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Pathogenesis of systemic sclerosis

Debendra Pattanaik[1,2], Monica Brown[3], Bradley C. Postlethwaite[1] and Arnold E. Postlethwaite[1,2]*

[1] *Department of Medicine, Division of Connective Tissue Diseases, The University of Tennessee Health Science Center, Memphis, TN, USA,* [2] *Department of Veterans Affairs Medical Center, Memphis, TN, USA,* [3] *Section of Pediatric Rheumatology, Department of Pediatrics, The University of Tennessee Health Science Center, Memphis, TN, USA*

Systemic scleroderma (SSc) is one of the most complex systemic autoimmune diseases. It targets the vasculature, connective tissue-producing cells (namely fibroblasts/myofibroblasts), and components of the innate and adaptive immune systems. Clinical and pathologic manifestations of SSc are the result of: (1) innate/adaptive immune system abnormalities leading to production of autoantibodies and cell-mediated autoimmunity, (2) microvascular endothelial cell/small vessel fibroproliferative vasculopathy, and (3) fibroblast dysfunction generating excessive accumulation of collagen and other matrix components in skin and internal organs. All three of these processes interact and affect each other. The disease is heterogeneous in its clinical presentation that likely reflects different genetic or triggering factor (i.e., infection or environmental toxin) influences on the immune system, vasculature, and connective tissue cells. The roles played by other ubiquitous molecular entities (such as lysophospholipids, endocannabinoids, and their diverse receptors and vitamin D) in influencing the immune system, vasculature, and connective tissue cells are just beginning to be realized and studied and may provide insights into new therapeutic approaches to treat SSc.

Keywords: systemic sclerosis, scleroderma, innate immunity, adaptive immunity, vasculopathy, fibrosis, animal models

Introduction

Systemic sclerosis (SSc, scleroderma) is a complex connective tissue disease of unknown etiology with multiorgan involvement and heterogeneous clinical manifestations. The clinical and pathologic manifestations of the disease are the result of three distinct processes: (1) innate and adaptive immune system abnormalities leading to production of autoantibodies and cell-mediated autoimmunity, (2) microvascular endothelial cells (MVEC) and fibroproliferative vasculopathy of small vessels, and (3) fibroblast dysfunction leading to excessive collagen (CI) and other matrix components accumulation in skin, blood vessels, and internal organs (1, 2).

The incidence of SSc is about 20 cases per million populations per year and the prevalence is more than 250 patients per million populations in USA (3). Major organ involvement leads to decreased survival in SSc. Pulmonary fibrosis [interstitial lung disease (ILD)] and pulmonary arterial hypertension (PAH) cause more than half of all SSc-related deaths (3). However, patients with SSc live longer and cardiac deaths are increasing.

Progressive thickening and fibrosis of skin secondary to excessive CI accumulation is the most evident and universal finding and can be associated with varying degrees of fibrosis of internal organs. Vascular dysfunction and abnormalities are often seen, and can precede organ involvement by several years (4).

Disease manifestations vary from limited skin involvement with minimal systemic involvement [limited cutaneous (lc) SSc] to widespread skin involvement accompanied by internal organ involvement [diffuse cutaneous (dc) SSc]. These two forms differ mainly in regards to extent of skin involvement, autoantibody association, and the pattern of organ involvement (**Table 1**) (5). Given the heterogeneity of clinical symptoms and signs, American College of Rheumatology (ACR)/EULAR recently developed new classification criteria (6). The new classification criteria would improve sensitivity, which would lead to earlier diagnosis, and it also incorporates the autoantibodies that are commonly used for diagnostic purposes.

It is widely believed that SSc develops in an individual with a "permissive" genetic makeup. Genetic associations of SSc are

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Abbreviations: 2-AG, 2-arachidonoyl glycerol; α2AP, α2-antiplasmin; AA, African American; ACA, anticentromere antibodies; Acea, eicosatetraenamide; ACR, American College of Rheumatology; ADCC, antibody-dependent cellular cytotoxicity; AEA, anandamide; AFA, anti-fibrillarin antibody; AIF1, allograft inflammatory factor-1; AKT, protein kinase b; ALAS2, delta-aminolevulinate synthase 2 protein; ALK, activin receptor-like kinase; ANA, antinuclear antibodies; APC, antigen-presenting cells; ARA, anti-U3 ribonucleoprotein; αSMA, α smooth muscle cell actin; ATA, anti-DNA topoisomerase I antibody; ATGL, adipose triglyceride lipase; aTregs, activated Tregs; αZAP, α zinc-finger alpha protein gene; BANK1, B-cell scaffold protein with ankyrin repeats 1; bFGF, basic fibroblasts growth factor; BiP, immunoglobulin-heavy-chain binding protein; BLM, bleomycin; BMP, bone morphogenic protein; BP, binding protein; Bregs, B regulatory cells; Cav-1, caveolin-1; CB1/CB2, cannabinoid receptors 1 and CB2 ditto; CBP, CREB-binding protein; CCR1, C–C chemokine receptor type 1 protein or gene; CD40L, CD40 ligand; CGRP, calcitonin G-related peptide; CGS, candidate gene study; cGVHD, chronic graft-versus-host disease; CI, type I collagen; CII, type II collagen; CIII, type III collagen; COX-2, cyclooxygenase 2 (i.e., prostaglandin synthase 2); CpG, C phosphate G; cScl-GVHD, chronic scleroderma graft-versus-host disease; CSK, C-src tyrosine kinase; CTGF, connective tissue growth factor; CV, type V collagen; CXCL4, angiostatin, platelet factor-4; DAMPs, damage-associated molecular patterns; dcSSc, diffuse cutaneous systemic scleroderma; DNAM-1, DNAX accessory molecule-1 (i.e., CD226); ECM, extracellular matrix; EC(s), endocannabinoid(s); ECS, endocannabinoid system; EDNR-A or -B, gene that encodes endothelin receptor type A or B; Egr-1, early response gene 1; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; EVI, evenness interrupted, in reference to cell surface multipass transmembrane protein; FAAH, fatty acid amide hydrolase; FasL, Fas ligand; FGF, fibroblast growth factor; FH-1, human fetal lung fibroblast line; Fli(1), friend leukemia integration; Fra-2, Fos-related antigen-2; FTY720, fingolimod; GATA-3, GATA binding protein-3; GMCSF, granulocyte monocyte-colony stimulating factor; GPCRs, G-protein-coupled receptors; GVHD, graft-versus-host disease; GWAS, genome-wide association studies; hCMV, human cytomegalovirus; HGF, hepatocyte growth factor; HLA, human leukocyte antigen; HMGB-1, high-mobility group box-1 protein; HMW-MAA, high molecular weight melanoma-associated antigen; HSP, heat-shock protein; IC, intracellular; ICAM-1, intercellular adhesion molecule-1; IFIT1, IFIT2, and IFITM1, interferon-regulated genes; IFN, interferon; IFNRI, type 1 interferon receptor; IGF, insulin-like growth factor; IGF-BP-5, insulin-like growth factor binding protein-5; ILCs, innate lymphoid cells; ILD, interstitial lung disease; IP, interferon-inducible protein; IPAH, idiopathic PAH; IPF, idiopathic pulmonary fibrosis; IRAK1, interleukin-1 receptor associated kinase 1; IRF, interferon regulatory factors; IRFSNP, interferon regulator factor, SNP (see entry); ITGAM, integrin αM; ITGAX, integrin αX; JAMs, junctional adhesion molecules; JNK, c-Jun N-terminal kinase; KCNA5, potassium voltage-gated channel, shaker-related subfamily, member 5 gene; kDA, kilo Dalton, measurement of; LAP, latency-associated peptides; lcSSc, limited cutaneous systemic scleroderma; LLC, large latent complex; LPA, lysophosphatidic acid; LPS, lipopolysaccharides; LTBP, latent TGF-β1-binding protein; MAGL, monoacylglycerol lipase; MAPK, mitogen-activated protein kinase; MCP-3, monocyte chemoattractant protein-3; MCR1, melanocortin receptor 1, gene found in yeast; MECP2, methyl-CpG-binding protein 2 gene; MHC, major histocompatibility complex; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; miRNA, micro RNA (miR); MMP, matrix metalloproteinase; mPGES-1, microsomal prostaglandin E2 synthase 1; mRNA, messenger RNA; MRSS, modified Rodnan skin score; MS, multiple sclerosis; MVEC, microvascular endothelial cells; MW, molecular weight; NAG-2, MVEC surface protein tetraspan novel antigen-2; NLR, a pattern recognition system (NOD)-like receptor family; NO, nitric oxide; NOD, nucleotide-binding and oligomerization domain; NOTCH, neurogenic locus notch homolog 4 protein encoded by the NOTCH4 gene, and an evolutionarily conserved pathway in multicellular organisms that regulates cell-fate determination during development and maintains adult tissue homeostasis; NSIP, non-specific interstitial pneumonia; PAI, plasminogen activator inhibitor; PAH, pulmonary arterial hypertension; PAMPs, pathogen-associated molecular patterns; PAR, protease-activated receptor; PASMC, pulmonary artery smooth muscle cells; PBMC, peripheral blood mononuclear cell; PDGF/PDGFR, platelet-derived growth factor/receptor; PECAM, platelet/endothelial cell adhesion molecule; PG, prostaglandin; PGF, placental growth factor; PG-Gs, microsomal prostaglandins; PI-3, phosphatidylinositol 3; PLD4, phospholipase D family member 4; PPARγ, peroxisome proliferation-activated receptor gamma; PRR, a pattern recognition receptor system in cell cytoplasm; PSD3, pleckstrin and Sec7 domain containing 3 gene; PTEN, phosphatase and tensin homolog; PTPN22, protein tyrosine phosphatase non-receptor type 22; PXK, *paraxylene–orthoxylene* (phox homology) domain containing serine/threonine kinase; RA, rheumatoid arthritis; RANTES, regulated upon activation, normal T-cell expressed and secreted; RGS-5, regulator of G protein signaling 5; RLRs, RIG-I-like receptors; RORγt, RAR-related orphan nuclear receptor gamma transcription factor; RP, Raynaud's phenomenon; R-Smads, receptor-regulated Smads; rTregs, resting Tregs; S1P/S1P$_1$, sphingosine 1-phosphate (type 1 receptor); scl GVHD, sclerodermatoses GVHD; Serpine 1, plasminogen activator inhibitor; Siglec-1, CD169, sialoadhesin; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; Smad, small mother against decapentaplegic family of transcription factors; Snail-1, a protein of the C2H2-type zinc-finger family that regulates transcription; SNPs, single nucleotide polymorphisms; sRAGE, soluble advanced glycation end products; SSc, systemic scleroderma; STAT, signal transducer and activator of transcription; TG, transgenic; TGF-β, transforming growth factor beta; TIMP-1 or -2, tissue inhibition of metalloproteinase-1 or -2; TLRs, toll-like receptors; TNFAIP3, tumor necrosis factor alpha-induced protein-3; TNFSF4, tumor necrosis factor superfamily member 4 gene; TNIP1, TNFAIP3 interacting protein-1; tPA, tissue t plasminogen activator; Tregs, T regulatory cells; TRPV, transient receptor potential vanilloid; Tsk-1 (Tsk-1/+), tight skin; TSP-1 or TSP-2, thrombospondin-1 or -2; UPAR, urokinase-type plasminogen activator receptor; VDR, vitamin D receptor; VE, vascular endothelial; VEGF, vascular endothelial growth factor; VitD, Vitamin D; VW, von Willebrand Factor; Wnt, proteins that form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis; WT, wild-type.

TABLE 1 | Important differences between limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc).

Features	lcSSc	dcSSc
Skin	Skin thickening occurs late, limited to the distal part of upper and lower extremities, face, neck, and upper chest. Telangiectasias and calcinosis are common. Tendon friction rub not seen	Skin thickening occurs early, moves up to proximal part of extremities and trunk. Telangiectasias and calcinosis may occur late in disease. Tendon friction rub present
GI	Esophageal dysmotility is more common than small and large intestine involvement	Esophageal dysmotility is frequently seen. Small and large intestinal involvement is more common
Pulmonary	Pulmonary fibrosis is less frequent and less severe. Frequent and severe pulmonary hypertension is more common	Pulmonary fibrosis is more common and severe. Pulmonary hypertension is less frequent
Kidney	Renal crisis uncommon	Renal crisis is more frequent
Autoantibody association	Anticentromere antibodies (ACA) are predominant	Anti-DNA topoisomerase I antibody (ATA) (Anti-Scl-70) antibody is predominant Anti-RNA polymerase antibody is more common

summarized below. A triggering event such as an infection or environmental toxin has been implicated as starting the processes that lead eventually to SSc in individuals with a permissive genetic background. The realization that an "interferon (IFN) signature" exists in most patients with SSc implies activation of the innate immune system and lends validity to the long-held suspicion that infections (such as with cytomegalovirus, Epstein-Barr virus, and more recently *Toxoplasma gondii*) could be SSc triggers in receiving more attention and a re-examination (7, 8). There is mounting evidence that the microbiota may play a role in development of autoimmunity, an area that is unexplored in SSc (9). Analysis of skin transcriptome has identified high levels of *Rhodotorula* sequences in dcSSc patients (10).

No animal model develops SSc that faithfully replicates human SSc, and this has impeded our understanding of the disease. There are many unresolved questions related to the etiopathogenesis of SSc. For example, it is unclear whether the innate/adaptive immune system abnormalities, vasculopathy, and fibroblast dysfunctions are separate, unrelated processes or are mechanistically linked, which of the three processes is of utmost importance and how interaction among the three processes leads to the development of the disease. These three processes will be discussed.
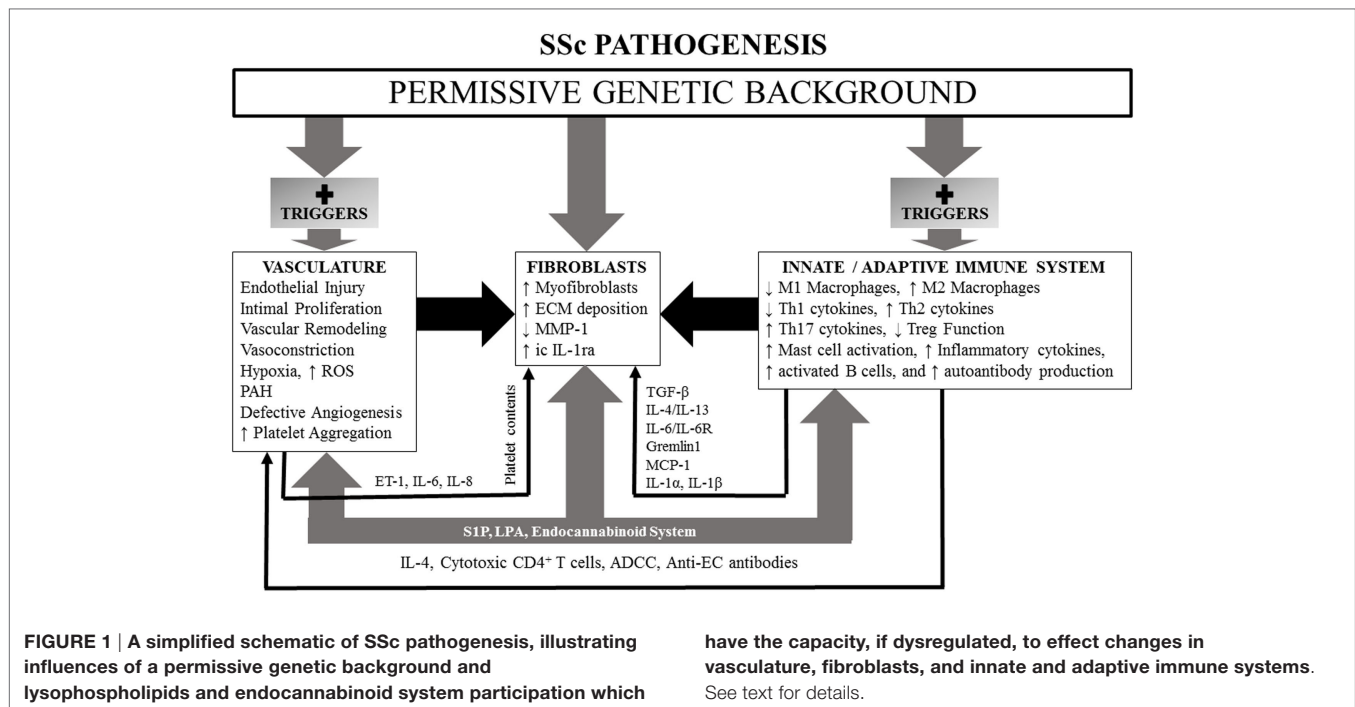
We first review evidence for genetic abnormalities in SSc since they can influence responses of the innate and adaptive immune systems, vascular function, connective tissue metabolism, and fibroblast function. Since the innate and adaptive immune systems are the first to respond to environmental triggers, be they infections or toxins in nature, and through generations of cytokines, chemokines, and growth factors that can affect function of vascular and connective tissue cells, we discuss them next. The vascular abnormalities and fibrosis in SSc are then discussed. The endocannabinoid system (ECS) (which influences functions of the immune system, vasculature, and fibroblasts) may be dysregulated in SSc as suggested by recent studies of SSc dermal fibroblasts. We have included a discussion of this important system with special emphasis on potential ECS targets that might offer new therapeutic approaches for management of SSc. Lysophospholipids [lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P)] and their different receptors (which regulate immunity, vascular physiology, and fibrosis) are dysregulated in SSc and likely contribute to the pathogenesis of the disease. Vitamin D (VitD) status also impacts function of most cell types and likely influences pathogenesis and

clinical features of SSc. An overall scheme of SSc pathogenesis is illustrated in **Figure 1**.

