact macrophage activation through IL-6 secretion and stimulation of STAT3 and Akt pathways [81]. Myofibroblasts produce various cytokines and chemokines to aid in the recruitment and facilitate the function of innate immune cells [82] Myofibroblasts secrete IL-8 and CCL2, chemoattractant for neutrophils. Neutrophil extracellular traps (NET) promote fibroblast proliferation and differentiation *in vitro* driving ECM production [83]. Interactions between fibroblasts and innate immune cells often result in the amplification of ECM synthesis.

Fibroblasts can bridge the gap between innate and adaptive immune responses through expression of CD40 and other co-stimulatory molecules, in addition to producing cytokines that polarise T cells. CD40 is expressed by dermal and pulmonary fibroblasts and is up-regulated in SSc [84]. Fibroblast CD40 can co-stimulate T lymphocyte proliferation, facilitating interactions between fibroblasts and T cells in skin [84]. Generally, CD40-CD40L interaction facilitates cross-talk between fibroblasts and immune cells, while engagement of CD40 on fibroblasts causes upregulation of ICAM-1 and VCAM-1 [85]. The CD40/CD40L pathway can promote fibrogenesis by activating both fibroblasts and immune cells. Fli1^{+/-} fibroblasts promote Th2 and Th17-like differentiation of CD4⁺Foxp3⁺ Tregs *in vitro* during co-culture assays, through the release of both IL-33 and IL-6 [56]. Fli-1 was found to bind the promoter regions of both IL-33 and IL-6 in human dermal fibroblasts, suggesting fibroblasts are driving disease pathogenesis by skewing Treg phenotype. Activated fibroblasts express SDF-1 (CXCL12) a ligand for CXCR4 and T cell expression of CXCR4 is upregulated by fibroblasts through TGF- β signalling [86]. SDF-1-CXCR4 signalling also promotes the migration and activation of B cells. The activation of fibroblasts can limit T cell apoptosis [87] thereby enhancing T cell persistence at sites of inflammation. Moreover CXCL4 appears to link inflammation and fibrosis in SSc by epigenetically regulating the differentiation of immune cells [88].

Unconventional T cells: ‘Adaptate’ Immunity in SSc?

Gamma-delta T cells ($\gamma\delta$ T) cells represent a relatively small subset of T cells in the peripheral blood, expressing TCR receptors composed of γ and δ chains. $\gamma\delta$ T cells often display tissue specific localisation [89]. $\gamma\delta$ T cell-mediated immune responses result in the production of various cytokines (IFN- γ , TNF- α , IL-17) depending on the

insult [89]. In systemic sclerosis, patients with disease duration of less than 3 years and positive for anti-Scl-70 antibodies had reduced circulating $\gamma\delta$ T cells [90]. Human skin fibroblasts displayed increased proliferation *in vitro* following co-culture with $\gamma\delta$ T cell supernatants when compared to conventional T cell supernatants [91]. In the bleomycin model of lung fibrosis $\gamma\delta$ TCR^{-/-} mice exhibit progressive fibrosis and reduced levels of chemokine CXCL10 *in vivo* [92]. iNKT cells promote the development of autoreactive B cells and can ‘jump-start’ and/or modulate an adaptive immune response [93]. Reduced numbers of these cells have been detected in the circulation of SSc patients [94]. Decreased numbers of these unconventional T cells in the periphery could be reflective of their recruitment into the tissue, suggesting a role in the early pathogenesis of the disease. Interestingly, these cells can serve both innate and adaptive functions making them attractive candidates for further investigation SSc.

Adaptive immunity in SSc

The precise mechanisms responsible for autoimmunity and progression towards fibrosis have yet to be fully elucidated. Autoreactive T cells and autoantibodies produced by B cells have been identified as key players in this process [95]. Hyper-reactive B cells are accompanied by increased levels of B-cell activating factor (BAFF) in SSc patients [96]. Reduced numbers of circulating regulatory B cells (Bregs) have also been detected [97], supporting a shift from tolerance to autoimmunity. A variety of studies have implicated Th1 cells, Th2 cells, Th17 cells, Th22 cells, regulatory T cells (Tregs), T follicular helper (Tfh) cells, and CD8⁺ T cells in SSc pathogenesis [98-104]. Antigen specific oligoclonal T cells indicate an antigen specific immune response [105] arising due to a breakdown in self-tolerance and induction of T cell activation and

secretion of pro-inflammatory and pro-fibrotic cytokines [106]. As previously mentioned, reduced numbers of ‘non-conventional’ T cell subsets, capable of serving both innate and adaptive function, have been identified in SSc [107]. Studies *in vitro* demonstrated evidence of a contact dependent cross-talk between B cells and fibroblasts regulating ECM production [6, 108]. The Th1 cytokines, IFN- γ and IL-12, limit fibrosis [108] whereas the prototypical Th2 cytokines, IL-4, IL-5, and IL-13, promote fibrogenesis [109]. A Th2-type signature is present in the affected organs and serum of SSc patients [98-100]. We have previously demonstrated that SSc patient T cell-derived IL-13 activates fibroblasts by promoting collagen secretion [110]. The current paradigm implies Th1 responses limit, while Th2 responses promote fibrogenesis. T helper (Th) cells are involved in the early inflammatory and late fibrotic phase, these cells can interact with and promote differentiation of fibroblasts.