Genetics of SSC

Genetic influences have long been suspected to impact SSc. In families with a history of SSc, the incidence of disease can range from 1.5 to 1.7% (11). Having a family history of SSc increases the risk of developing disease 15–19-fold in siblings and 13–15-fold in first-degree relatives (11–13). Over the last decade, candidate gene study (CGS) approach and genome-wide association studies (GWAS) have been used to identify genetic associations that confer susceptibility to SSc. CGS and GWAS have allowed for the identification of genetic variations [single nucleotide polymorphisms (SNPs)] that are likely to be involved with the pathogenesis of scleroderma. CGS analyses SNPs to determine if the gene has association with a disease or a disease trait. The SNPs being studied have been selected based on their known association with other autoimmune diseases or on their possible functional relevance in the disease pathogenesis. GWAS arrays on the other hand, use tagSNPs to scan the entire genome to identify millions of SNPs. It takes into consideration the haplotype structure of the population being studied. Unlike CGS, GWAS identifies SNPs in a non-hypotheses-driven manner and allows for the identification of newly identifiable genes that were not previously identified in the disease. As regards to SSc, GWAS has confirmed major histocompatibility complex (MHC) II region as being most significant in this disease. Both CGS and GWAS have identified multiple genes that have been found to have firm associations in the pathogenesis of SSc.

Performing a GWAS can be very costly. Recently, the immunochip consortium was developed and implemented the immunochip analysis assay. The immunochip array provides high-density mapping of autoimmune diseases-associated loci using a custom SNP genotyping array (14). It was designed to increase efficiency of mapping autoimmunity risk loci and to reduce the cost of mapping (15). The immunochip uses variants from across 186 known autoimmunity risk loci and places them on an Illumina Infinium array platform. The platform contains 196,524 different variants of autoimmunity risk loci that may have functional significant effects in diseases like SSc. It also identifies variants with lower penetrance using a cost efficient strategy (14). Many of these genes have been

FIGURE 1 | A simplified schematic of SSc pathogenesis, illustrating influences of a permissive genetic background and lysophospholipids and endocannabinoid system participation which have the capacity, if dysregulated, to effect changes in vasculature, fibroblasts, and innate and adaptive immune systems. See text for details.

firmly established in the pathogenesis of SSc. In this review, we will focus on genetic associations in MHC – human leukocyte antigen (HLA, **Table 2**), non-HLA genetic SNP (Table 3), and microRNAs (miRNAs) (**Table 4**). We will focus on the most relevant associations first and then discuss others that may have modest effects on SSc.

HLA Association with SSc

The HLA-1 complexes HLA-A, -B, -C, and -G and HLA class-II complexes HLA-DP, -DQ, and -DR have all been identified in SSc susceptibility (11, 17, 108, 109) (**Table 2**). HLA class-II is the most significant region associated with SSc (14). *HLA-DRB1*01*, *HLA-DRB1*11*, *HLA-A*30*, and *HLA-A*32* have SSc susceptibility, while *HLA-DRB1*07*, *HLA-B*57*, and *HLA-Cw*14* are protective against SSc (17). *HLA alleles DRB1*0802* and *DQA1*0501* are associated with increased mortality (110). Clinical features of disease, disease phenotype, and SSc-specific autoantibodies have been distinguished based on HLA subtypes (**Table 1**). In a GWAS study that included 5471 SSc patients of European ancestry, *HLA-DQB1* locus was associated with anticentromere antibodies (ACA), *HLA-DPA1/B1* loci with anti-DNA topoisomerase I antibody (ATA), and neurogenic locus notch homolog 4 (NOTCH4) with ACA and ATA (24). In another study that included SSc patients of African American (AA) and Hispanic descent, *DRB1*1104*, *DQA1*0501*, *DQB1*0301*, and *DQB1* had strong positive association in SSc patients of Hispanic and of European ancestry (24, 31). *DRB1*0404*, *DRB1*11*, and *DQB1*03* alleles are associated with anti-U3 ribonucleoprotein (ARA) in this subpopulation (24). In this same subpopulation, *DRB1*0701*, *DQA1*0201*, *DQB1*0202*, and *DRB1*1501* had a negative or protective association against SSc (27). These studies have also identified *DRB1*11* with association

with ATA and *DRB1*01*, *DRB1*04*, and *DRB1*0501* have association with ACA (31). *HLA-DPB1* and *HLA-DPB2* SNPs rs7763822/rs7764491 and rs3117230/rs3128965 have strong association with ATA or ACA positivity (25). In AA patients with SSc, *DRB1*0804*, *DQA1*0501*, and *DQB1*0301* are associated with SSc (28), and have a higher frequency of ARA or anti-fibrillarin antibody (AFA) positivity (111).

*HLA-DRB1*1101*, **1104*, **1501*, and **0802* (commonly associated with the dcSSc subset) show the amino acid sequence [67]FLEDR[71] in their β chain, whereas *HLA-D Q β1* alleles **D301*, **0302*, **0401*, **0402*, **0601*, and **0602* (commonly associated with SSc) show a [71]TRAELDT[77] motif on their β chain (29). In a study in French SSc patients with European ancestry, both FLEDR and, to a lesser degree, TRAELDT were associated with dcSSc (29). Addition of a tyrosine at position 30 strengthened the TRAELDT association with dcSSc (29). Further analysis showed that the FLEDR motif had the highest association with SSc patients who were ATA positive, while TRAELDT had lesser association in this subset (29). The TRAELDT association with ATA positivity and dcSSc were not dependent entirely on FLEDR (29). The authors concluded that double dose of the shared epitope, as well as compound heterozygosity, may confer a higher risk for development of SSc.

HLA-DPB1 and *-DPB2* are reported to have strong susceptibility with SSc in the Korean and Chinese populations (25). Subtypes *DPB1*1301* and *DPB1*0901* were most common in Korean patients with SSc, while *DPB1*03:01*, *DPB1*13:01*, *DQB1*03:03*, *DQB1*05:01*, and *DQB1*06:11* were significantly increased in the Chinese SSc patient population (26). Those who carried the *DPB1*03:01* had a higher chance of developing pulmonary fibrosis verses those who carried *DPB1*04*, and those SSc patients were more likely to be ACA positive (112). *DQB1*03:03* and *DQB1*05:01* were strongly associated with

TABLE 2 | HLA genes associated with SSc.

HLA-associated genes	Population (n = SSc)	Disease phenotype and/or clinical features	Autoantibody association	Reference
HLA CLASS I GENES ASSOCIATED WITH SSc				
A*30	Brazil (141)	Pulmonary fibrosis	ATA	(16, 17), (16, 18–22), (17), (17),
B*13	European ancestry (95)	PAH		(16), (18), (17), (23)
B*35	Brazil	PAH		
	Native Indian	dcSSc		
	Hispanic			
B*62	European ancestry (95)	dcSSc and high skin scores, pulmonary fibrosis		
B*65		PAH		
C*04	Brazil	PAH		
Cw4	Native Indian	Pulmonary fibrosis	dcSSc pulmonary fibrosis ATA	
Cw*0602	European ancestry (95)	Pulmonary fibrosis	ARA	
G	European ancestry	Lower vascular cutaneous	dcSSc	
	African	ulcers telangiectasias		
	Brazilian	arthropathy		
HLA CLASS-II GENES ASSOCIATED WITH SSc				
DPA1/B1	European ancestry (5,471)	Pulmonary fibrosis	ATA	(24–26)
DPB1*1301	Korean Chinese (338)		ACA, ATA, and dcSSc	
*0901			ATA	
DPB1/B2 SNPs				
rs3128930				
rs7763822/rs7764491	European ancestry (1,107)		ACA	
rs3117230/rs3128965				
rs3117230	Caucasian			
rs7763822	African American		ACA	
rs7764491	Hispanic		ATA	
rs3117230				
rs312965				
rs3128965A				
DPA1/B1				
DQA1	African American	Pulmonary fibrosis	ATA/ACA	(16, 18, 19, 26–30)
*0501	Native American		dcSSc	
DQB1			ACA	
*03:01	European ancestry		Anti-U1RNP/	
*03:03	Korean/Chinese		ATA	
*04:00	Hispanic			
*05:01	Italian			
*06:11	Brazilian		ATA	
*26:00	Native American			
[71]TRAELDT[77]	French (282)		dcSSc ATA	
DRB1	European ancestry			(17–19, 29–34)
	Hispanic			
	Italian			
*01			ACA	
*0404			ACA	
*05			ACA	
*07				
*0804	African American (278)		AFA/ARA	
*11		Increased skin score, pulmonary fibrosis	ATA	
*1104			ACA	
			ATA	
*1502	Thai (50)	No association PF, DU, sclerodactyly, myositis, SICCA	ATA	
*1602	Native American (32)	dcSSc PF	ATA	
*0407	European ancestry	Renal crisis dcSSc		
	African American			
	Hispanic (1,517)			
*1304	European ancestry			
	African American			
	Hispanic (1,517)			
DRB5*01:02	Thai (50)		ATA	
[67]FLEDR[71]	French (282)	dcSSc	ATA	

(Continued)

TABLE 2 | Continued

HLA-associated genes	Population (n = SSc)	Disease phenotype and/or clinical features	Autoantibody association	Reference
NOTCH4	European ancestry (5,471)		ACA ATA	(24)
GENES WITH NEGATIVE ASSOCIATION FOR SSc				
C*03	Brazilian Spanish	PAH	ATAq	(16, 27, 30, 33)
DQA1	European ancestry (1,300)			
*0201	Hispanic			
*0501	Italian Spanish (940)	dcSSc		
DQB1	European ancestry			
*0202	Hispanic			
*04	Brazilian	dcSSc		
DRB1	Brazilian			
*01	Spanish			
*04	Thai			
*0701				
*1501				

TABLE 3 | Non-HLA genes associated with SSc listed from largest to smallest SSc population analyzed.

No.	Non-HLA-associated genes	Other diseases identified	Study type	Population (n = SSc)	Polymorphism/SNPs (OR)	Disease association	Proposed function	Reference
1	BANK1	SLE	Case–control meta-analysis cohort	European ancestry (21,568)	rs10516487*G (1.12), rs17266594*T (1.14), rs3733197* A (0.73), AA (0.41), AG (0.85)	dcSSc ATA+	Mobilization of calcium from intracellular stores in B-cell receptor	(35–37)
2	IRF5	SLE, RA, UC	GWAS meta-analysis immunochip array case–control cohorts	European and Asian ancestry (15,251)	rs2004640 (0.84), rs2004640*TT (1.56), rs10488631 (1.63), rs4728142 (1.22)	dcSSc, lcSSc, ATA, ACA, interstitial lung, longer survival mild PF	Regulate IFN-gene expression and inflammatory cytokine production. Stimulate TLR expression	(38–42), (42–44), (14, 45, 46)
3	PXK	SLE	Immunochip pan-meta-analysis GWAS	European ancestry (12,685)	rs2176082 (1.21), rs4681851 (1.58)	SSc, ACA	Degradation and trafficking of epidermal growth factor	(14, 47)
4	STAT4	SLE, RA, primary biliary cirrhosis	GWAS immunochip meta-analysis cohort, case–control cohorts	Multi-ethnic ancestry (10,696)	rs7574865 (1.29), additive effect with IRF5 rs2004640 (1.72-2.752), rs11889341 (1.33), rs7574865*, TT vs. GG (0.49), TT vs. TG (0.48), TT + TG vs. GG (0.74), T vs. G (0.72), rs10168266*, CC (0.69), T (1.44)	lcSSc, ACA, fibrosing alveolitis, increased in patients who carry TBX21 CC genotype, dcSSc, ATA, pulmonary fibrosis	T-cell signaling and differentiation; signaling IFN1; regulate cytokine signals	(38, 48–54)
5	PTPN22	DM-1, RA, SLE	Meta-analysis case–control cohorts	Multi-ethnic and European ancestry (10,204)	PTPN22 CT/TT (2.21) higher risk of SSc than PTPN22 CC (1.70), PTPN22, C1858T [rs2476601* T (1.15)], [1858 T (1.147)], 1858 C	ATA and ACA, ACA, protective	T-cell receptor signaling	(55–58)
6	TNFSF4	SLE	Meta-analysis cohort	European ancestry (10,093)	rs1234314 (1.15), rs12039904 (1.18), rs2205960*AA (1.33), rs844648 (1.10), rs844644 (0.91)	dcSSc, lcSSc and ACA+, protective in all sub-groups of SSc except ARA+	B-cell proliferation and differentiation T-cell stimulation and survival	(48, 59–61)
7	BLK	SLE	Case–control meta-analysis	European and Asian ancestry (9,305)	rs2736340 (1.27), rs13277113 (1.16), C8orf13-BLK, and BANK1 additive effect, FAM167-BLK	dcSSc, dcSSc, dcSSc, lcSSc	Disruption in B-cell gene expression and abnormal NFκB signaling	(48, 62–64)

(Continued)

TABLE 3 | Continued

No.	Non-HLA-associated genes	Other diseases identified	Study type	Population (n = SSc)	Polymorphism/SNPs (OR)	Disease association	Proposed function	Reference
8	IL-2, IL-2RA	SSc	Case–control cohort	European (7,516)	IL-2: rs6822844 (0.86), rs907715 (0.91), rs2069762*A- (1.06), rs6822844*T- (0.86), rs683545*G (0.93), rs907715*T (0.91); IL-2RA genes: rs11594656, rs2104286 (1.30), rs12722495	SSc, lcSSc, dcSSc, and lcSSc when ACA+	T-cell proliferation and turning off T-cell response	(65, 66)
9	DNASE1L3	SLE, RA	Immunochip	European ancestry (7,169)	rs35677470 (2.03)	ACA	Defective apoptotic DNA breakdown	(14, 46)
10	JAZF1	SLE	GWAS pan-meta-analysis	European ancestry (6,835)	rs1635852 (1.13)	SSc	Repress transcription	(47)
11	KIAA0319L	SLE, dyslexia	GWAS pan-meta-analysis	European ancestry (6,835)	rs2275247 (1.49)	lcSSc	Protein coding	(47)
12	IL-12Rβ2	Psoriasis, Behcet's disease, primary biliary cirrhosis	GWAS	European ancestry (6,250)	rs3790567 (1.17), rs2305743* A/G (0.81), rs8109496* C/G (0.82), rs436857* A/G (0.81), rs11668601* C/T (0.84)	SSc	Stimulates IFN production and TH1 differentiation	(67)
13	IRF8	SLE	Cohort meta-analysis	European and Asian ancestry (6,201)	rs11642873 (0.75), rs2280381 (1.36)	lcSSc	Regulate IFN-gene expression and inflammatory cytokine production. Stimulate TLR expression	(24, 68)
14	CD247	SLE	GWAS	European ancestry (6,080)	rs2056626 (0.78)	SSc-G minor allele protective effect	T-cell signaling and activation	(49, 69)
15	ATG5	SLE	Immunochip	European ancestry (5,850)	Intron rs9373839* G (1.19)	SSc	Autophagy vesicle formation	(14)
16	IL-12A-SCHIP	Primary biliary cirrhosis, idiopathic pulmonary fibrosis	Immunochip	European ancestry (5,850)	rs77583790 (2.81), intergenic between SCHIP1 and IL-12A	LcSSc	Stimulates IFN production and TH1 differentiation	(14)
17	CSK	SLE, RA	GWAS	European ancestry (5,270)	rs1378942 (1.2)	SSc	Cell regulation, differentiation, migration, and immune response	(70)
18	PSD3	Hepato-cellular carcinoma	GWAS	European ancestry (5,270)	rs10096702 (1.18)	SSc	Binding protein for signal transduction	(66)
19	IL-12Rβ1	Psoriasis, Behcet's disease, primary biliary cirrhosis	Immunochip	European ancestry (5,052)	rs2305743 (0.81), rs436857	SSc	Stimulates IFN production and TH1 differentiation	(71)
20	IRAK1	SLE	Cohort meta-analysis	European ancestry (4,873)	rs1059702*TT (1.43)	dcSSc, ATA, SSc-related fibrosis alveolitis	Influence T-cell receptor signaling and TLR activation. Regulator of NFκB by way of X chromosome	(42, 48, 72)
21	IL-21	RA, SLE, DM-type 1, Graves' disease, celiac disease	Case–control	European ancestry (4,493)	rs6822844 (0.86), rs907715 (0.91), allelic combination: rs2069762* A- (1.06), rs6822844* T- (0.97), rs6835457*G- (0.93), rs907715* T (0.91), rs6822844* T (0.84)	lcSSc, ACA+, global SSc, dcSSc, lcSSc, protection: SSc, lcSSc, and ACA+	B-cell differentiation and regulates TH17 development	(65)

(Continued)

TABLE 3 | Continued

No.	Non-HLA-associated genes	Other diseases identified	Study type	Population (n = SSc)	Polymorphism/SNPs (OR)	Disease association	Proposed function	Reference
22	TNIP1	RA, SLE, psoriatic arthritis	GWAS cohort	European ancestry (4,389)	rs9275224 (0.69), rs6457617 (0.69), rs9275245, rs3130573 (1.12) located in PSORC1C1 gene, rs2233287 (1.19), rs4958881 (1.19), rs3792783 (1.19)	Global SSc, global SSc except ACA+: dependent on HLA II, global SSc	Negative regulatory of NFκB	(48, 73, 74)
23	MIF	SLE, psoriatic arthritis, Lofgren's syndrome, inflammatory bowel disease	Cohort	European ancestry (4,286)	MIF-173 (1.10)	dcSSc	Activates innate immunity and sustains cellular response	(60, 75)
24	NFkβ1	Behcet's disease, Grave's disease, Hashimoto thyroiditis	GWAS	European ancestry (4,156)	rs1598859 (1.19)	SSc	Controls inflammation, transcription regulator	(66, 76)
25	CD226	SLE, DM-type 1, multiple sclerosis	Cohort	European ancestry (4,131)	rs763361* T (1.02), rs34794968 (0.90), rs727088 (1.02)	dcSSc, ATA+, ILD, pulmonary fibrosis	Co-stimulator of T cells and T-cell adhesion	(77–80)
26	PPARγ	RA, psoriatic arthritis, DM-type 1	GWAS	European ancestry (3,989)	rs310746 (1.28)	SSc	Blocks TGF-β, mediated fibrosis	(81–83)
27	ITGAM	SLE	Case–control cohort meta-analysis	European ancestry (3,735)	rs1143679* A (1.12)	SSc	Regulates neutrophil and monocyte cell activation and adhesion. Phagocytosis of complement-coated particles	(47, 84, 85)
28	TNFAIP3	SLE, RA, celiac disease, multiple sclerosis	Cohorts case–control	European and Asian ancestry (3,365)	rs5029939* G (2.08), rs6932056 (1.69), rs117480515* A (3.20), rs117480515* A (3.94)	dcSSc, fibrosing alveolitis, PAH, SSc, polyautoimmune subset	Regulate immune system signaling by regulating ubiquitin	(43, 48, 68, 86)
29	IRF7	SLE	Case–control	European ancestry (2,316)	rs1131665 (0.87)	ACA	Regulate IFN-gene expression and inflammatory cytokine production. Stimulate TLR expression	(87)
30	IL-23R	Inflammatory bowel disease, psoriasis, AS	Case–control	US – multi-ethnic and European ancestry (2,134)	rs11209026*GG (0.81), (Arg381 Gln), rs11465804*TT (0.83)	ATA, protection against PAH, dcSSc, ATA, protection against PAH	Promotes TH17 expansion	(28, 88, 89)
31	TLR2	Inflammatory bowel disease, multiple infections	Cohort	European ancestry (1,170)	rs5743704 (2.24), (Pro63 His)	dcSSc, ATA+, PAH	Pathogen recognition and direct immune response	(90)
32	CD87(UPAR)	Vascular disease, paranasal disease	Cohort	European ancestry (732)	rs344781*GG (1.96)	SSc-associated digital ulceration, PAH, ACA, dcSSc, lcSSc	Promotes extracellular matrix and vascular remodeling	(91)
33	PLD4	RA	Cohort	Japanese (730)	rs2841277 (1.29), rs2841280* G (1.29), (minor)	lcSSc, dcSSc, protective SSc	Phagocytosis of microglia	(68)
34	MMP-12	Kidney disease, skin diseases, arthro-sclerosis	Cohort	Italian ancestry (250)	rs2276109*AA (2.44)	dcSSc, lcSSc, ATA+, pulmonary fibrosis	Inhibits endothelial cell proliferation and angiogenesis	(92)

ACA, while *DQB1*06:11* was associated with ATA positivity and a marginal association with pulmonary fibrosis. *DQB1*03:01* had an increase frequency of anti-U1RNP positivity in Chinese patients with SSc (26).

The role of HLA II in Italian and Spanish SSc patients has also been examined. *HLA-DRB1*1104*, *DQA1*0501*, and *DQB1*0301* haplotypes are overexpressed in this patient population (30). Carrying the *HLA-DQB1*03* and *HLA-DRB1*11* alleles are risk factors for developing SSc in this subset of patients. Having the *HLA-DRB1*0701* allele was protective (30). *HLA-DRB1*1104* allele has association with ATA, while *HLA-DQB1*0501* in ATA patients is protective (30). ACA-positive patients expressed *HLA-DRB1*01* and *-DQB1*05*. Patients who had pulmonary fibrosis were found to have an association with *DRB1*11* (32).

*HLA-A*30* and *-DQB1*04* alleles were found to relate to SSc susceptibility in a subset of Brazilian patients (16). In patients who had PAH, *HLA-B*35*, and *C*04* were associated as risk genes for this complication, while *C*03* was protective (16). *HLA-DRB1*15:02* and *DRB5*01:02* are associated with ATA positivity in SSc Thai patients. There were no associations seen between these genes and other clinical manifestations of disease including pulmonary fibrosis, digital pits, sclerodactyly, myositis, or SICCA symptoms. *DRB1*04* was protective in this patient population (33).

In a population of French SSc patients of European ancestry, amino acid sequences [67]FLEDR[71] shared by *HLA-DRB* was associated with ATA positive and dcSSc. Amino acid sequence [71]TRAELDT[77] shared by *HLA-DQB1* showed weak association in dcSSc patients with positive ATA (29). A higher prevalence of SSc has been identified in the Choctaw Indian population in comparison to non-full-blooded Choctaws, other Native Americans, as well as the general population (18, 19). Multiple genetic loci located on chromosome 6 near the HLA complex have been identified and may contribute to the high prevalence of disease (19). *HLA-B35*, *Cw4*, *DRB1*1602*, *DQA1*0501*, and *DQB1*0301* are strongly associated with SSc in the Choctaw Indian population who present clinically with dcSSc, pulmonary fibrosis, and ATA positivity (18).

*HLA-B*62* and *HLA-DRB1*07* correlate with diffuse skin involvement while high skin scores correlate with *HLA-DRB1*11* (17). *HLA-B*62* and *HLA-Cw*0602* has association with pulmonary fibrosis, while *HLA-B*13* and *HLA-B*65* with PAH (17). *HLA-B*35* is associated with a high risk of developing PAH in systemic sclerosis by influencing the production of endothelin-1 (ET-1) and decreasing endothelial nitric oxide synthase (eNOS) (18, 20–22). HLA-G is expressed in skin of patients with systemic sclerosis. Its presence is associated with having lower vascular cutaneous ulcers, telangiectasias, and inflammatory arthropathy (23).

Non-HLA-Associated Genes

Multiple studies including GWAS, meta-analysis, and recently immunochip array analysis have repeatedly shown that modifications in *CD247*, interferon regulatory factor 5 (*IRF5*), and signal transducer and activator of transcription protein 4 (*STAT4*) genes are associated with SSc susceptibility (**Table 3**). Many autoimmune disorders share a common genetic background. Both systemic lupus erythematosus (SLE) and SSc share many clinical features and genetic components. Disease sample size and lack of statistical power limits the ability to determine which genes may contribute to autoimmunity. Combined analysis of different autoimmune diseases increase sample size and allows for statistical power to identify genetic variants that effect disease. Using a GWAS pan-meta-analysis approach allows for the detection of new genetic susceptibility loci, as determined by Martin et al. (47). In the Martin et al. study, GWAS pan-meta-analysis approach for SSc and SLE identified and validated three new susceptibility genes for SSc [*KIAA0319L*, paraxylene–orthoxylene domain containing serine/threonine kinase (*PXK*), and *JAZF1*] (47). Genes related to cellular response to IFNγ and the nervous system was overrepresented in both SLE and SSc. In SSc, genes related to cell signaling, migration, and adhesion were over-represented (47). In this section of the review, we will discuss Non-HLA-associated genes reported to be associated with SSc. In **Table 3**, we have listed the non-HLA SSc-associated genes in order of decreasing SSc sample size analyzed.

Autophagy Protein-5

Autophagy protein-5 (ATG5) is an ubiquitin ligase protein that assists in autophagosomal elongation that mediates pathogen clearance; allowing for the degradation of unwanted cytoplasmic material. It has a role in the development of both the innate and adaptive immune system (14). Variations in *ATG5* are associated with susceptibility in SLE and childhood and adult asthma (14). Variants located within *ATG5* intron rs9373839 G minor allele have been identified as SSc susceptibilities (14). The location of this variant may suggest that distant genes may affect downstream the function of *ATG5*.

B-Cell Scaffold Protein with Ankyrin Repeats 1

B-cell scaffold protein with ankyrin repeats 1 (BANK1) exerts influence in B-cell receptor-induced calcium mobilization from intracellular (IC) stores. It has been identified in SLE as a susceptibility gene. There is an increased risk for developing SSc with *BANK1* haplotype G–C compared to A–T haplotype (35). *BANK1* variants rs3733197 G alleles, rs10516487, rs10516487*G, and rs17266594*T are strongly associated with diffuse dcSSc and ATA autoantibodies (36).

B-Lymphocyte Kinase

B-lymphocyte kinase (BLK) encodes B-cell signal transducer and functional variant C8orf13-BLK. Disruption in *BLK* may result in abnormal B-cell gene expression and altered NFκB signaling (48). *C8orf13-BLK* has been identified in multiple studies as a risk gene for SSc (62–64). *C8orf13-BLK* variant rs2736340 and rs13277113 are associated with SSc and dcSSc (63). An additive effect between *C8orf13-BLK* and *BANK1* increases susceptibility to dcSSc (62). Two haplotype blocks (*FAM167A* and *BLK*) have also been identified. Allele rs13277113*A in the *BLK* block is significantly associated with SSc (64). This association was observed despite autoantibody profile or disease classification (dcSSc or lcSSc) (64).

CD247

CD247 encodes T-cell receptor zeta (CD3ζ), which functions in the assembly of TCR–CD3 complex and its transport to the cell surface, thereby playing a crucial role in cell signaling (49). Variants of *CD247* may lead to impaired immune response and dysregulation of T-cell activation. *CD247* has been associated with susceptibility to SLE. *CD247* rs2056626 (in addition to *IRF5*, MHC, and *STAT4*) were identified as susceptibility genes for SSc in multiple studies. The G minor allele of this variant has a protective effect (49, 69). This variant was not found to have an association with SSc or disease subtypes in a Hans Chinese cohort, suggesting that the association may be ethnicity-dependent (113).

c-SRC Tyrosine Kinase

c-SRC tyrosine kinase (CSK) is important for cell regulation, differentiation, migration, and immune response. *CSK* inactivates src kinases by phosphorylating tyrosine at the C-terminus. In fibrosis, srk kinases regulate FAK needed for integrin signaling and fibroblast adhesion to extracellular matrix (ECM). Incubating fibroblasts with inhibitors of *CSK* decreases COLIAI and COLIA2. Polymorphisms in *CSK* prevent or inhibit the phosphorylation of src leading to fibrosis (70). Polymorphism in the intron of the *CSK* gene is associated with SSc. Variant rs1378942 is associated with overall SSc (70).

Deoxyribonuclease 1-Like 3

A member of the human DNase 1 family, deoxyribonuclease 1-like 3 (DNASE1L3) is secreted by macrophages and is found in the liver and spleen (14). During apoptosis, *DNASE1L3* has a role in the fragmentation of DNA. It also generates double-strand breaks in immunoglobulin-encoding genes. In regards to autoimmune susceptibility, *DNASE1L3* is found to be associated with susceptibility to SLE and rheumatoid arthritis (RA). Using the immunochip array, *DNASE1L3* SNP rs35677470 was identified as a risk for SSc and ACA positivity. These authors identified a substitution in amino acid Arg to Cys at position 206 on exon 8 of *DNASE1L3* protein resulted in the loss of a hydrogen bond. The amino acid substitution in this position may cause the protein to become inactive suggesting a potential role for SNP rs35677470 in autoimmunity due to defective apoptotic DNA breakdown (14).

Interferon-Regulated Genes and PAH

Multiple studies using GWAS, meta-analysis, and immunochip analysis assays have confirmed the involvement of IFN in SSc susceptibility. The identification of multiple variants in IFN genes in association with SSc, SSc lung disease, and SSc mortality highlights the significance of the IFN pathway in the development and progression of SSc. IFN modulate differentiation, survival, proliferation, and cytokine production by T and B cells and dendritic cells. IFN stimulate the expression of toll-like receptors (TLRs) 3, 7, and 9. IFN genes were overexpressed in peripheral blood mononuclear cells (PBMCs) from patients with SSc and SLE. Higher IFN scores correlated with ATA, anti-U1RNP, lymphopenia, and IFNα/IFNβ receptor 2 (*IFNAR2*) missense mutation rs7279064 GG or GT (114). Other variants in the IFN pathway have also been well established in SSc. Polymorphisms

in *IRF5*, *IRF7*, and *IRF8* have been identified. *IRF5* mediates IFN activity and is an important inflammatory signaling pathway. Polymorphisms in IRF5 are associated with SLE, RA, ulcerative colitis, and others. Regulation in immune reaction to infections by *IRF5* is activated by TLRs 7 and 9. In SLE, *IRF5-transportin-3* gene (*TPO*) rs4728142 correlates with *IRF5* expression leading to increased binding of zinc-finger BD 3 (ZBTB3) affecting both RNA transcription and DNA binding (115). In SSc, *IRF5* rs200460 is associated with dcSSc, lcSSc, ATA, and ACA. The strongest association is with ATA and ILD (38). It is linked to overall mortality independent of disease type or serology (39). A Han Chinese cohort of 424 SSc patients identified rs2004640*TT genotype as being significant in this population. This variant is associated with pulmonary fibrosis and ATA positivity (40). *IRF5* rs4728142 is predictive of longer survival and milder pulmonary fibrosis. The association is independent of age of disease onset, autoantibody profile, or disease type (41). *IRF7*: Interferon regulatory factor 7 (IRF7) activates type IFN genes in response to DNA/RNA immune complexes and viral infections. *IRF7* associates with susceptibility to SLE. Multiple variants in the *IRF7* genes confer susceptibility to SSc. *IRF7* rs1131665 is associated with SSc-associated ACA positivity. The variants identified were replicated in a Spanish cohort (87). *IRF8*: Multiple studies have identified *IRF8* association with SSc and rs11642873 with lcSSc (24). *IRF8* rs2280381 has been identified as SSc susceptible gene in a Japanese cohort (68).