T Cells

T lymphocytes are a key stimulus in promoting vascular abnormality and become activated *via* the production of autoantibodies and cytokines. T cells are detectable within the periphery and in the inflammatory infiltrate of and affected organs [4, 8]. In the skin, matrix producing fibroblasts are often found in close proximity to infiltrating immune cells [111]. Expression of an oligoclonal TCR receptor suggests T cell proliferation and expansion in response to a specific antigen [105]. CD4+CD8+ double positive T cells are present in the skin and peripheral blood of SSc patients and produce high levels of IL-4 [112]. In the periphery this may represent an activated effector memory phenotype [113, 114], while in the tissue CD4+CD8+ T cells may have a regulatory phenotype.

CD8 T cells: Effector, Memory and Cytotoxic Functions in SSc

A CD8⁺T cell gene expression signature was identified in SSc patient skin [115] and the SSc lung contains increased numbers of CD8⁺T cells [116]. Fuschiotti and colleagues identified a subset of skin resident effector/memory CD8⁺ T cells in the lesional skin of patients with early/active dcSSc [117]. Circulating and skin resident CD8⁺ T cells produced high concentrations of the pro-fibrotic cytokine IL-13 correlating with the extent of skin fibrosis and ECM production by dermal fibroblasts *in vitro*. Li *et al.*, detected activated effector memory CD8⁺CD28⁻ T cells in both blood and skin of patients with early dcSSc. These cytotoxic cells express skin-homing receptors and are present at increased frequency compared to healthy controls [118]. CD8⁺CD28⁻ lymphocytes proliferate in response to IL-2 and IL-15 and are susceptible to apoptosis [119], demonstrating an increased migratory capacity. Klein *et al.*, showed that SSc CD8⁺ T cells displayed specific patterns of chemokine receptor expression correlating with distinct cytokine profiles [120]. Effector CD8⁺ T cells express the cytolytic effector molecules perforin and granzyme B (GZB), while CD8⁺CD226⁺ T cells produce more IL-13 [121]. This was positively correlated with cytotoxic capacity and ability to induce endothelial cell apoptosis. IL-13 signals through the receptor IL-13R α 1, resulting in downstream activation of STAT6 signalling. IL-13R α 1 is highly expressed by fibroblasts and mononuclear cells in the skin of patients with early dcSSc [117]. CD8⁺ T cells drive pathogenesis of SSc through pro-inflammatory and pro-fibrotic activity in addition to cytotoxic damage to endothelial cells. Cytotoxic CD8⁺ T cells (CTL) are present in the skin infiltrate during the earlier timepoints of active disease whereas CD4⁺ T cell subsets are detected later in disease progression of cutaneous SSc [117].

CD4+ CTLs in SSc

Recently, Maehara *et al.*, identified the presence of CD4+ CTL as the dominant infiltrating T cell subset in the skin of previously untreated dcSSc patients [103]. These cells were visualised directly within the lesion by staining for CD4 and GZB displaying a cytotoxic profile accompanied by elevated metabolic activity and clonal expansion. CD4+ CTL in the blood of dcSSc patients have an activated effector phenotype, losing expression of co-stimulatory receptor CD28. Interestingly, CD4+ CTLs produced the pro-fibrotic cytokine IL-1 β and there was a positive correlation between CD4+ CTL expansion and the number of activated myofibroblasts in the skin [103].

Th9, Th22 and Th17

CD4+T cells can express IL-9 and Th0 (naïve) cells are capable of differentiating into Th9 cells and participate actively in the induction of tissue fibrosis [122]. IL-9 expression is enhanced by cytokines (IL-4, TGF β and TSLP) and induced by the activation of ETS transcription family member PU.1 [123]. Elevated expression of Th9 polarising cytokines has been detected in patients with SSc [124]. A study by Yanaba *et al.*, detected increased serum IL-9 in SSc patients associated with decreased frequency and severity of pulmonary fibrosis [125], the authors suggested a protective role for IL-9 in the pathogenesis of SSc. Conflicting reports were published by Jiang *et al.*, showing IL-9 levels in the serum were higher in patients with CTD-ILD and associated with the severity of pulmonary fibrosis [122]. IL-9 levels inversely correlated with IFN γ production and positively correlated with IL-4 levels. This study contained but was not limited to SSc-ILD patients and it should be noted that contrasting findings could be explained by the presence of other CTD-ILD pathologies.