Attention has focused on the possible contribution of the immune system to pathogenetic processes in PAH, especially innate immunity and IFNs (116, 117). Type I IFNs are implicated by the association of use of IFNα in the treatment of hepatitis and of IFNβ in the treatment of multiple sclerosis (MS) with development of PAH (118, 119). Diseases in which there is an "IFN signature" (such as SLE, SSc, and infection with HIV) are associated with development of PAH (120–124). Furthermore, IFNα and IFNγ added to cultures of human pulmonary artery smooth muscle cells (PASMC) primed with TNFα or to cultures of human lung MVEC or human lung fibroblasts, cause release of the potent vasoconstrictor, ET-1, and of IFN-inducible protein-10 (IP-10) (117). In a series of 128 SSc patients with PAH and 35 patients with no PAH, the SSc patients with PAH had higher levels of IP-10 and ET-1 in their sera compared to SSc patients without PAH or compared to healthy controls. More SSc patients with PAH had detectable levels of IFNα and IFNγ in their sera than SSc patients without PAH (117). In this series of SSc patients, levels of TNFα, IL-12p70, IL-6, IL-1α, and IL-8 were significantly higher in sera in SSc patients with PAH when compared to SSc patients without PAH (117). Additional studies of this patient group revealed that serum levels of IP-10 in the SSc-PAH patients correlated with pulmonary vascular resistance, and levels of brain natriuretic peptide in serum, and serum IP-10 levels in the SSc-PAH patients inversely correlated with cardiac index and 6-min walks test (117). Sections of lung from patients with idiopathic PAH (IPAH) or with SSc-PAH expressed higher levels of type I interferon receptor 1 (IFNR1) in endothelium, smooth muscle layer, vascular interstitium, and in intravascular inflammatory cells as assessed by immunohistochemistry and Western blotting (117). While the above studies strongly implicated type I

IFN as playing a pathogenic role in SSc-PAH and IPAH, further evidence was substantiated in the type I interferon α receptor 1 knockout mouse which was found to be resistant to experimental hypoxic PAH induction. These mice did not have elevated serum levels of ET-1 when compared to wild-type (WT) control mice (117). Analysis of PBMC from patients with SSc revealed CD169/sialoadhesin (*Siglec-1*) and other IFN-regulated genes were over-expressed in patients with dcSSc, whereas patients with lcSSc with PAH overexpressed *IL-13RA1*, intercellular adhesion molecule-1 (*ICAM-1*), *C–C chemokine receptor type 1 protein or gene* (*CCR1*), *JAK2*, and *melanocortin receptor 1* (*MCR1*) (123, 125, 126). IL-13 was also elevated to higher levels in sera of patients with lcSSc with PAH, and *MCR1* was induced on CD14⁺ monocytes suggesting monocytes are activated in lcSSc patients with PAH of an alternative (i.e., IL-4/IL-13) rather than classical [i.e., IFNγ/lipopolysaccharides (LPS)] pathway (123). The identification of multiple IFN genes having association in SSc, SSc lung disease, and mortality highlights the significance of the IFN pathway in the development and progression of SSc.

Interleukin-1 Receptor Associated Kinase 1

Interleukin-1 receptor associated kinase 1 (*IRAK1*) gene is located on the Xq28 and is in the same haplotypic block with methyl-CpG-binding protein 2 gene (*MECP2*). *IRAK1* encodes a serine/threonine protein kinase that regulates NFκB through T-cell receptor signaling and TLRs/IL-1R activation. It also plays a role in IFN induction. IRAK1 has been identified in SLE as a susceptibility gene (42, 72). In SSc, *IRAK1* rs1059702*TT is associated with dcSSc, SSc-related fibrosing alveolitis, and ATA positivity (42, 72). The presence of the T allele may contribute to disease severity, and presence of *MECP2* rs17435 may explain the association of *IRAK1* variant rs1059702 with this subset (42, 72).

IL-2/IL-12 Genetic Susceptibility

Variants in interleukin-2 receptor α (IL-2A), *IL-12R* (*IL-12Rβ1* or *IL-12Rβ2*) have been reported to be associated with SSc. *IL-2* plays a role in immune system homeostasis and self-tolerance. It facilitates B-cell immunoglobulin production and induces natural killer cell proliferation and differentiation (65). The binding of *IL-12* to its receptors stimulates IFN production and promotes TH1 differentiation. IL-12 signals through STAT pathway and a defect in either *STAT4* or *IL-12R* could influence SSc pathogenesis. Variant SNP rs77583790 found in the intergenic region between *SCHIP1* and *IL-12A* was found to be associated with lcSSc (14). *IL-12Rβ1* and *IL-12Rβ2* recruit tyrosine kinases and activate transcription of other genes. Polymorphisms in IL-*12Rβ1* and *IL-12Rβ2* have been identified in psoriasis, Behcet's disease, and primary biliary cirrhosis (67). Two studies were conducted to investigate the role of *IL-2* in SSc. ILR2 gene variants: rs11594656, rs2104286, and rs12722495 were associated with SSc, lcSSc, and ACA positivity. The associations are strongly dependent on ACA since removal of ACA from the analysis resulted in loss of association, and the strongest association with ACA positivity was with rs2104286, with associations of the other IL-2 RA gene variants being lost after conditioning to rs2104286 (66). Polymorphism in rs2104286 has the strongest association with

ACA while rs6822844 and rs907715 have association with SSc and lcSSc (66). *IL-12Rβ1* rs2305743 and rs436857 were found to be associated with SSc (71). Polymorphisms in these receptors may affect the binding of transcription factors decreasing the expression of IL-12. *IL-12Rβ2* rs3790567 is associated with SSc. *IL-12Rβ2* gene maps close to the *IL-23* coding region, the association between rs3790567 was not found to be dependent on *IL-23* (67). *IL-2/IL-21*: *IL-21* affects the innate and adaptive immune response playing a role in the differentiation of B cells into plasma cells and regulation of TH17 development (65). Polymorphism in the *IL-2/IL-21* region is associated with lcSSc and global SSc. *IL-2/IL-21* variant rs682284 is strongly associated with multiple autoimmune diseases and is considered an autoimmune susceptibility locus (127). The rs907715 minor allele and rs682284 have association with SSc. Variant rs6822844 influences lcSSc and ACA positivity (65). The allelic combination of rs2069762*A–rs6822844*T–rs6835457G–rs907715*T is associated with dcSSc and lcSSc (65). The T allele for rs6822844 acts as a protective for lcSSc and ACA positivity.

IL-23

IL-23 promotes the expansion of TH17. *IL-17* and *IL-23* are elevated in the plasma of SSc patients (28). Polymorphism in *IL-23R* is associated with SSc and ATA positivity. *IL-23R* variant rs11209026*GG (Arg381 Gln variant) has association with ATA positivity and rs11465804*TT is associated with dcSSc and ATA positivity. The major alleles rs11209026*G and 11465804*T were decreased in patients with PAH, suggesting that the major allele is protective against PAH (28).

Integrin αM

Integrin αM (ITGAM) β2 is a leukocyte-specific integrin that regulates neutrophil and monocyte cell activation and adhesion. It allows for phagocytosis of complemented-coated particles. Deficiency in ITGAM results in increased IL-6 production by antigen-presenting cells (APC) (128). Pooled meta-analysis, subsequent independent meta-analysis, and GWAS looking at shared risk polymorphisms for SLE and SSc confirmed *ITGAM* variant rs1143679 were associated with susceptibility to SSc (47, 84, 128).

Juxtaposed with Another Zinc-Finger 1

Juxtaposed with another zinc-finger 1 (*JAZF1*) encodes a nuclear protein with zinc-fingers that functions to repress transcription. It has been associated with bone morphogenesis and CI deposition (47). *JAZF1* has been identified as an SLE-associated locus, and a recent GWAS pan-meta-analysis has confirmed *JAZF1* rs1635852 association with SSc (47).

KIAA03192L

KIAA03192L has been identified in polycystic kidney disease and dyslexia as a disease susceptibility gene. It is expressed in macrophages and natural killer cells in mice and in CD33⁺ myeloid cells and CD14⁺ monocytes in humans. *KIAA03192L* is overexpressed in PBMCs of SLE patients. In SSc, *KIAA03192L* variant rs2275247 is associated with lcSSc (47).

Protein Tyrosine Phosphatase Non-Receptor Type 22

Protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) plays a critical role as a gatekeeper for T-cell receptor signaling. It encodes the protein tyrosine phosphatase lymphoid tyrosine phosphatase in T-cells and acts to inhibit T-cell signaling through dephosphorylation of substrates. Polymorphism in *PTPN22* has been associated with type 1 DM, RA, and SLE. Earlier studies looking at the relationship between *PTPN22* and SSc failed to show an association between *PTPN22* and SSc (129, 130). Larger studies in SSc patients showed association with *PTPN22* Ct/TT genotypes with both ATA and ACA positivity. The T allele associated with ATA positivity and the CC genotype with both ACA and ATA positivity (55). Meta-analysis confirmed *PTPN22* rs2476601*T and the minor allele 1858T are associated with SSc and ACA positivity (56, 57). Haplotype 1858C allele was protective in a French cohort (58).

Paraxylene–Orthoxylene (Phox Homology) Domain Containing Serine/Threonine Kinase

Paraxylene–Orthoxylene domain containing serine/threonine kinase is a protein that plays a role in the ligand-induced internalization, degradation, and trafficking of epidermal growth factors. Variation in PXK is association with SLE susceptibility where it is found to alter B-cell receptor internalization (131). *PXK* rs2176082 and rs4681851 are associated with SSc and rs2176082 has association for ACA positivity. The association of rs2176082 is related to *DNASE1L3* (14, 47).

Signal Transducer and Activator of Transcription Protein 4

Signal transducer and activator of transcription protein 4 is critical for T-cell signaling and differentiation (132–134). *STAT4* is involved in effecting a Th1 cytokine response by transmitting signals from IL-2, IL-12, and IL-23 receptors and in signaling after type 1-IFN engages its receptor (135, 136). The role of *STAT4* in fibrosis was assessed in scleroderma mouse models. To assess the contribution of *STAT4* to bleomycin (BLM)-induced skin fibrosis and fibrosis of skin in (tight skin) Tsk-1/+ mice, BLM was injected for 3 weeks into *STAT4*$^{-/-}$ and *STAT4*$^{+/+}$ mice. *STAT4*$^{-/-}$ mice were crossed with Tsk-1/+ mice, and skin fibrosis was assessed (137). The deletion of *STAT4* significantly reduced skin fibrosis in the BLM model but not in the Tsk-1/+ model (137). In the BLM model, it was noted that there were decreased numbers of inflammatory cells including T cells and proliferating T cells and decreased quantity of IL-6, IL-2, TNFα, and IFNγ in lesional skin of *STAT4*$^{-/-}$ vs. *STAT4*$^{+/+}$ mice (137).

Signal transducer and activator of transcription protein 4 is considered an autoimmunity loci since its association has been firmly confirmed in SLE, RA, primary biliary cirrhosis, and SSc (48). SNP rs7574865 is associated susceptibility to lcSSc and ACA positivity (50, 51). SNP rs7574865 and rs10168266 were associated with dcSSc, ATA positivity, and pulmonary fibrosis in a Chinese cohort (52). Variant rs7574865*T allele has an additive effect with IRF5 rs2004640 seen in fibrosing alveolitis (38). Gene–gene interactions between *STAT4* and polymorphism in the transcription factor T-bet show increased susceptibility to SSc. Transcription

factor *T-bet* [(T-box expressed in T cells) (*TBX21*)] is an important transcriptional activator of Th1 differentiation effecting Th1/Th2 balance. Polymorphisms in *TBX21* have associations with RA, asthma, and type 1 DM. TT genotype of *TBX21* variant rs11650354 confers susceptibility to SSc in a recessive manner while *STAT4* variant rs11889341 A allele is associated with an increased risk of SSc in a dominant pattern. *STAT4* genotype increased the SSc risk in the presence of *TBX21 CC* genotype (53). Plasma levels of circulating IL-6 and TNF were increased in SSc patients who carry the *TBX21 CC* genotype where as those who carry the TT genotype show increased circulating IL-2 and IL-5 suggesting that patients who carry the CC genotype have a prominent pro-inflammatory cytokine profile (53). Gene expression profile from whole blood RNA of SSc patients suggest a role for type 1-IFN and pro-inflammatory cytokines in the CC genotype and of the T-cell pathway in the TT group (53).

Tumor Necrosis Factor Alpha-Induced Protein-3

Tumor necrosis factor alpha-induced protein-3 (*TNFAIP3*) encodes ubiquitin-modifying protein A20 and has a critical role in the regulation of immune signaling pathways.

Polymorphism in *TNFAIP3* is associated with SLE, RA, and celiac disease. *TNFAIP3* rs117480515, rs5029939*G allele, and rs6932056 carry an increase of susceptibility to SSc (43, 68). TNFAIP3 SNP and rs5029939*G is associated with dcSSc, fibrosing alveolitis, and PAH (43). The rs117480515*A allele is associated with SSc polyautoimmune subset (86).

Tumor Necrosis Factor Superfamily Member 4 Gene

Tumor necrosis factor superfamily member 4 gene (*TNFSF4*) encodes for the T-cell co-stimulatory molecule, OX40 ligand. *TNFSF4* has a role in B-cell proliferation and differentiation and T-cell proliferation. Ox40–OX40L promotes generation of Th2 cytokines. It has been identified as a susceptibility gene for SLE. TNFSF4 SNPs variant rs1234314, rs2205960, rs844648, rs12039904, rs1234317, and rs10912580 have been identified as susceptibility genes in SSc and are associated with lcSSc- and ACA-positive SSc patients in multiple French European studies (120–122). The minor allele rs1234314 has association for lcSSc, ACA, and ATA, while rs844648 confirmed association with dcSSc and ARA. Variant rs844648 was found to be protective in all SSc sub-groups except ARA+. In women, rs2205960*TT/GT and rs844648*AA associates with increased risk for SSc (59). These studies suggest *TNFSF4* as a susceptibility gene for SSc.

TNFAIP3 Interacting Protein-1

TNFAIP3 interacting protein-1 (*TNIP1*) gene interacts with A20 binding protein (BP) and inhibits TNF-induced NFκB-dependent gene expression; thereby negatively regulating NFκB. Mutations in this gene have been associated with RA, SLE, and psoriatic arthritis. *TNIP1* gene and protein expression was reduced in lesional skin tissue and cultured fibroblasts from SSc patients. *In vitro*, *TNIP1* had inhibitory effects on inflammatory cytokine-induced CI production (73). *TN1P1* SNP rs2233287, rs4958881, and rs3792783 are associated with global SSc (74). A two-staged GWAS showed strong linkage disequilibrium in the *HLA-DQB1*

gene: rs9275224, rs6457617, and rs9275245. Within the MHC region, there was association with rs3130573 located in the *PSORC1C1* gene. *PSORS1C1* also show susceptibility in global SSc except for ACA positivity patients but this association is dependent on HLA class-II (74).

CD87 (UPAR)

Urokinase-type plasminogen activator receptor (UPAR) promotes ECM and vascular remodeling. It regulates growth factor activation and is responsible for cell adhesion, migration, and proliferation (91). *UPAR* rs344781*G allele is associated with SSc-related digital ulcers, pulmonary artery hypertension, ACA positivity, and lcSSc (91). Genotype rs344781*GG is identified as an independent risk factor for SSc-related digital ulcers and PAH (91). *CD226*: acts as a co-stimulator of T cells and plays a role in T-cell adhesion. It is expressed on NK cells, monocytes, platelets, and B and T cells (77). It has been correlated with susceptibility to SLE, type 1 diabetes, thyroid disease, and MS (78–80). In SSc, the *CD226* T allele of rs763361 may contribute to disease severity due to its association with multiple SSc subsets including dcSSc, ATA positivity, and ILD (80). *CD226* haplotype SNP rs763361, rs34794968, and rs727088 correlates with pulmonary fibrosis (77). *MIF*: Macrophage migration inhibitory factor (MIF)-173 acts upstream, activates innate immunity, and sustains cellular and inflammatory responses. MIF induces endothelial adhesion and induces fibroblast proliferation that may contribute to vasculopathy (135). MIF-173 is lower in lcSSc. *In vitro*, *C7 MIF* encoded fibroblasts produced more MIF than non-stimulated fibroblasts (75). In an American and European study that included 3,800 SSc patients, MIF was found to have higher association with dcSSc compared to controls and lcSSc (75, 138). *MMP-12*: matrix metalloproteinase-1 (MMP-1) rs2276109*AA genotype has significant association in dcSSc, lcSSc, ATA positivity, and pulmonary fibrosis in an Italian SSc population (92). *NFkB1* gene SNP rs1598859 is associated with overall SSc disease (70). *PLD4*: phospholipase D family member 4 (PLD4) was identified as a susceptibility gene for SSc in Japanese (68). *PPARγ*: peroxisome proliferation-activated receptor gamma (*PPAR*-γ) when engaged by ligands of different types blocks transforming growth factor (TGF)-β mediated fibrotic responses *in vitro* in cultured fibroblasts and in various fibrotic animal models *in vivo* (81, 82). PPARG rs310746 is associated with SSc (83). *PSD3*: involved in signal transduction pathways and IC signaling. Polymorphism in the *PSD3* gene rs10096702 is associated with overall SSc (70). *TLR2*: subcutaneous injections of TLR ligands into the skin of SSc results in a significant inflammatory reaction resulting in SSc skin changes (90). *TLR2 pro63* His is associated with dcSSc, PAH, and ATA positivity (90). *TLR5* and 10 expression were increased in SSc fibroblasts *in vitro* and *in vivo* (139).

Vascular Related Genes

Endothelin-1 is one of three isoforms and is synthesized by vascular endothelial (VE) cells, fibroblasts, bone marrow mast cells, neutrophils, macrophages, and cardiac myocytes (140). Various triggers induce synthesis of ET-1 including TGF-β and other growth factors, cold exposure, low shear stress, hypoxia, and angiotensin II (140); but its synthesis is reduced by nitric oxide (NO), natriuretic peptides, increased blood flow, and prostacyclin

(141). ET-1 is also degraded by MMP-1, which is reduced in SSc (140). Two types of receptors for ET-1 (ETα and ETβ) are variably expressed on endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, tissue fibroblasts, neutrophils, mast cells; and monocytes and ET receptor engagement on these cells triggers a variety of pro-inflammatory or fibrotic response, including vasoconstriction of vasculature (140). ET-1 increases surface expression of ICAM-1 on fibroblasts, stimulates CI synthesis, promotes formation of myofibroblasts, and facilitates binding of T cells to fibroblasts (140, 142). ET-1 acts as a downstream mediator of TGF-β, and its induction by TGF-β in fibroblasts is via small mother against decapentaplegic (Smad)-independent signaling that involves c-Jun N-terminal kinase (JNK) and activin receptor-like kinase (ALK)5 pathways (143). Polymorphisms of ET-1 receptors are associated with SSc. For example, there is an association of *EDNRB* polymorphisms and dcSSc and *EDNR-A* polymorphism with anti-RNA polymerase autoantibodies in SSc (140). Polymorphisms were also described in the promoter of the NOS2 gene that confers susceptibility to PAH in SSc (144). Potassium voltage-gated channel shaker-related subfamily 5 (*KCNA5*) has a role in the regulation of vascular tone. It is inhibited by hypoxic conditions leading to vasoconstriction. *KCNA5* may have a protective role against PAH-associated SSc, this protective role was identified with variant rs10744676 (145).

MicroRNAs

MicroRNAs are translational regulators of gene expression and also destabilize messenger RNAs (mRNAs) of target genes (146). MiRNAs are tissue- and cell type-specific short, single-stranded non-coding RNAs that function to modulate gene expression (**Table 4**). MiRNA bind to the 3′ untranslated region of mRNA of the target gene and mediate post-transcriptional regulation. Once bound, they either cause translational repression of the target gene or induce the degradation of the gene (147–149). In SSc, several miRNAs are associated with TGF-β and CI expression. In comparison to normal skin tissue, Zhu et al. (93, 147) found that skin from patients with lc and dc SSc expressed miR-21, miR-31, miR-146, miR-503, miR-145, and miR-29b. In these patients, miR-21 was increased in both tissue and fibroblasts whereas miR-145 and -29b were decreased. These miRNAs targeted the TGF-β pathway – including Smad7, Smad3, and COL1A1. TGF-β stimulation resulted in increased miR-21 expression and decreased expression of Smad7, while the upregulation of miR-145 was associated with a downregulation of Smad3 message. These same authors found that overexpressing miR-21 in fibroblasts decreased Smad7 but knocking down the expression of miR-21 increased Smad7 expression (93). miR-21 was also found to have increased expression in BLM-induced skin fibrosis. Reporter gene assay analyses revealed that the target gene for miR-21 is Smad7, while the target gene for miR-145 is *Smad3* (93, 94).

Ninety-five miRNAs were analyzed in the sera of SSc patients and healthy controls. This analysis revealed that miR-30b was significantly downregulated in SSc patients and the modified Rodnan skin score (MRSS) inversely correlated with the level of miR-30b (95). Downregulation was also seen in the skin of scleroderma patients and BLM-treated sclerotic skin (95). Transfection studies

TABLE 4 | MicroRNAs in SSc.

Micro RNA (miR)	Implications for SSc pathogenesis	Reference
miR-21 ↑ in SSc skin and fibroblasts	↑ by TGF-β, ↓ Smad7	(93, 94)
miR-145 ↓ in SSc skin and fibroblasts	Smad3 is its target gene	(93, 94)
miR-29b ↓ in SSc skin and fibroblasts	Suppressor of fibrosis	(94)
miR-30b ↓ in SSc skin and fibroblasts	Suppressor of PDGFR-β	(95)
miR-29a ↓ in SSc sera and skin	Suppressor of CI and CIII synthesis by fibroblasts, miR-29a is reduced by TGF-β, PDGF-β, and IL-4. Lower serum miR-29a associates with PAH	(96–98)
miR-196a ↓ in dcSSc sera	Expression of miR-196a reduced by TGF-β. May regulate CI synthesis. ↓ miR-196 = ↑ MRSS, ↑ digital pitting, and scars	(99, 100)
miR-150 ↓ in SSc fibroblasts and sera	Reduces fibroblast CI, pSmad3, and integrin expression	(101)
miR-7 ↓ in localized scleroderma skin and fibroblasts	Reduces CI synthesis by fibroblasts. Regulated by TSP2	(102, 103)
miR-let-7a ↓ in SSc and localized scleroderma skin and sera	Reduces CI synthesis by fibroblasts	(104)
miR-129-5p ↓ in SSc	Suppressor of COL1A1 expression in fibroblasts	(105)
miR-142-3p ↑ SSc in sera	May regulate αV integrin, which may recruit and activate small latent complex that regulates autocrine TGF-β activity	(106)
miR-92a ↑ in SSc sera and fibroblasts	May downregulate MMP-1	(107)
miR-21 ↑ SSc in fibroblasts	miR-21 is upregulated by TGF-β and decreases expression by Smad7	(93, 94)

showed that miR-30b affects platelet-derived growth factor/receptor (PDGFR)-β expression by suppressing this receptor (95). In their evaluation of 15 SSc patients and 15 normal subjects, Koba et al. (150) found that miR-206 and miR-21 were useful in distinguishing patients with SSc from normal subjects (150).

miRNA-Targeting CI

The expression of miR-196a was investigated in SSc both *in vitro* and *in vivo*. *In vivo* miR-196a was detected in the serum of SSc patients. Patients who had measurable lower levels of miR-196a had dcSSc compared to lcSSc. Lower levels of miR-196a was also associated with higher prevalence of pitting digital scars and more fibrotic skin as measured by MRSS (99). *In vitro*, the expression of miR-196a was normalized by TGF-β small interfering RNA (siRNA) in SSc fibroblasts, and the addition of miR-196a inhibitor to these fibroblasts resulted in the downregulation of CI. When the inhibitor was added to normal fibroblasts, there was an overexpression of CI (99). These results suggest that miR-196a may regulate CI expression.

Micro-RNA-29 (miR-29) is a TGF-β associated miRNA and is linked to fibrosis likely by interaction with several extracellular genes including ELN, FBN1, COL1A, COL1A2, and COL3A1 (151, 152). TGF-β/Smad3 signaling appears to negatively

regulate miR-29 (153). Support for this relationship was the finding that in BLM pulmonary fibrosis mouse model, Smad3 was upregulated while miR-29 was downregulated in contrast to results with Smad3⁻/⁻ mice, which were protected from BLN pulmonary fibrosis and miR-29 was upregulated (153). In addition, therapeutic delivery of miR-29 to mice using Sleeping Beauty transposon-mediated gene transfer protected mice from developing BLM-induced lung fibrosis (153). MiR-29a has the ability to bind to the *3′UTR of COL1A1* and *COL1A2* (96, 154). Maurer et al. (97) found that miR-29a was strongly downregulated in SSc fibroblasts and skin sections when compared to healthy controls (97). SSc fibroblasts, in which miR-29 was overexpressed, exhibited decreased expression and protein levels of CI and CIII, while knockdown of miR-29 in normal fibroblasts increased CI production. Levels of miR-29 were reduced in normal fibroblasts when these fibroblasts were cultured with TGF-β, PDGF-β, or IL-4 (97). These studies confirm that miR-29a directly regulates CI. Serum levels of miR-29a were investigated to determine its potential role as a biomarker in SSc. In 61 patients with SSc, approximately 40% of which had dcSSc, miR-29a was found to be upregulated and not downregulated as expected in the serum of these patients. Patients with scleroderma spectrum disorder (SSD) are those who did not fulfill the ACR diagnosis criteria for SSc but who may develop scleroderma in the future. In these patients, miR-29a was downregulated compared to healthy controls, dcSSc, and lcSSc patients (96). Decreased serum levels of miR-29a may also be associated with higher right ventricular systolic pressure and PAH (96).

MiR-150 expression is decreased in SSc fibroblasts and sera. Normal fibroblasts that were transfected with miR-150 inhibitor had induced expression of type 1 CI, pSmad3, and integrin (101). Forced expression of miR-150 in SSc fibroblasts resulted in downregulation of CI, pSmad3, and integrin (101). In patient sera, lower expression of miR-150 correlated with severe clinical disease (101).

Skin and fibroblasts from localized scleroderma showed decreased levels of miR-7 compared to keloid skin and normal skin *in vivo* and *in vitro* (102). Normal fibroblasts that were transfected with miR-7 inhibitor exhibited upregulation of COL1A2 (102).

Skin and sera from SSc and localized scleroderma patients showed a downregulation of miR let-7a when compared to normal and keloid skin (104). CI was reduced by the overexpression and inhibition of miR let-7a in human and mouse skin fibroblasts (104). Intermittent overexpression of miR let-7a by intraperitoneal injections reduced dermal fibrosis in the BLM skin model (104).

MiR-129-5p is a regulator of *COL1A1* (154) and is downregulated in SSc (105). Nakashima et al. (105) found that, in their 20 patients with SSc, IL-17A expression was increased in the involved skin and sera, but IL-17R type A was decreased in SSc fibroblasts when compared to normal (105). IL-17A reduced protein expression of type I CI α1 chain [α1(I)] and connective tissue growth factor (CTGF). IL-17A also induced the expression of miR-129-5p (105). In the presence of IL-17A, miR-129-5p is increased with α1(I) and CTGF. The authors suggest that since SSc fibroblasts have intrinsic activation of TGF-β, TGF-β suppresses IL-17A, in addition to miR-129-5p with resultant CI accumulation (105).

MicroRNA-29a and miRNA-196a are low in SSc fibroblasts and can suppress CI gene expression, suggesting the low-level expression of the miRNAs permit CI to be upregulated by TGF-β and other mediators in SSc fibrogenesis (97, 99). Levels of other miRNAs have been found to differ in patients with SSc compared to healthy controls as follows: serum miR-142-3p was higher in SSc patients than healthy controls (106); levels of miR-21 were increased, whereas levels of miR-145 and miR-29b were decreased in SSc lesional fibroblasts (94); miR-92a is more elevated in sera and SSc lesional fibroblasts than in normal healthy controls and may downregulate MMP-1 (107); and levels of miRNA-7 were found to be reduced in sera and lesional fibroblasts from patients with localized scleroderma and may regulate CI expression (102). MiR-150 regulates β3 integrin expression and was found to be downregulated in lesional SSc dermal fibroblasts compared to healthy donor fibroblasts (101); miR let-7a was found to be decreased in sera and lesional fibroblasts from patients with SSc or localized scleroderma (104); and miR-21 was found to be upregulated in SSc lesional dermal fibroblasts (93).

Discoidin domain receptor 2 (DDR2) and thrombospondin-2 (TSP2) were both found to be decreased in SSc dermal fibroblasts (103, 104). In SSc dermal fibroblasts, *DDR2* mRNA and protein levels were suppressed, but the knockdown of TGF-β in these fibroblasts resulted in increased expression of *DDR2* (104). In normal fibroblasts, *DDR2* knockdown increased miR-196a expression with resultant decrease in CI. This was not seen when *DDR2* was knocked-down in SSc fibroblasts (104). In SSc, fibroblasts, knocking down *DDR2* did not affect TGF-β signaling or miR-196a expression, suggesting that intrinsic expression of TGF-β causes the downregulation of *DDR2* in SSc fibroblasts (104).

Thrombospondin 2 mRNA expression and protein levels are decreased in SSc fibroblasts when compared to controls but were upregulated in conditioned medium from SSc fibroblasts (103). Knockdown of *TSP2* in dermal fibroblasts caused decreased expression of CI and increased miR-7 expression (103). SSc dermal fibroblasts show an increased expression of miR-7 (103) suggesting that a negative feedback mechanism may exist between *TSP2* and miR-7 (103).

Matrix metalloproteinase-1 was downregulated when normal dermal fibroblasts were overexpressed with miR-92a (107). In 61 patients with SSc, medium serum levels of miR-92a were elevated. This upregulation was constitutively also found in SSc dermal fibroblast, but when these fibroblasts were transfected with siRNA of TGF-β, the expression of miR-92a was decreased (107). These studies suggest that miR-92a ability to affect *MMP-1* suggest that miR-92a may be a target for *MMP-1*.

Hair miRNA
MicroRNA from the hair shaft and roots was studied. Hair-miR-196a was found to be significantly decreased in SSc patients (100). Hair miR-29a was obtained from 20 SSc patients, 5 dermatomyositis, and 13 controls to determine its usefulness as a biomarker. Hair miR-29a was significantly lower in SSc patients, and the decreased levels were associated with a higher prevalence of phalangeal contractures (98). We may see more studies using hair miRNAs to assess biomarkers and disease phenotypes.

Immune System in SSc Pathogenesis

Role of Innate Immune System
Engagement of the innate immune system depends on 13 different TLRs, which are not antigen-specific but instead recognize patterns and which segregate on the basis of the nature of the ligands they encounter such as distinct molecular patterns in particular pathogens, in endogenous cellular constituents, or in cellular products of the host [reviewed in Ref. (155)]. Considerable evidence suggests that *TLR2* and *TLR4* expressed on cells and IC *TLR3*, *7*, *8*, and *9* have particular relevance to SSc pathogenesis. For example, a rare functional polymorphism (Pro[631] His) in TLR2 (which has bacterial peptidoglycan, lipoprotein, and lipoteichoic acid and yeast-derived zymosan as natural ligands) is associated with ATA positivity and enhanced IL-6 production by dendritic cells when engaged by a TLR2 ligand (90, 155). TLR4 endogenous ligands [including fibronectin, hyaluronan fragments, heat-shock protein (HSP) 70, HSP9, high-mobility group box-1 (HMGB-1), and S100A proteins] could engage TLR4 (which is increased in SSc skin and lungs) and synergize with TGF-β to increase fibroblast CI production (155–160). Importantly, HSP70, HMGB-1, and hyaluronan are elevated in SSc sera or tissues (161–163). Of interest, elevated HMGB-1 and soluble advanced glycation end products (sRAGE) levels in sera of patients with SSc correlated with more internal organ involvement, immunological abnormalities, and total MRSS but correlated negatively with lung function (161). Double-stranded RNA is recognized by TLR3, single-stranded RNA, and imidazoquinoline compounds by TLR7 and TLR8, whereas unmethylated CpG oligonucleotide sequences are recognized by TLR9 and some of these ligands are present in SSc (164, 165).

Siglec-1 (CD169, sialoadhesin) is a marker for macrophage activation and its expression was found to be increased CD14^{+} monocytes in peripheral blood and on macrophages in dermis of lesional skin of a subset of patients with SSc (125). Furthermore, Siglec-1 was induced in peripheral blood CD14^{+} monocytes from normal donors when cultured with IFNα, TLR3, 7, or 9 agonists but not by TLR2 or 4 (125). In the skin, activated macrophages expressing Siglec-1 may also release cytokines or growth factors that are able to stimulate fibroblasts or myofibroblasts to synthesize CIs and other matrix components (125). In addition, sera containing autoantibodies from patients with SSc induce high levels of IFNα in normal monocytes that is inhibited by pretreatment of the sera with bafilomycin and RNA-degrading enzymes, suggesting that the immune complexes in SSc sera contain RNA that can bind IC TLRs (166). While other agents (e.g., IL-4, LSP, IFNβ, IFNγ) might also induce Siglec-1 expression on monocytes/macrophages in SSc, these findings are compatible with the notion that generations of IFNα by activation of IC TLRs 3, 7, or 9 agonists might be ongoing in a subset of SSc patients (125, 167).