IL-9 is expressed mainly by infiltrating immune cells in SSc skin, these cells were identified as Th9 cells by co-localization with transcription factor PU.1 [126]. This study found increased frequencies of IL-9 producing cells in patients with diffuse disease and the number of IL-9-producing cells correlated with mRSS score; however no correlation was seen with pulmonary hypertension. More recently, Wohlfahrt *et al.*, demonstrated that PU.1 is highly expressed in fibroblasts of SSc biopsy samples and overexpression of PU.1 caused phenotypic switching of fibroblasts from an inflammatory to a matrix producing state [127]. The pathogenic role for PU.1 was verified in the BLM model of lung and skin fibrosis, additionally a fibroblast specific knockout of PU.1 was shown to reduce fibrosis.

Th22

Th22 cells are a T helper cell subset characterised by production of IL-22. Activated T cells produce IL-22 while the IL-22 receptor is expressed by non-hematopoietic cells [128]. Increased frequency of circulating Th22 cells in SSc correlated with expression of skin homing receptor CCR6 [129] and SSc-ILD was associated with increased numbers of IL-22 producing cells. IL-22 deficient mice have more pronounced fibrosis in the BLM model and anti-IL22 treatment exacerbated the fibrotic phenotype of WT mice [130]. Significant increases of IL-22 transcript have been found in lesional skin of SSc patients compared to healthy controls, suggesting the presence of a distinct population of IL-22 producing cells [131]. IL-22+ cells are increased in the dermis of SSc skin compared to controls, the majority of IL-22+ cells in the dermis are T cells, while dermal fibroblasts express IL-22R subunits. IL-22 stimulation did not induce collagen production by dermal fibroblasts *in vitro* but instead elevated CCL2 levels [128].

Th17

Th17 cells are a T helper cell subset characterised by production of IL-17 and expansion of these cells is promoted by IL-23. Elevated levels and frequencies of IL-17A and/or Th17 cells have been reported in serum, peripheral blood, BAL and tissue of SSc patients [129, 132, 133]. In mouse models of SSc IL-17A plays a pro-fibrotic role. IL-17A deficiency protects against skin and lung fibrosis in the BLM model, reducing collagen deposition [134, 135] and decreasing dermal thickening in the TSK1 model [135]. Further studies using the BLM model identified IL-17A and IL-1 β dependent regulation of BAFF in pulmonary fibrosis [136] and higher frequencies of circulating and tissue infiltrating Th17 cells [137]. Serum levels of IL-17A in BLM treated mice correlated with the degree of tissue inflammation and fibrosis, while anti-IL17 treatment reduced fibroblast proliferation and collagen production [137]. Blocking IL-17 activity blunted IL-1 mediated fibrosis, reducing the clinical severity of graft-versus-host disease (GVHD) [138], while in the BLM model downstream IL-17 activity was associated inflammation and collagen deposition.

The role of IL-17A in studies involving human samples, particularly SSc dermal fibroblasts, is less clear. Several studies reported pro-fibrotic effects of IL-17A on fibroblasts *e.g.* increased pro-inflammatory cytokine production (CCL2, IL-8, IL-6), proliferation and collagen deposition [133, 139-141]. Dysregulated expression of IL-17 receptors by SSc dermal fibroblasts compared to healthy controls has also been reported [140]. The number of Th17 cells in tissue correlated with disease activity and IL-17 from SSc patients promoted collagen production, this response was blocked by an IL-17 neutralising antibody [141]. A recent investigation revealed that co-culture of T cells and autologous SSc skin fibroblasts promoted fibroblast apoptosis, this was attenuated by recombinant IL-17A treatment [142]. Contrastingly, several studies state that

IL-17A negatively correlates with disease activity and identify a protective role for IL-17A in SSc. IL-17A prevented fibroblast-myofibroblast transition by TGF β and inhibited collagen production by SSc fibroblasts *in vitro* [143]. IL-17A may exert context dependent negative regulation of TGF- β dependent SMAD signalling [144]. A recent investigation using 3D-organotypic skin equivalents by Chizzolini and colleagues showed that while IL-17A promoted the expression of pro-inflammatory genes, there was no effect on collagen production [145]. Additionally, IL-17A attenuated TGF β mediated Wnt signalling and collagen production in this model. IL-17A exerts effects independently of stromal cells and can promote the production of high affinity antibodies by B cells, further investigation into the role of IL-17A in SSc using more physiologically relevant models is required.

Th9, Th22 and Th17 cell subsets are found in the peripheral blood, serum and skin lesions of SSc individuals and participate in disease pathogenesis through cross-talk with non-haematopoietic stromal cells. Infiltrating cells are exposed to a variety of cytokines and chemokines and tissue dependent-trans-differentiation can promote fibrogenesis. This necessitates further research into the tissue specific functions of these cells and their characteristic cytokines. Treg plasticity may account for increased frequency of Th17 cells in the periphery and in the tissue. T cell response may be polarized toward the Th17 compartment, furthermore altered Th17 and Treg subpopulations have been identified in individual SSc donors [146]. Yang *et al.*, reported increased expression Th17 cells and their hallmark cytokines in the peripheral blood, serum and skin [141]. Liu *et al.* found that CD4⁺CD25⁺FoxP3^{lo}CD45RA⁻ non-regulatory T cells produced high levels of IL-17 representing a transitional phase where Tregs are converted to Th17 cells [147].