Interferon Signature in SSc
Interferons are multifunctional cytokines that are responsible for inducing cellular resistance to viruses. IFN-α, -β, and -ω are type 1-IFNs. There is evidence for a prominent IFN signature in SSc. For example, peripheral whole blood cells in 50% of SSc patients have increased expression of IFN-regulated genes and lung tissues

from SSc patients with ILD have increased IFN and IFN-regulated gene expression (168, 169). It appears that the IFN signature in SSc discussed below may arise from activation of TLRs expressed on the surface of cells by infectious agents or by endogenous proteins, RNA, DNA, and other cellular products that can trigger IC TLRs summarized above. IFN regulatory factors (IRF) coordinate the expression of IFN and IFN-inducible genes that help regulate the innate and adaptive immune responses (169, 170). Thus far, *IRF5*, *IRF7*, and *IRF8* appear to be relevant to SSc (169) (see **Table 3**).

IRFSNP Associations

IFN regulatory factor 5, a major regulator of type 1-IFN, induces the transcription of IFN-α and other pro-inflammatory cytokines, is involved in TLR signaling, and is critical for activation of IFN-associated genes (109, 169) (see **Table 3**). *IRF5* has association with SLE (171–173), and multiple studies have shown SNPs of *IRF5* are associated with SSc susceptibility. *IRF5* rs2004640*TT was found to have a strong association with dcSSc, fibrosing alveolitis, antinuclear antibody (ANA), and ATA positivity in a French cohort (38). In addition to rs2004640, these same authors found an association between rs3757385 and rs10954213 variants and SSc (43). In this study, *IRF5* haplotype "R" was identified as a risk while haplotype "P" was protective (43). A Japanese case–control association study with 281 SSc and 477 controls found that rs2004640, rs10954213, and rs2280714 were all significantly associated with SSc, with rs2280714 having the strongest association with SSc, and these SNPs were significantly enriched in dcSSc and ATA-positive patients (45). Carmona et al. found that SNPs rs10488631, rs2004640, and rs4728142 showed strong associations in SSc global disease, and that association of rs20004640 was dependent on rs4728142 (174). rs728142*A-rs2004640*T haplotype explained this association suggesting that all three haplotypes provide an additive effect (174). In another study, *IRF5* SNP rs4728142 was found to be predictive of longer survival in SSc patients with ILD (41). *IRF7* is upregulated in peripheral blood cells from patients with early SSc and is associated with ACA-positive SSc (175). *IRF8* is induced by IFNγ and modulates TLR signaling (24). Polymorphism rs11642873 in the *IRF8* gene was found to be associated with lcSSc (24). *IRF8* SNP rs2280381 was found to have association with SSc in a Japanese population consisting of 415 SSc and 16,891 controls with a replication study consisting of 315 SSc (68). While associations of the above variations in IRF genes with certain manifestations do not establish cause and effect, they suggest genes that regulate IFN expression and downstream effects may play a central role in determining disease severity and specific organ involvement.

Inflammasome and SSc

The cytoplasm of cells also contains another pattern recognition receptor (PRR) system called the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family that recognize IC motifs and, when activated via the "inflammasome" involves NFκB and mitogen-activated protein kinase (MAPK), which in turn stimulates production of pro-inflammatory cytokines IL-1B and IL-18. Polymorphisms of one of the NOD family members, *NLRP1*, are associated with ILD and ATA positivity in patients with SSc (176). Relevance of the NOD family to SSc

was further evidenced by studies showing inhibition of inflammatory activation-reduced IL-1β and CI production by SSc lesional fibroblasts and studies in *NALP3* null mice showing they were resistant to lung fibrosis (177, 178). *NLRP3* and pro-inflammatory cytokines (IL-1β and IL-18) were found to be increased in skin biopsies of patients with dcSSc or lcSSc compared to age-matched control and correlated with MRSS (179).

Transitioning from Innate to Adaptive Immunity

Rather than two separate and mutually exclusive immune systems, it is being realized that there is likely an ongoing interplay between the innate and adaptive immune systems (180). Attention has focused on innate lymphoid cells (ILCs) that are involved not only in immediate immune host defense but also in maintaining homeostasis of mucosal and lymphoid tissue (180, 181). Three different types of ILCs have been described to-date: *ILC1*, *ILC2*, and *ILC3* (181). These ILCs do not express somatically rear-ranged antigen receptors, but express MHC Class-II and possess transcription factors and cytokine profiles reminiscent of Th cells (181, 182). ILC1s, like Th1 cells, utilize T-bet and produce IFNγ; ILC2s, like Th2 cells, utilize GATA-binding protein-3 (GATA-3) and produce IL-5, IL-9, and IL-13; and ILC3s, like Th17 cells, utilize RAR-related orphan nuclear receptor gamma transcription factor (RORγt) and produce IL-17A and IL-22 (181). ILCs express TLRs and IL-1 receptor, and ILC2s and ILC3s can act as APC similar to dendritic cells (181, 183, 184). In mouse models, ILC3s were shown to promote antigen-specific CD4+T cells and antigen-specific T-cell-dependent B-cell antibody production (181). What role ILCs play in innate and adaptive immunity in SSc remains to be defined and ongoing research should eventually better elucidate how ILC effect transition from innate to adaptive immunity.

Dendritic cells by using surfaces and IC PRRs play key roles in linking innate immune response to adaptive immune responses by identifying antigens from pathogen-associated or damage-associated molecular patterns (PAMPS or DAMPS) by using TLRs, NLRs, RIG-I-like receptors (RLRs), and receptors for advanced glycation end products (RAGE) (185). The identified antigens are then processed and the information is presented to T cells in the context of MHC-II/antigen complex binding the T-cell receptor, CD86/CD80 costimulation of T-cell CD28, followed by release of cytokines from dendritic cells that affect T-cell differentiation and effect Th1, Th2, Th17, and T regulatory (Treg) cell differentiation (185, 186).

Adaptive Immunity in SSc

A number of observations over several decades strongly implicate a major role for the adaptive immune system in SSc pathogenesis. These include the development of features of SSc in chronic graft-versus-host disease (cGVHD) in humans, which is largely mediated by donor T cells and reversal of fibrosis and vasculopathy after autologous hematopoietic CD34+ stem cell treatment of patients with SSc (187, 188).

Immunohistochemical analysis of skin of patients with SSc shows perivascular and tissue accumulations of activated *CD4+ T cells*, *monocytes*, and *CD4+CD8+ double positive T cells* that express high levels of IL-4 (189, 190). DNAX accessory molecule-1 (DNAM-1) modulates adhesion; co-stimulates

T lymphocytes; expresses on most CD4^{+} and CD8^{+} T cells, NK cells, monocytes, platelets, and some B cells; and is found to be expressed on inflammatory cells in biopsies of lesional skin of patients with SSc (191).

Autoantigens Recognized by SSc T Cells

Of particular significance is the finding in lesional SSc skin sites of *Vdelta1+/gamma/delta T cells* that express HLA-DR and CD49d, suggesting that they have homed to these locations and expanded (192). Furthermore, analysis of T-cell repertoire in different skin locations from the same patient is compatible with clonal expansion of T cells to a widely distributed and persistent antigen (193). A variety of autoantigens that elicit T-cell responses in patients with SSc are widely distributed in tissues, have been described, and include types I, II, and V CIs (CI, CII, CV); laminin; low molecular weight (MW) *N*-sulfated heparin sulfate; 3500 MW RNA antigen; elastin; and DNA topoisomerase I (189, 194–198). Of potential relevance is the finding that the CI-specific CD25^{+}CD4^{+} T cells isolated from SSc PBMC have a memory (CD45R^{+}) phenotype (195). Most patients with SSc have production of IFNγ by their PBMCs when cultured with CI or constituent α1 and α2 chains, which can be reduced by inducing immune tolerance via chronic administration in a dose-dependent manner by oral bovine CI (199, 200). In a double-blind, randomized clinical trial of daily oral bovine CI or placebo for 12 months, patients with dcSSc ≥3 years duration, patients receiving oral bovine CI had a significant improvement in MRSS compared to the placebo-treated patients (201). These studies suggest CI might be a widely distributed relevant antigen in SSc.

Microchimerism in SSc

Fetal–maternal and maternal–fetal microchimerisms have been proposed as mechanisms triggering autoimmunity in SSc and other autoimmune diseases (202–204). This microchimerism, in susceptible individuals, could initiate a type of cGVHD producing SSc with the microchimeric cells acting as effectors or as targets of an immune response (204). It is noteworthy that, in women with SSc who have given birth to male children, male offspring Th2-oriented T cells that express high levels of IL-4 are found in these women's skin and blood (205).

CD4^{+} Regulatory T Cells and CD4^{+} Th17 T Cells in SSc

The dysregulation in SSc of Th17 and/or Tregs (mostly CD4^{+}CD25^{+}Foxp3^{+}) has been reported by several groups. Different (and contradictory) results have been reported that seem to be dependent to some extent on how Tregs are defined by flow cytometry. Tregs have been found to be increased in the blood of SSc patients but have defective suppressive function (206). Papp et al. (207) reported decreased percentages and suppressive function of CD4^{+}CD25^{+}Tregs but increased percentage of Th17 cells in blood of SSc patients (207). Klein et al. (208) reported SSc patients had elevated CD4^{+}D24^{+}Foxp3^{+}Tregs in lesional skin but normal percentages in the peripheral blood (208). Slobodin et al. reported an increased number of Tregs in the blood of SSc patients but no concomitant increase in TGF-β or IL-10 production by CD4^{+}T cells (209). Fenoglio et al. found SSc patients had reduced frequency in blood and reduced suppressive function of CD4^{+}CD25^{+}Tregs and increased Th17 cell expansion after polyclonal or antigen-specific stimulation of SSc PBMC (210). Finally, Mathian et al. analyzed circulating activated (a)Tregs (CD4^{+}CD45RA[(]CD25^{bright} T cells) and resting (r)Tregs (CD4^{+}CD45RA^{+}CD25^{+} T cells) in controls and SSc and found decreased frequency but normal suppressive function of both types of Tregs and in the lesional skin found no CD4^{+}Foxp3 mRNA in SSc compared to normal donor skin (211).

Abnormalities in Treg numbers or function could facilitate development of adaptive immune responses to autoantigens in SSc. Mast cells and S1P which are increased in SSc are two potential antagonists for proper development and function of Treg cells, as both have the capacity to inhibit Tregs (212–214). Furthermore, both S1P and mast cells enhance generation of Th17 cells (213, 215). The field of Tregs is still evolving and future studies with better markers for Treg subsets will need to be performed to better characterize this role in SSc.

Possible Influence of Vitamin D Deficiency and Lysophospholipids on Immune Dysregulation in SSc

Vitamin D insufficiency/deficiency has been implicated in triggering and enhancing a number of autoimmune diseases. Low serum 25(OH)D concentrations have been reported to be more common in patients with SSc than in healthy controls. Furthermore, 25(OH)D levels have been reported to negatively correlate with several laboratory and clinical parameters in European Disease Activity Score, Raynaud's phenomenon (RP), erythrocyte sedimentation rate, systolic pulmonary artery pressure, MRSS, and positively correlate with carbon monoxide diffusion lung capacity (216–218). A number of effects of 1,25(OH)$_2$D3 on immune cells have been reported that could explain its ability to decrease autoimmunity and, conversely, how VitD deficiency contributes to increased autoimmunity [these are summarized in Ref. (219)]. For example, effects of 1,25(OH)$_2$D3 on APC include: (1) downregulation of MHC class-II molecule expression in APC; (2) downregulation of surface expression of co-stimulatory receptors (CD40, CD80, and CD86) and other maturation-induced proteins (CD1a, CD83); (3) inhibition of dendritic cell maturation, induction of tolerogenic DC that are able to induce Treg cells; (4) inhibition of IL-12 p70 release from DC; and (5) inhibition of pro-inflammatory cytokines in monocytes and macrophages (219). Effects of 1,25(OH)$_2$D3 on T cells include: (1) inhibition of antigen-specific and lectin-stimulated T-cell activation and progression from G1a to G1b proliferation; (2) inhibition of IL-12, IFNγ, IL-2 release; (3) stimulation of IL-4, IL-5, and IL-10 production; and (4) inhibition of Fas ligand (FasL) expression by activated T cells (219). The effect of 1,25(OH)$_2$D3 on B cells is to inhibit production of IgA, IgE, IgG, and IgM and in NK cells to inhibit IFNγ production (219, 220).

Administration of VitD3 in escalating daily doses of 2000 U (2000 U for the first month, then 4000 U for the second month, and 8000 U for the third month) to healthy VitD-deficient individuals induced increased frequencies of CD38^{+} B cells and reduced frequencies of CD4^{+}IFNγ^{+} and CD4^{+}IL-17^{+} T-helper cells (221). Treatment of SLE patients with hypovitaminosis D with 100,000 U of VitD3 weekly for 4 weeks and then monthly

for 6 months resulted in an increase in naïve CD4^{+} T cells and CD3^{+}CD4^{+}CD25^{hi}CD127^{-}Foxp3^{+}Tregs and decreases in CD19^{+} B cells, anti-ds DNA antibody titers, and proteinuria (222). Similar studies with high-dose VitD supplementation have not been reported in patients with SSc, but the above studies in SLE and normal hypovitaminosis individuals demonstrate the potential for immune modulation by high-dose VitD supplementation that might decrease autoimmunity in patients with SSc.

Lysophosphatidic acid and *S1P* levels are increased in sera of patients with SSc, suggesting they may play a role in different aspects of the disease (214) [reviewed in Ref. (223)]. Platelets, macrophages, dendritic cells, mast cells, and endothelial cells are sources of LPA and S1P, and these cells (plus T cells and B cells), NK cells, fibroblasts, and other cells express various types of LPA and S1P G-protein-coupled receptors (GPCRs) [reviewed in Ref. (223)]. PPARγ, which resides intracellularly and counters TGF-β fibrogenesis, is also an additional receptor for LPA (224). In addition to S1P being able to "disarm" Foxp3 Tregs mentioned above, S1P and LPA regulate the function, migration, and trafficking of all lymphoid cells and monocyte/macrophage/dendritic cells with S1P also being able to sequester T cells in the thymus and peripheral lymphoid organs, resulting in some instances in lymphopenia, which is frequently found in patients with SSc (225–227). By acting on APC, S1P and LPA each can suppress development of Th1 T-helper cells, but they have different effects on Th2 T-helper cells in that S1P suppresses their development while LPA fosters their development (228). Th2 T-helper cell predominance is a feature of some patients with SSc with production of IL-4 and IL-13, which facilitate development and expansion of B cells and autoantibodies that are common features of SSc. Lysophospholipids need further study in SSc, given the potential to regulate immunity.

Vascular Abnormalities in SSc

Vascular dysfunctions and abnormalities leading to RP, digital ulcers, and nail-fold capillary abnormalities usually are among the earliest and key manifestations of SSc. The various vascular abnormalities are summarized in **Table 5**. Postmortem examination reveals the vascular changes in SSc are more typical of a vasculopathy than of a vasculitic process – given the paucity of inflammation in the vessel wall with widespread systemic intimal proliferation in the pulmonary, coronary, and the renal arteries (229). Patients with SSc who develop PAH and renal crisis exhibit vascular lesions characterized by classic concentric intimal proliferation, marked luminal obstruction, lymphocyte infiltration, and relative paucity of plexiform lesions (230–233).

Earliest signs of vascular dysfunction include impaired vascular tone and vascular permeability (234). Impaired balance of vasoconstrictor substances (e.g., ET) and vasodilator substances (e.g., NO), plays important roles in vascular dysfunction. Platelet activation and enhanced coagulation with reduced fibrinolysis also contribute to the vasculopathy in SSc. Abnormalities in the vascular system can be seen in clinically normal skin of SSc patients (235). Large gaps between endothelial cells, vacuolization of endothelial cell cytoplasm, and loss of membrane-bound storage vesicles are some of the earliest detectable changes in the endothelial cells (235–237). In a 20-year follow-up study, sequential changes can be

TABLE 5 | Key vascular abnormalities of SSc.