Regulatory T cells (Tregs)

Tregs are characterised by expression of the CD4 T cell co-receptor and CD25, the α -chain of the IL-2 receptor. There are contrasting findings regarding the frequency of Tregs in peripheral blood and tissue of SSc patients. Some studies report reduced frequency [146, 148, 149] whereas others report no change or increased frequency [150, 151]. Elevated frequency of Tregs was detected in patients with a high interstitial lung disease score and correlated with disease activity and progression [150, 151]. Lower levels of skin resident Tregs in patients with advanced SSc may be a direct result of Treg transformation into pathogenic Th17 and/or Th2 effector T cells. SSc Tregs have impaired functional capacity and fail to produce inhibitory cytokines (IL-10, IL-35, TGF β) to suppress CD4/CD8 effector T cells [146, 149]. Reduced numbers in the periphery may result from decreased Treg development, reduced proliferation and/or survival of these cells. Elevated IL-1 β and IL-6 in the serum, BAL, and lesional skin can promote Th17 cell differentiation with pro-inflammatory or pro-fibrotic activity [120, 141]. Increased frequency of Tregs in the periphery correlates with lung involvement and reduced frequency of Tregs correlates with early disease.

MacDonald *et al.*, demonstrated Treg cells from SSc affected skin produced large amounts of IL-4 and IL-13 [152]. This study identified IL-33 as a regulator of skin Treg Th2 differentiation. Recent findings by Sato and colleagues identified tissue localised trans-differentiation of Tregs into Th2-like cells, regulated by activated IL-33 expressing fibroblasts present in lesional skin [56]. Previous studies detected elevated IL-33 levels in serum of systemic sclerosis patients in early disease [7]. The IL-33-receptor ST2 is increased in endothelial cells, lymphocytes, and fibroblasts/myofibroblasts of the skin and lung [153]. Taken together these findings implicate IL-33 as a key regulator of very early pathogenic events in SSc. IL-33 is

responsible for recruitment and stimulation of ST2-expressing cells (immune cells, fibroblasts and/or myofibroblasts).

T follicular helper cells

T follicular helper (Tfh) cells are a subset of CD4⁺ T cells that provide help to B cells, facilitating proliferation, isotype switching and somatic hypermutation, resulting in prolonged/long-lasting antibody responses [154]. Inducible co-stimulatory molecule (ICOS) is up-regulated on activated T helper cells and expressed at high levels by T follicular helper cells in the skin of SSc patients [155, 156]. In tissue the proximity of Tfh to germinal centres in secondary lymphoid organs enables interaction with B cells. A circulating Tfh subset has been identified that expresses CXCR5, PD-1 and ICOS [157]. These cells help B-cell differentiation into plasma cells *via* IL-21 secretion. Numbers of circulating Tfh are elevated in the blood of patients with SSc [158]. Tfh-associated cytokine IL-21 and its receptor (IL-21R) are also upregulated in patients with SSc [159]. In the BLM model, ICOS deficiency protects against skin and lung fibrosis [160]. A subset of infiltrating CD4⁺ ICOS⁺ PD-1⁺ Tfh-like cells were detected in the GVHD mouse model and in lesional skin of SSc patients. [104]. The presence of these cells correlated with disease activity. Removal of the ICOS⁺ T cell subset dysregulated fibroblast matrix production and reduced inflammation [104]. This indicated the presence of an IL-21-IL-21R signalling axis, facilitated by stromal expression of the IL-21 receptor. A recent study by Richard *et al.*, showed Tfh cells from patients with SSc induced B-cell differentiation into plasmablasts secreting Ig *via* IL-21, this was less pronounced in healthy controls [158]. These findings indicate that IL-21 or JAK1/2 blockade by ruxolitinib may tilt the balance of Tfh cells toward inhibiting autoantibody production and may prove beneficial in SSc patients.

551 **B cells**

552 B cells have important roles in the production of autoantibodies, cytokine production
553 and presentation of antigens. B cells are present in the inflammatory infiltrate of SSc
554 lesional skin [161]. The presence of B cells within the infiltrate is associated with early
555 diffuse skin disease and correlates with skin progression [6]. SSc patients have
556 unusually high numbers of activated peripheral B cells compared to healthy controls
557 [96], this is accompanied by a reduced frequency of regulatory B cells (Bregs) [97]. B
558 cells from SSc patients demonstrate up-regulated CD19 expression this is a crucial
559 regulator of B cell activation. B cells contribute to cutaneous and pulmonary fibrosis
560 through local effector function while regulatory B cells play a protective role.