Presence of proliferative vasculopathy with intimal proliferation in peripheral, pulmonary, coronary, and renal arteries in the absence of inflammation is a hallmark feature of scleroderma

Endothelial cell damage is a key and early process. It precedes fibrosis and particularly involves the arterioles

Early detectable changes in the endothelial cells include disappearance of membrane-bound vesicles, vacuolization of endothelial cell cytoplasm, and gaps between endothelial cells

Defective angiogenesis is an early event in the form of drop out of capillaries and abnormal capillary architecture without a compensatory process

There are conflicting reports regarding the presence and role of circulating endothelial progenitor cells in SSc

There is dysregulation of coagulation and fibrinolysis process

Platelets show enhanced aggregability to various triggers such as type I collagen and adenosine etc…, and are activated throughout the clinical course of SSc

LPA and S1P could potentially contribute to the vasculopathy via endothelial cell activation, neointimal formation, vascular leakiness, increased vasoconstriction, cardiac fibrosis, and hypertension

seen in capillaries (4) in skin, which include capillary enlargement, capillary loss, and telangiectasia. Further morphologic changes in vessel wall occur including fibrosis. Such capillary changes are wide spread in internal organs (e.g., lungs, heart, kidneys, and muscles) (238). Intimal proliferation and accumulation of proteoglycans in the arterioles and small arteries are also common (239, 240). The operative mechanisms that lead to this widespread vasculopathy in SSc of unknown, but animal models and *in vitro* studies have provided some clues.

Mechanism of Vascular and Endothelial Cell Injury in SSc

The etiology of the initial vascular damage in SSc is not known and is a topic of speculation. Infectious agents, cytotoxic T cells, NO-related free radicals, and autoantibodies against endothelial cells have all been implicated (234). Endothelial cell dysfunction, neural abnormalities, and various other intravascular defects likely contribute to the impaired vascular flow (241).

Endothelial Cell Injury

Evidence suggests that endothelial cell injury is an early and central event in the pathogenesis of SSc vasculopathy, and viral agents [especially human cytomegalovirus (hCMV)], cytotoxic T cells, antibody-dependent cellular cytotoxicity (ADCC), anti-endothelial cell antibodies, and ischemia-reperfusion injury are all suggested mechanisms for endothelial cell damage (234, 242). Levels of antibodies to hCMV are increased in patients with SSc which is reminiscent of the association of hCMV antibodies with vascular intimal proliferation and vasculopathy in patients with graft rejection and coronary artery bypass restenosis (243). In addition, there is evidence of binding of some ATAs to an epitope in hCMV-derived UL94 protein which happens to also show homology to MVEC surface protein tetraspan novel antigen-2 (NAG-2) (243). Apoptosis of MVEC can be effected by purified anti-UL94 peptide antibodies (244). Cytotoxic CD4^{+} T cells induce MVEC apoptosis via *in vitro* Fas-related pathway in contrast to CD8^{+}

T cells, NK, and LAK cells which utilize the granzyme/perforin system (243). ADCC to MVEC is operative in many patients with SSc (243). Anti-endothelial cell antibodies are commonly found in sera from patients with SSc and are capable of inducing MVEC apoptosis directly *in vitro* (245). Ischemia and reperfusion injury (especially associated with attacks of RP) is accompanied by upregulation of expression of junctional adhesion molecules (JAMs). This upregulation indicates endothelial dysfunction and allows attachment of platelets and neutrophils to the endothelium that is thought to lead to MVEC injury through production of superoxide radicals (which limit release of vasodilation substances such as NO and prostacyclin) (243, 246, 247). The major evidence for the presence of the endothelial injury in SSc is high serum levels of circulating von Willebrand (VW) factor, ET-1, increased levels of circulating viable and dead endothelial cells, and soluble JAM-A and JAM-C (234, 247–251). Subendothelial tissue forms a nidus for platelets to aggregate and initiates fibrin deposition and intravascular thrombus formation (1). The role of endothelial apoptosis is not clear. Sgonc et al. (252) demonstrated endothelial cell apoptosis in the University of California at Davis chicken lines 200/206, which spontaneously develop an SSc-like disease (252). Apoptotic endothelial cells may contribute to tissue injury when engulfed by immature dendritic cells and macrophages, which subsequently present cellular antigens to CD8$^+$ T cells, causing further tissue injury (253). These apoptotic endothelial cells can also activate the alternate complement pathway and coagulant pathway leading to vasculopathy (254, 255). Proof that there is ongoing endothelial apoptosis in SSc is thus far lacking, and Fleming and Wanless (256) failed to detect apoptotic endothelial cells in their study, although they did demonstrate loss of VE-cadherin, which regulates endothelial barrier function and found evidence of IFNα signaling (256). IFNα signaling suggests endoplasmic reticulum stress and the unfolded protein response in these cells (257, 258).

Defective Angiogenesis

The remarkable loss of capillaries and small vessels in patients with SSc suggests a defect in the process of angiogenesis. Tissue ischemia usually leads to the expression of angiogenic growth factors [e.g., vascular endothelial growth factor (VEGF)], which causes vasodilatation, proliferation, and migration of endothelial cells and stabilization of the lumina to form new vessels (259). Plasma levels of VEGF are elevated in SSc, and this could stimulate angiogenesis (260). Levels of other proangiogenic factors [e.g., PDGF, placental growth factor (PGF), and fibroblast growth factor 2 (FGF-2)] are also considerably elevated in the plasma of SSc patients (261). Expression of VEGF and its receptors, *VEGFR1* and *VEGFR2*, are increased in skin of SSc patients (260, 262, 263). In addition to elevated level of VEGF, other proangiogenic mediators (such as ET-1, adhesion molecules, and chemokines) are found in the circulation of SSc patients (264). Elevated levels of antiangiogenic factors such as *angiostatin*, *platelet factor-4* (also called CXCL4), thrombospondin-1 (*TSP-1*), and *IL-4* have been described in patients with SSc (264, 265).

Defective Vasculogenesis

The role of vasculogenesis in SSc is not clear, and there are conflicting reports regarding the presence and role of circulating endothelial progenitor cells in SSc (266). Increased levels of circulating endothelial progenitor cells have been demonstrated which supports their mobilization from bone marrow (267). However, in another study, there were substantially reduced numbers of bone marrow-derived circulating endothelial precursors compared to healthy subjects or patients with RA. The lowest number of these cells was observed in SSc patients with active fingertip ulcers, and this may suggest inadequate recruitment of these precursor cells and impaired vascular repair mechanisms (268). Atorvastatin can be effective in RP – perhaps by increasing the number of circulating endothelial progenitor cells, which suggests a role of endothelial progenitor cells in vascular dysfunction (269). Apoptosis of endothelial progenitor cells by a circulating factor has been implicated as the potential mechanism for the reduced number of circulating precursor cells in SSc (270). Mesenchymal stem cells might be another source of endothelial progenitor cells. In SSc, the angiogenic potential of these cells is reduced (271). This suggests that endothelial repair may be affected by unknown SSc disease effects on the bone marrow.

Pericytes mediate vascular maturation and stabilization during angiogenesis (272). They can further differentiate into vascular smooth muscle cells, fibroblasts, and myofibroblasts (273–275). Pericytes express *PDGFR*-β, and high molecular weight melanoma-associated antigen (HMW-MAA) in vascular lesions in SSc patients with associated RP and ANA (276). Another marker of angiogenic pericytes is regulator of G protein signaling (RGS-5), which is highly expressed in SSc vasculature (277). The exact role of RGS-5 is not clear, but it can negatively regulate vessel maturation (278). Pericytes proliferate and contribute to increased vascular wall thickness, which is characteristic of SSc vasculopathy (279).

Endothelial to Mesenchymal Cell Transition in the Pathogenesis of SSc Vasculopathy

There is subendothelial accumulation of activated fibroblasts or myofibroblasts and production of excessive CI and ECM components in blood vessels of SSc patients (1). During this process, endothelial cells lose their specific markers such as *VE-cadherin* and *VW factor* and acquire a mesenchymal phenotype expressing α smooth muscle actin (αSMA), Vimentin, and CI. It is postulated that endothelial cells might transform into mesenchymal cells induced by local growth factors and cytokines (1). The exact molecular mechanism and the cytokines involved are not known, but TGF-β has been implicated. There are recent reports of TGF-β being involved in various disease processes such as endothelial to mesenchymal transformation (280–284). Li and Jimenez (285) further examined the role of TGF-β in the transformation process and the signaling pathways involved (285) in a murine pulmonary endothelial cell model. They concluded that TGF-β could lead to mesenchymal transformation of the endothelial cells. They further demonstrated that the transformation is associated with strong upregulation of transcriptional repressor snail-1 and is mediated by the c-abl kinase and protein kinase C-δ. Snail-1 is a zinc-finger transcription factor that forms a complex with Smad3/Smad4 (1). Snail-1 induces numerous transcriptional events that could lead to expression of a mesenchymal phenotype. Besides this, Wnt signaling as well as NOTCH signaling pathways might be involved in this endothelial–mesenchymal transformation process (1).

Other potential mediators of this transformative process include PDGF (286), VEGF (287), insulin-derived growth factor (288), CTGF (289), ET-1 (290), and miRNAs (291, 292). Endothelial to mesenchymal cell transition is an interesting concept but needs further study to determine what role, if any, it plays in SSc vasculopathy.

Circulating Mediators of Vasculopathy

Higher levels of *ET-1* have been observed in patients with scleroderma renal crisis, lung fibrosis, PAH, and RP (293). Increased ET-1 expression is associated with increased ET-1B receptor in the skin and lung tissue of SSc patients (294).

In SSc, there is a reduction in *eNOS* gene expression and *NO release* in SSc and MVEC derived from lesional and non-lesional skin biopsies in the steady-state and after shear stress (295). This is probably associated with deficient endothelium-dependent relaxation in SSc (296). Impaired NO results in alteration of vascular tone, enhancement of platelet aggregation, and increased susceptibility of endothelial cells to oxidative injury. NO also limits cytokine-induced endothelial cell activation and monocyte adhesion and inhibits the endothelial cell release of IL-6 and IL-8, which are important inflammatory cytokines (297). Further, NO inhibits vascular smooth muscle cell proliferation through elevation of cyclic GMP and inhibition of mitogenic proteins, TGF-β and PDGF. Therefore, impaired NO production in SSc may contribute to the pathogenesis of arteriolar intimal proliferation and may have a prominent role in pathophysiology of the disease.

Coagulopathy in Systemic Sclerosis

Coagulation and fibrinolysis processes are dysregulated as evidenced by presence of microvascular thrombosis and enhanced fibrin deposition frequently seen in the vasculature of SSc patients. The loss of balance between fibrinolysis and coagulation contributes to vessel engulfment with fibrin and breakdown of vessel patency (298). The authors demonstrated impairment of fibrinolysis and activation of the coagulation pathway in a study of 29 patients (298). Activation of the coagulation system, as well as elevated levels of fibrinogen and VW factor, has been demonstrated in patients with SSc (299–302). Reduction of fibrinolysis, expressed as defective tissue t plasminogen activator (tPA) antigen release and/or elevated tPA inhibitor (PAI) antigen, supports existence of heterogeneous hypofibrinolytic pattern in SSc (303).

Plasmin has both pro-fibrotic and anti-fibrotic properties [pro-fibrotic by activating TGF-β and anti-fibrotic by activating both hepatocyte growth factor (HGF) and MMPs] (304, 305). Plasmin is inactivated via formation of a complex with α2-antiplasmin (α2AP), and elevated levels of plasmin-α2AP are associated with several fibrotic conditions including SSc (306). α2AP promotes fibrosis by activating phospholipase A_2 by binding to adipose triglyceride lipase (ATGL) to generate $PGF_2α$, which in turn stimulates production of TGF-β (307). Levels of α2AP are elevated in lesional BLM skin in mice, which is induced by CTGF via extracellular signal-regulated kinase 1/2 (ERK 1/2) and JNK pathways (308). α2AP induces αSMA⁺ myofibroblasts *in vitro* and mice with deletion of α zinc-finger alpha protein gene (αZAP) exhibit less infiltration of myofibroblasts at the site of BLM injections in the skin (308). Plasmin increases ECM degradation, and inhibition

of plasmin of α2AP decreases ECM degradation, which could be another mechanism by which α2AP could promote fibrosis.

Platelet Abnormalities in SSc

Chronic activation of platelets and their released products could contribute to the vascular, immunologic, and connective tissue pathology of SSc (309). SSc platelets show *enhanced aggregation* to various triggers [e.g., CI, adenosine diphosphates, 5-hydroxytryptamine (309–311), ET-1, S1P, and LPA (223)]. ET-1 and S1P cause vasoconstriction by engaging $S1P_2$ and $S1P_3$ receptors (312). In the human fetal lung fibroblast line (FH-1), S1P utilizes $S1P_1$ receptors to inhibit TGF-β1-induced αSMA expression while utilizing $S1P_3$ receptors to stimulate αSMA expression (313). Sera from patients with SSc have elevated levels of arachidonoyl-LPA and S1P (214). LPA induces platelet aggregation, vascular smooth muscle proliferation, and neointima formation, which can induce vasospasm and RP (314–317).

The various platelet-derived factors include: *inflammatory mediators* [NO, serotonin, thromboxane A_2, prostaglandin (PG) D_2, PGE_2, PGF_2, 12-hydroxyeicosatetraenoic acid, β thromboglobulin, neutrophil-activating peptide-2, platelet factor-4, platelet activating factor, adenosine, histamine, P-selectin, CD40 ligand (CD40L), dinucleoside polyphosphates, 2-arachidonyl glyceride, MMP-27], *chemokines* [macrophage inflammatory protein (MIP-1α); monocyte chemoattractant protein-3 (MCP-3); IL-8; and regulated upon activation, normal T-cell expressed and secreted (RANTES)], *cytokines* [IL-1β and granulocyte monocyte-colony stimulating factor (GMCSF)], and *growth factors* [(PDGF) A, B, C, D, TGF-β1 and 2, epidermal growth factor, VEGF-A and C, brain-derived neurotrophic factor, insulin-like growth factor-1 (IGF-1), basic fibroblasts growth factor (bFGF), HGF, and CTGF] (309). Platelets from scleroderma patients overexpress a specific non-integrin *65-kDa receptor for CI*, phosphatidylinositol (PI)-3 secondary to increased nitrotyrosylation and increased protein kinase B (Akt) activity (309, 318). Overexpression of these mediators is induced by cytokines produced by T cells and monocytes activated by autoantigen such as CI that (in turn) changes the phenotype of megakaryocytes (318). The platelets store numerous *fibrogenic mediators* and contribute to chronic tissue fibrosis in SSc by release into tissue of TGF-β1, TGF-β2, PDGF-A, B, C, D, LPA, S1P, adenosine, bFGF, CTGF, and IGF-1. These aforementioned mediators have many biological properties and effects on a host of cells that could also facilitate and contribute to autoimmunity and fibrosis (5).

Animal Models Resembling SSc Vasculopathy

Animal studies in mice recapitulate some of the vasculopathy of SSc. Mice with a conditional deletion of *Fli1* develop systemic vascular lesions characterized by capillary dilation, vascular fragility, stenosis of arterioles, increased vascular permeability, micro-aneurysms, decreased expression of platelet/endothelial cell adhesion molecule (PECAM)-1, PDGF-β, and S1P type I receptor ($S1P_1$) and increased endothelial cell MMP-9 expression (319).

Caveolin-1 (cav-1) is one of three membrane proteins that coat caveolae which are plasma membrane invaginations important in clustering together of receptors that can influence signal

transmission of the specific receptor ligand (320). *Cav-1* is involved in internalization and degradation of TGF-β receptors, thereby reducing signaling by TGF-β (321, 322). There is decreased expression of *cav-1* in lesional skin and lungs of patients with SSc and in lungs of patients with idiopathic pulmonary fibrosis (IPF) (323, 324). *Cav-1* null mice develop PAH and right and left ventricular enlargement and failure (325). However, in contrast to *cav-1* null mice with PAH, in human IPAH, there is an apparent increase in cav-1 expression in the PASMC compared to healthy controls and that the over expression of *cav-1* increases capacitive Ca^{++} entry and DNA synthesis in PASMC (326). The cav-1 null mice also develop pulmonary fibrosis, raising questions regarding the etiology of the PAH in this model which is yet to be clearly defined. In a French and Italian SSc population, *Cav-1* rs959173C showed protective association with SSc and lcSSc (327). The rs959173C protective allele is associated with increased CAV-1 protein expression (327).

Fos-related antigen-2 (Fra-2) transgenic (TG) mice develop microvascular and proliferative vasculopathy and express *Fra-2* in vascular structures (endothelial cells and vascular smooth muscle cells) similar to its expression in skin of SSc patients (328). An early event in the *Fra-2* TG model is apoptosis of endothelial cells (328). The *Fra-2* TG mice also developed pulmonary vascular lesions resembling SSc-associated PAH and later developed dermal and pulmonary fibrosis resembling the "non-specific interstitial pneumonia" (NSIP) (328). These results suggest *Fra-2* might be involved in pathogenesis of SSc vasculopathy and to-date this is the only mouse model that manifests both vasculopathy and fibrosis with features shared by the human SSc disease.