561 The role of fibroblasts in activating/differentiating B cells through BAFF production
562 has been demonstrated in rheumatoid arthritis (RA) but is less clear in SSc.
563 BAFF/BAFF-R signalling enhances B-cell survival, growth and metabolic fitness. In
564 RA fibroblast-like synoviocytes (FLS) can promote class-switch recombination leading
565 to hypersomatic mutation of B cells [162]. This occurs in a BAFF dependent manner
566 and requires direct contact between FLS and B cells. FLS express the membrane bound
567 form of BAFF following stimulation with IFN- γ /TNF- α and promote B cell survival
568 [163]. A study by Francois *et al.*, found that contact between SSc dermal fibroblasts
569 and B cells promoted collagen production, however SSc fibroblasts did not secrete
570 BAFF or express the BAFF receptor [6]. Paradoxically, BAFF levels are elevated in
571 SSc patients following treatment with rituximab [164].

572 Rituximab is the standard of care in addition to chemotherapy in patients with
573 high-grade B-cell non-Hodgkin's lymphoma (NHL). Rituximab is a human/murine
574 chimeric mAb that specifically targets the transmembrane protein CD20 on B cells,
575 leading to a significant depletion of peripheral cancerous/autoreactive B cells [165].

Systematic reviews by Vettori *et al.*, and Colaci *et al.*, [166, 167] have reported an association between non-Hodgkin's lymphoma (NHL) and SSc. The aberrant levels of BAFF in NHL may be released by malignant B cells or by supporting stromal cells (lymph node or bone marrow derived stromal cells) [168]. Stromal cells are present within the microenvironmental niche occupied by malignant B-cells, promoting cell growth and survival. Alternatively the association of SSc and NHL may be linked to the emergence of a malignant clone from a pool of hyper-stimulated B cells [169].

Regulatory B cells (Bregs)

In addition to providing help to T cells, B cells can dampen T-cell driven immune responses. In SSc these regulatory B cells (Bregs) are functionally deficient and total numbers are greatly reduced [97]. This reduction persists throughout disease duration and is associated with autoantibody production and pulmonary fibrosis. In humans regulatory B cells represent a very small proportion (less than 1%) of total peripheral blood B cells [170]. SSc patients with pulmonary fibrosis have an impaired ability to produce IL-10 following TLR-9 stimulation and levels of IL-10-positive Bregs inversely correlate with disease activity [171]. In SSc mice, IL-10 producing Bregs are suppressed (GVHD model) and the absence of these cells promoted severe disease [172]. A recent investigation by Matsushita *et al.*, showed increased skin fibrosis in B cell specific IL-10-deficient mice following bleomycin challenge [173]. Exogenous BAFF treatment reduced Breg numbers in mice while treatment with a BAFF antagonist had no effect on Bregs but instead suppressed effector B cells (Beffs). It is suggested that patients who respond to autologous stem cell transplant 'reset' their immune system and that Breg cell numbers and functionality are restored.

Effector (Beff) and Memory B cells

SSc patient B cells produce high levels of IL-6 and BAFF [96]. IL-6 is capable of inducing Tfh and Th17 differentiation from naive CD4⁺ T cells and plasma cell differentiation. Le Huu *et al.*, showed that administration of an anti-IL-6 antibody reduced skin fibrosis in mice, a study by Matsushita *et al.*, showed reduced skin fibrosis in B cell specific IL-6-deficient mice [172, 173]. This study also found that BAFF inhibition altered the balance of regulatory and effector B cells, showing the BAFF inhibitor/antagonist functioned by reducing the numbers of IL-6+Beffs. GM-CSF+ Beff cells are increased in SSc patients [174]. These cells co-express high levels of both TNF- α and IL-6 and belong to an effector/memory B cell subset. Memory B cells (CD19⁺CD27⁺) are present at reduced numbers in SSc and have increased expression of activation markers (CD80, CD86, and CD95) in addition to increased susceptibility to apoptosis [175]. CD27⁺ memory B cells can rapidly differentiate, present antigens and secrete cytokines. B cells display a number of antibody independent effector functions, such as antigen presentation to effector T cells *via* MHCII, thus promoting the release of inflammatory cytokines. Peripheral B lymphocytes from SSc patients can secrete IL-6 and TGF β and activate fibroblasts *in vitro* [176]. IL-6+Beff cells can promote fibroblast collagen production *via* direct interaction with fibroblasts, while paracrine signalling and cytokine production by Beffs can activate macrophages. Ibrutinib is a Bruton's tyrosine kinase (BTK) inhibitor, treatment of hyperactivated effector Beffs with Ibrutinib reduced pro-fibrotic cytokines IL-6 and TNF- α [177]. Treatment with Ibrutinib did not alter production of regulatory IL-10. Depletion of specific B cell subsets may represent an attractive therapeutic avenue; however this approach is currently limited by the existing markers of B cell subsets and/or specific activation states. Unlike Foxp3⁺ T cells Bregs don't exist as a distinct

transcriptional phenotype. IL-10 producing Bregs do not appear as a separate B cell lineage and instead represent an activation or differentiation state of several traditional B cell subsets [165]. IL-10-dependent and IL-10-independent mechanisms should also be investigated. Studies by Debes and colleagues detected IL-10-positive B cells in healthy/normal skin (uninflamed human and mouse skin), experiments utilising IL-10 reporter mice demonstrated that even in the absence of inflammation B cells can transcribe IL-10 [178]. IL-10 may have an important function in the steady state to suppress inflammation and maintain tissue homeostasis.

Emerging SSc Therapies: the fibroblast and beyond

Rituximab

Use of monoclonal antibody (mAb) therapy, to selectively deplete B cells, is a well-established treatment for B-cell malignancies. B cell depletion and rituximab treatment has shown efficacy in murine models of fibrosis [43].

A small randomized trial examined the efficacy of rituximab in SSc-ILD, this study compared rituximab plus "standard therapy" with standard therapy alone [179].

Improvements in lung function (diffusing capacity of the lungs for carbon monoxide (DLCO) and forced vital capacity (FVC)) were observed in rituximab treated patients.

A follow up study suggested lung improvement persisted, for up to 2 years, with prolonged treatment compared to baseline (two additional cycles of rituximab at 12 and 18 months) [180]. An investigation by Bosello *et al.*, identified long term efficacy of B cell depletion accompanied by improvement in skin score and significant improvement in severity indices after 12 months compared to baseline. B-cell depletion also seemed to preserve the pulmonary function [181]. A phase 2 observational trial was conducted by the European Scleroderma Trials and Research network (EUSTAR), this trial

consisted of 254 SSc patients treated with rituximab and matched with untreated SSc patients [182]. Rituximab use was associated with a good safety profile and significant improvement in skin fibrosis was observed, but no changes were detected in lung fibrosis. Potential impact on stability of lung function will need to be investigated using a placebo-controlled randomized trial.

Rituximab appears to have general anti-fibrotic effects, exerting influence on the vasculature through stabilization and generation of capillaries [183] and acting directly on dermal myofibroblasts to downregulate the expression of the PDGF receptor [184]. Rituximab can inhibit pro-fibrotic Wnt signalling *via* modulation of TGF β signalling and up-regulation of Dkk-1 expression by dermal fibroblasts [185]. Additionally, B cell depletion with rituximab reduced IL-4 and CD40L expressing T cells in the skin and peripheral blood of SSc patients [186]. This may indicate a beneficial role for rituximab in SSc pathogenesis through the inhibition of Th2 cytokines.

Abatacept

Abatacept is a CTLA4 immunoglobulin fusion protein that inhibits T-cell activation by binding to CD80 and CD86 on APCs, thus blocking the required CD28 interaction between APCs and T cells. Genetic polymorphisms in co-stimulatory molecules *e.g.* CTLA-4 have been detected in SSc patients [187]. CTLA4-Ig treatment may limit the fibrotic process in SSc patients by downregulating the number of circulating fibrocytes [188]. Abatacept showed a general improvement in mRSS score compared to placebo with significant improvement in patients with a ‘normal-like’ or ‘inflammatory’ skin gene profile, but not in patients with a ‘fibrogenic’ profile [189]. Transcriptional analysis of patient skin biopsies with diffuse SSc showed improvement in skin score and downregulation of inflammatory pathways following treatment with abatacept. A

preliminary report of two patients with SSc-ILD treated with abatacept revealed improvement in lung function of both patients (DLCO, FVC) [190]. The ASSET trial is a placebo-controlled trial of abatacept in patients with early dcSSc. This study found abatacept was well-tolerated in patients, however significant change in mRSS score versus placebo was observed [191]. Secondary outcome measures, including gene expression subsets did show evidence in support of abatacept. It should be noted that whilst early studies suggested no change in mRSS, a quantifiable skin measurement, there were clinically meaningful effects. A study by Maehara *et al.*, examined pre-and post-treatment skin biopsies of 4 patients with early diffuse SSc, enrolled in the ASSET trial [103]. Abatacept reduced the accumulation of CD4+ CTLs in the skin of patients with active SSc (3/4 patients). Abatacept treatment might block the differentiation of naive and memory CD4+ T cells, and potentially CD8+ T cells, into effector cells by interfering with co-stimulation.

Tocilizumab

IL-6 drives fibrosis by activating STAT3 and dermal fibroblasts from SSc patients display increased STAT3 activation at a variety of sites *e.g.* endothelium, fibroblasts, keratinocytes and immune cells [192, 193]. Dermal fibroblasts from patients with SSc constitutively express more IL-6 than those from healthy controls while IL-6 antibody treatment blocks collagen release by dermal fibroblasts [194]. Elevated IL-6 expression in patient skin biopsies and serum predicts ILD progression in SSc [195].

Tocilizumab (TCZ) is a humanized IL-6 receptor antibody and an antagonist of the IL-6 receptor [196]. Tocilizumab binds to both the soluble IL-6 (sIL-6) receptor and membrane-bound IL-6 (mIL-6) receptor, completely inhibiting the binding of IL-6 to the IL-6 receptor [196]. The results of the phase 2/3 double-blind randomized

placebo-controlled study (faSScinate) with tocilizumab showed no statistically significant differences. However, a trend of reduction in skin scores ($p = 0.06$) and stabilization of FVC compared to placebo ($p = 0.09$) was observed [197]. Interestingly, experiments involving matched skin biopsy samples identified significant alterations in dermal fibroblast transcriptional profiles [198]. Gene expression profiles of skin biopsies from TCZ treated patients had reduced TGF β -regulated genes and molecular pathways compared to placebo. The results of a recently completed phase III trial of tocilizumab (focuSSced trial; NCT02453256) study indicated while tocilizumab therapy did affect skin scores, progression of lung fibrosis in these patients may be delayed. Further enrichment of patients with high IL-6 and/or STAT3 may have improved the rate of responders.

Pirfenidone

Pirfenidone is an antifibrotic agent that is approved for use in idiopathic pulmonary fibrosis (IPF) [199] though the exact mechanism of action is yet to be identified. Proposed mechanisms include the modulation of TGF β signalling and pirfenidone was shown to attenuate TGF β 1 induced collagen 1 production in lung fibroblasts [200]. Pirfenidone prevented fibrocyte migration by limiting production of CCL2 and CCL12 [201] and inhibited pro-fibrotic and pro-inflammatory cytokines, such as TGF- β 1, IL-1 β and IL-6 [202]. Furthermore, pirfenidone reduced human lung fibroblast proliferation and myofibroblast differentiation *via* inhibition of TGF- β -induced SMAD3 phosphorylation [203].

The safety and tolerability of pirfenidone in SSc-ILD was evaluated in the LOTUSS study [204], pirfenidone was generally well-tolerated in SSc patients despite underlying gastrointestinal disease. A recent single-centre randomised control trial investigating

the safety and efficacy of pirfenidone in SSc-ILD failed to find a significant beneficial effect of the drug over placebo [205]. The SLS-III trial is ongoing (NCT03221257) and will compare the effect of pirfenidone and mycophenolate mofetil (MMF) with MMF and immunosuppressive therapy alone. It is hoped the rapid anti-fibrotic effect of pirfenidone will complement the delayed anti-inflammatory and immunosuppressive effects of MMF to produce a more rapid or greater improvement in lung function.

Nintedanib

Nintedanib is an inhibitor of multiple tyrosine kinases approved for use in IPF as it slows disease progression [206]. Nintedanib attenuates fibrosis *in vivo* in multiple mouse models; *e.g.* BLM induced skin fibrosis,, GVHD model and the TSK1 model [207]. Nintedanib works by inhibiting the pro-fibrotic functions of FGF, PDGF and VEGF by eliciting potent blockade of growth factor receptor kinase activity [208]. It also reduces in the number of infiltrating inflammatory cells in the lungs [209]. Approval for use in SSc-ILD was based on the SENSICIS study, a large randomized trial investigating the use of nintedanib in the treatment of SSc-ILD [209]. The relative reduction in rate of annual FVC decline was comparable to results seen in the trials of nintedanib in IPF (44% versus 49%) [206]. There was no significant difference in mRSS score. These findings suggest the use of nintedanib in SSc-ILD may reduce the rate of ILD progression. However, further investigation is needed to identify the patients with SSc-ILD most likely to benefit from nintedanib and an extended trial (NCT03313180) investigating long term safety is ongoing.

Lenabasum

Lenabasum is a synthetic cannabinoid receptor type 2 (CB2) agonist that can selectively activate the CB2 receptor in immune cells and fibroblasts [210]. Lenabasum triggers cellular signals that reduce inflammation and prevent fibrosis *via* activation of CB2 [211]. This has shown beneficial effects in animal models of skin and lung fibrosis [212, 213]. A recent phase II clinical trial with Lenabasum in early diffuse SSc patients compared to placebo showed large and statistically significant improvement in the CRISS (Combined Response Index in diffuse cutaneous Systemic Sclerosis) score and histological improvement [214]. Lenabasum is also being evaluated in the Phase 3 RESOLVE-1 trial (NCT03398837) in patients with dcSSc. Results are eagerly anticipated.

776 **Table 2: Relevant ongoing phase II/III clinical trials in Systemic Sclerosis**

777 **targeting inflammatory and fibrotic components of disease pathophysiology**

778

Study Drug	Mode of action	ClinicalTrials.gov identifier	Phase
	Inflammation/Immune activation		
Rituximab*	CD20 targeting monoclonal antibody	NCT01862926	III
Lenabasum	Agonist to the cannabinoid receptor type 2	NCT03398837	III
Brodalumab	Anti- interleukin-17 mAb, acts by inhibiting IL-17 receptor rather than the molecule	NCT03957681	III
Bermekimab	MABp1 is a human monoclonal antibody, cloned directly from human B lymphocytes, that specifically targets and neutralizes IL-1 α	NCT04045743	II
Tofacitinib	Pan inhibitor of the Janus Kinase (JAK) family of kinases with a high degree of selectivity against JAK1 and JAK3	NCT03274076	II
	Fibrosis		
Nintedanib	Growth factor receptor tyrosine kinase inhibitor	NCT03313180	III
Pirfenidone**	Anti-fibrotic and inhibitor of inflammatory cytokines, given with mycophenolate mofetil	NCT03856853	III
Romilkimab	Bispecific IgG4 antibody that binds and neutralizes both circulating interleukin-4 and interleukin-13	NCT02921971	II
GSK2330811	Humanized IgG1 κ monoclonal antibody, functionally blocks human oncostatin-M from binding to the gp130 receptor	NCT03041025	II

779 *Rituximab can affect vasculature and exerts general anti-fibrotic effects

780 ** Pirfenidone is also anti-inflammatory

781

Summary and Conclusions

SSc is a complex and intriguing disease. It is clear that the interplay between innate and adaptive immunity in the disease is complex. Alterations in the quantity and phenotype of multiple immune cells are characterised in the disease, particularly early on. Fibroblasts display diverse functionality in SSc through the production of ECM, acting as ‘immune sentinels’ *via* the release of cytokines and thorough direct and indirect interactions with immune cells. This ongoing communication further drives disease pathogenesis and highlights the importance of bi-directional communication between immune and stromal cells. It may be that at different stages of the disease; late versus early for example, different context dependant pathways are prominent meaning that therapeutic targeting is lost in the ‘window of opportunity’ akin to rheumatoid arthritis. Novel targets are being continuously developed, drugs that target both immune and stromal cells show potential for meaningful and/or tangible therapeutic benefit. For example, the tyrosine kinase inhibitor nintedanib has recently been approved for treatment of SSc-ILD and drugs such as lenabasum appear close to being clinically effective. Better understanding of the disease pathogenesis and molecular phenotyping means we can enrich the patients most likely to benefit from specific targeting. What was once seen as an ‘untreatable’ disease is now looking towards being a ‘treatable’ disease; at least in some endotypes.

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Figure Legends

Figure 1: Bi-directional communication between cytokine producing T cell subsets and fibroblasts

Cytokines and growth factors released by T cells (solid black arrows) promote fibroblast activation and lead to myofibroblast transition through induction of pro-inflammatory and pro-fibrotic factors. Fibroblasts and myofibroblasts in turn (dotted line arrows) can promote T cell recruitment, polarization and trans-differentiation *via* release of cytokines, chemokines, growth factors and extracellular matrix production. Th: T-helper; Treg: regulatory T cell; IFN: interferon; IL: interleukin; TGF: transforming growth factor; TNF: tumour necrosis factor; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; ECM: extracellular matrix.

Figure 2: Fibroblast-B cell cross-talk in systemic sclerosis is mediated by effector B cells and IL-21 signaling

Pro-inflammatory Beff numbers are increased in SSc whereas the numbers of protective IL-10 producing Bregs are reduced. Cytokines produced by Beffs drive Th1 and Th17 activation, while Breg IL-10 is inhibitory. Beffs promote fibroblast/myofibroblast activation and production of ECM through release of cytokines IL-6, TNF α , GM-CSF and BAFF. Tfh cells help B cell differentiation into antibody producing plasma cells

via IL-21 secretion. SSc fibroblasts have increased IL-21R expression and IL-21 production promotes fibrosis by inducing expression of MMPs, IL-6, TGF β and CCL2 expression by myofibroblasts. Beff: effector B cell; Breg; regulatory B cell; Th: T-helper; Tfh: T follicular helper cell; IL: interleukin; TGF: transforming growth factor; TNF: tumor necrosis factor; CCL: C-C motif chemokine ligand; BAFF: B cell activating factor; GM-CSF: granulocyte-macrophage colony stimulating factor; MMP: matrix metalloproteinase; ECM: extracellular matrix.

Figure 3: Effects of emerging SSc therapies on pathogenic functions of immune and stromal cells

Dysregulated Th2 cytokine production in systemic sclerosis by T and B cells drives myofibroblast activation and ECM deposition. The myofibroblast further contributes to pathogenesis through the release of growth factors, pro-inflammatory and pro-fibrotic mediators. Potential cellular and molecular targets for therapy are indicated with brown arrows (solid blunt headed arrows: inhibitors; dotted blunt headed arrow: depletion antibody; double headed arrow: agonist). Romilkimab and Bermekimab can bind to and neutralize pro-inflammatory cytokines e.g. IL-4/IL-13 and IL-1 α . Rituximab acts by depleting B cells and can also inhibit Th2 cytokine production. Pirfenidone and nintedanib can inhibit the pro-fibrotic actions of TGF- β , inflammatory chemokines and growth factors respectively. GSK2330811 and Brodalumab function as inhibitors of the gp130 and IL-17 receptors. Lenabasum is an agonist of the CB2 receptor found on both immune and stromal cells. Th2 : T helper 2 , Th17 : T helper 17 ; IL: interleukin; TGF: transforming growth factor; TNF: tumour necrosis factor; CCL: C-C motif chemokine ligand; BAFF: B cell activating factor; GM-CSF: granulocyte macrophage colony

856 stimulating factor; ECM: extracellular matrix; OSM: oncostatin-M; CB2:cannabinoid

857 receptor 2.

858

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