Pulmonary Arterial Hypertension

Cellular Stress in SSc-PAH

Patients with lcSSc, who also have PAH, have the highest expression of the endoplasmic reticulum stress/unfolded protein response genes, *Activating Transcription Factor-4al-b*, a spliced form of X-box BP, and immunoglobulin-heavy-chain BP (257). In PBMC of the lcSSc patients, HSP gene (*DNAJB1*), and IFN-regulated genes (*IFIT1*, *IFIT2*, and *IFITM1*) were upregulated, but *IRF4* was downregulated compared to healthy controls (257). Further analysis showed that the severity of PAH (as reflected in pulmonary artery pressure) positively correlated with level of *DNAJB1* expression, while endoplasmic reticulum stress marker correlated with IL-6 levels in the whole lcSSc population (257).

Interferon Signature in SSc-PAH

Type I IFNs are implicated by the association of use of IFNα in the treatment of hepatitis and of IFNβ in the treatment of MS with development of PAH (118, 119). Diseases in which there is an "IFN signature" (such as SLE, SSc, and infection with HIV) are associated with development of PAH (120–124). Furthermore, IFNα and IFNγ (added to cultures of human PASMC primed with TNFα or to cultures of human lung MVEC or human lung fibroblasts) cause release of the potent vasoconstrictor, *ET-1*, and of *IP-10* (117). In a series of 128 SSc patients with PAH and 35 patients with no PAH, the SSc patients with PAH had higher levels of IP-10 and ET-1 in their sera compared to SSc patients without PAH or compared to healthy controls; more SSc patients with PAH had

detectable levels of IFNα and IFNγ in their sera than SSc patients without PAH (117). In this series of SSc patients, levels of TNFα, IL-12p70, IL-6, IL-1α, and IL-8 were significantly higher in sera in SSc patients with PAH when compared to SSc patients without PAH (117). Additional studies of this patient group revealed that serum levels of IP-10 in the SSc-PAH patients correlated with pulmonary vascular resistance, and levels of brain natriuretic peptide in serum, and serum IP-10 levels in the SSc-PAH patients inversely correlated with cardiac index and 6-min walks test (117). Sections of lung from patients with IPAH or with SSc-PAH expressed higher levels of IFNR1 in endothelium, smooth muscle layer, vascular interstitium, and in intravascular inflammatory cells as assessed by immunohistochemistry and Western blotting (117). While the above studies strongly implicated type I IFN as playing a pathogenic role in SSc-PAH and IPAH, further evidence was substantiated in the *type I IFN α receptor 1* knockout mouse which was found to be resistant to experimental hypoxic PAH induction. These mice did not have elevated serum levels of ET-1 when compared to WT control mice (117). Analysis of PBMC from patients with SSc revealed *Siglec-1* and other IFN-regulated genes were overexpressed in patients with dcSSc, whereas patients with lcSSc with PAH overexpressed *IL-13RA1*, *ICAM-1*, *CCR1*, *JAK2*, and *MCR1* (123, 125, 126). IL-13 was also elevated to higher levels in sera of patients with lcSSc with PAH, and *MCR1* was induced on CD14$^+$ monocytes suggesting monocytes are activated in lcSSc patients with PAH of an alternative (i.e., IL-4/IL-13) rather than classical (i.e., IFNγ/LPS) pathway (123).

Other Mediators and Gene Polymorphisms in SSc-PAH

Polymorphisms were described in the promoter of the *NOS2* gene that confers susceptibility to PAH in SSc (144).

In another report, patients with lcSSc with PAH, had higher levels of circulating monocyte-related cytokine mediators (TNFα, IL-1β, IL-6, and ICAM-1) and vascular injury markers (VEGF, VCAM-1, and VW Factor), and their PBMCs exhibited increased expression of mRNA for ICAM-1, IL-1β, JAK2, IFNGR1, IL-13Rα1, tissue inhibitor of metalloproteinase (TIMP)-2, delta-aminolevulinate synthase 2 protein (ALAS2), CCR1, and AIF1akt (126).

Urokinase-type plasminogen activator receptor, *CD87*: (discussed under "Genetics of SSc") SNP, *UPAR* rs344781G allele, is associated with SSc-related digital ulcers, pulmonary artery hypertension, ACA positivity, and lcSSc (91).

Sphingosine 1-phosphate and *LPA* may have effects on the vasculature in SSc that contribute to some of the abnormalities observed in the disease. For example, there is overexpression of VE-cadherin, IFNα signaling, and *Rgs-5*, which is associated with an antiangiogenic phenotype (188). Overexpression of *Rgs-5* may reduce signaling via S1P$_1$ receptor and increase S1P signaling through other S1P receptors that could reduce endothelial eNOS, increase vasoconstriction, increase vascular leakiness, and reduce angiogenesis [reviewed in Ref. (223)]. Furthermore, S1P may contribute to PAH by constricting pulmonary arteries while LPA may contribute to systemic hypertension, cardiac fibrosis, endothelial cell activation, and neointima formation (via PPARγ) [reviewed in Ref. (223)].

Lysophosphatidic acid, S1P, and other chemoattractants (such as TGF-β1, TGF-β2, IL-8, MCP-3, and other mediators released from aggregated/activated platelets adhering to damaged microvascular endothelium and diffusing into perivascular tissue) could establish chemotactic gradients that would promote outward transversal migration of monocytes, dendritic cells T and B lymphocytes, and NK cells resulting in perivascular accumulation of these cells to set the stage permitting innate and adaptive immune responses that lead to autoimmunity and fibrosis (223).

Fibrosis in SSc

Links to the Innate and Adaptive Immune Systems

Over three decades ago, it was recognized that human lymphocytes and monocytes (when stimulated by antigen or T-cell mitogen *in vitro*) elaborate soluble mediators (lymphokines, monokines, growth factors, chemokines, and cytokines) that induced fibroblast chemotaxis or (when added to cultures of human fibroblasts) induce fibroblast growth and synthesis of collagenase (MMP-1) and CI (329–340). These studies provided tangible evidence that immune cells are fully capable of modulating chemotaxis and growth of fibroblasts, as well as regulating synthesis of CI and CIII and the major CI degradative enzyme, MMP-1, by fibroblasts.

Later studies conducted with purified recombinant or natural cytokines, chemokines, and growth factors known to be synthesized by cells of the innate and adaptive immune system have allowed fibroblasts specific functions to be assigned to certain ones. *TGF-β1*, which is produced by most cell types but also by CD4+CD25+Foxp3 Tregs, monocytes/macrophages, mast cells, and platelets and *IL-4*, which is produced by Th2 cells, and mast cells received early attention as being potent stimulators of CI synthesis and chemotaxis by fibroblasts (341–345).

Cells of the innate and adaptive immune system elaborate a variety of cytokines and chemokines in addition to TGF-β and IL-4 (such as *IL-6*, *PDGF*, *IL-1*, *IL-13*, *IL-17*, *IL-5*, *MCP-1*, and *CTGF*) that have been found to be increased in serum or in tissues in which excess connective tissue matrix is accumulating in SSc. These cytokines/chemokines are at the interface between the immune system and fibroblasts.

Signal transducer and activator of transcription protein 4 is critical for T-cell signaling and differentiation (132–134). *STAT4* is involved in effecting a Th1 cytokine response by transmitting signals from IL-2, IL-12, and IL-23 receptors and in signaling after type 1 IFN engages its receptor (135, 136). The role of *STAT4* in fibrosis was assessed in scleroderma mouse models. The deletion of *STAT4* significantly reduced skin fibrosis in the BLM model but not in the Tsk-1/+ model (137). In the BLM model, it was noted that there were decreased numbers of inflammatory cells including T cells and proliferating T cells and decreased quantity of IL-6, IL-2, TNFα, and IFNγ in lesional skin of *STAT4*−/− vs. *STAT4*+/+ mice (137). In addition to having a role in SLE and RA susceptibility, *STAT4* has been identified as a susceptibility gene in SSc (50) (see **Table 3**).

Macrophage migration inhibitory factor-173 acts upstream and activates innate immunity. It plays a role in sustaining cellular and inflammatory response. It causes fibroblasts proliferation and acts as an antiapoptotic (135).

Links to Vascular Damage

Endothelin-1 is one of three isoforms and is synthesized by VE cells, fibroblasts, bone marrow mast cells, neutrophils, macrophages, and cardiac myocytes (140) (See discussion under "Genetics of SSc"). ET-1 is overexpressed in skin biopsies of patients with dcSSc (179).

Fos-related antigen-2, reviewed above, appears to have both vasculopathic and fibrogenic properties and may be a contributor to these processes in patients with SSc.

SSc Fibroblast Phenotype and Myofibroblasts

Earlier studies indicated that normal human dermal fibroblasts (grown for prolonged periods of time *in vitro* in the presence of culture medium supplemented with culture supernatants obtained by activating normal human donor peripheral blood lymphocytes and monocytes with T-cell mitogen *in vitro*) acquired a "scleroderma-like phenotype" that resembled cultured lesional SSc skin fibroblasts at the ultrastructure level with respect to excessive production of glycosaminoglycans (346). A phenotypic characteristic of cultured SSc lesional skin fibroblasts is that they produce reduced levels of *MMP-1*, an enzyme necessary for degradation of triple helical CI and CIII (347). Some SSc lesional fibroblasts regain production of MMP-1 after several subpassages, and when these fibroblasts lines are then cultured for 3 weeks with *IL-13* or *PDGF-BB*, then cultured in plain medium before TNFα stimulation, the production of MMP-1 in response to TNF-α stimulation is markedly reduced compared to normal donor fibroblasts similarly treated with IL-13 or PDGF-BB (348). These studies suggest that *in vivo* chronic exposure of SSc fibroblasts to certain cytokines, derived from activated lymphocytes and monocytes either in circulation or from lymphocytes/monocytes infiltrating SSc lesional skin, can induce an SSc fibroblast phenotype that persists in the absence of the cytokines for some period of time. Platelets that are being chronically activated/aggregated in patients with SSc may also contribute some cytokines/growth factors (e.g., PDGF-BB) that could contribute to induction of the scleroderma fibroblast phenotype (309).

Fibroblasts cultured from lesional skin biopsies of patients with SSc contain increased numbers of myofibroblasts and synthesize increased amounts of CI and *TIMP-1*, in contrast to fibroblasts grown from non-lesional SSc skin or skin of healthy controls (349). This increased CI production phenotype reverts toward normal as the SSc lesional fibroblasts in culture are passaged, as shown by LeRoy (350). The myofibroblasts in SSc lesional skin contain αSMA and fibronectin ED-A splice variant, the latter being a requirement for TGF-β1 to induce myofibroblast formation (275, 351). In normal wound healing, myofibroblasts contract the newly formed ECM, and their development and function are modulated by mechanical forces and stiffness of the ECM microenvironment (352). The origin of myofibroblasts in SSc lesional skin is not completely understood, but likely candidates include resident connective tissue fibroblasts, epithelial cells, pericytes, and circulating fibrocytes. Myofibroblasts are induced by a number of cytokines, growth factors, and other agents present in SSc tissue or serum, including: *TGF-β1*, *TGF-β3*, *IL-4*, *TNFα*, *IL-6*, *GMCSF*, *thrombin*,

bradykinin, *histamine*, *tryptase*, *oncostatin M*, *IL-13*, *PDGF-β*, *ET-1*, *TLR 2/1 ligands*, and the *lysophospholipids*, *S1P* and *LPA* (5, 353–355).

Levels of *IL-1α* are elevated in sera of patients with SSc, and SSc monocytes produces more. IL-1 than normal monocytes when stimulated *in vitro* (356, 357). IL-1α and -β stimulate proliferation of human dermal fibroblasts and upregulate production of CI, TIMP, PGE_2, MMP-1, and hyaluronan (358, 359). IL-1α and -β were observed to promote viability of cultured SSc lesional skin fibroblasts and myofibroblasts *in vitro* in the presence or absence of serum and directly induced expression of αSMA and N-cadherin (360). This suggests that IL-1 may contribute to the longevity of myofibroblasts in SSc skin.

Fibroblasts grown from SSc lesional skin biopsies constitutively overexpress IC IL-1α; and after stimulation *in vitro* with TNFα or IL-1β, both *icIL-1α* and *icIL-1* receptor protein antagonist (*icIL-1ra*) are markedly upregulated compared to normal donor fibroblasts (361). Overexpression of icIL-1α in normal skin fibroblasts also induces expression of icIL-1ra (361). When icIL-1ra is overexpressed in cultures in normal human skin fibroblasts via transfection with a viral vector (pLXSNicIL-1ra type 1), it induces a myofibroblast phenotype characterized by increased expression of α*SMA* and *PAI-1* (362).

Treatment of SSc lesional fibroblasts with IL-1α siRNA resulted in decreased proliferation and production of IL-6 and CI, whereas stably transfecting with icIL-1α induced proliferation and IL-6 and CI synthesis (363).

TGF-β Receptor-Smad Signaling in Fibroblasts

A great deal of effort has elucidated the complex receptor engagement and signaling of TGF-β and its 1, 2, and 3 isotypes that occur in mammals and which have been the topic of several recent reviews (364–367). TGF-β1, 2, and 3 are synthesized as *inactive propeptides* which have to be cleaved intracellularly by the protease, *farin*, to generate active 25 kDa MW, active TGF-β1, 2, or 3. The active TGF-β is bound by the cleaved amino terminal peptide called "latency-associated peptide" (LAP) and, in connective tissue, the latent TGF-β1-LAP complex is bound to latent TGF-β1-binding protein (LTBP), which is termed "large latent complex" (LLC) (365, 368). Latent TGF-β can be activated by interaction with *integrins* and by *several proteases* such as thrombin, plasma transglutaminase, cathepsin D, and plasmin (369). There are three classes of TGF-β receptors. *TGF-β receptor 1* has two forms: *ALK1* (found mainly in endothelial cells) and *ALK5* (which is present in most cells) (367). *TGF-β receptor 2* forms a heteromeric complex with type 1 receptors and phosphorylates it, setting in motion IC signaling via receptor-regulated Smads (R-Smads) which are type 1 receptor specific [i.e., ALK1 causes Smad1/5/8 phosphorylation while the predominant ALK5 causes Smad2/3 phosphorylation (367)]. The phosphorylated R-Smads complex with Smad4 and in the nucleus interact with co-activators [e.g., CREB-binding protein (CBP)/p300] and co-expressors (e.g., Ski/Sno) to transcriptionally activate or repress target genes (367). Inhibitory Smads (Smad 6 and 7) can bind to TGF-β type 1 receptors and to Smad4 or effect ubiquitination and proteasomal degradation (367). A

coreceptor called *endoglin*, of which there are two spliced variants called short and long forms, can (under different conditions by interacting with ALK1 or ALK5) decrease or enhance TGF-β signaling, respectively (367). *Betaglyan* ("type 3" TGF-β receptor) can also act as a coreceptor by facilitating TGF-β binding/interaction with type 1 and 2 TG-β receptors (370). *CTGF* can also interact with TGF-β type 1 and 2 receptors and facilitate Smad3 signaling, which has a pro-fibrotic effect (367). Other members of the TGF-β superfamily including *Activin* (*A*, *B*, and *AB*), bone morphogenic proteins (BMPs), and growth differentiation factors utilize components of the TGF-β receptor complex (366). In addition to the canonical Smad-dependent pathway described above, TGF-β can signal through non-canonical Smad-independent Wnt, MAPK, phosphatidylinositol-3-kinase/AKT, and Rho-like GTPase pathways (366). Activating transcription factor 3 (ATF3), which regulates oxidation and cellular stress, is upregulated in SSc dermal fibroblasts by TGF-β; and ATF3 suppresses TGFG-β-induced proliferative effects via interaction with Smad3 in a c-Jun-dependent manner (371).

Recently, it was reported that the fibrogenic effect of IL-6 in fibroblasts is brought about by binding of IL-6 to soluble IL-6 receptor (IL-6R) by a JAK1 and STAT3-dependent mechanism that is mediated through *Gremlin-1*, which utilizes TGF-β type 1 and 2 receptors and the TGF-β signaling pathway dependent on Smad3 that leads to CI gene expression, but is not dependent on TGF-β protein (372).

Transforming growth factor-β induces the early response gene (*Egr-1*), via a Smad-independent pathway via MEK1/2/ERK signaling (373). Overexpression of *Egr-1* induces CI gene upregulation (374). In addition, IL-13 and insulin-like growth factor-binding protein-5 (IGF-BP-5) have been shown to induce *Egr-1* expression by MAPK signaling pathway (375). Other extracellular signals which are relevant to SSc [such as PDGF, hypoxia, HGF, or LPS (bacterial LPS), oxidative stress, thrombin, LPA, ultraviolet light, cigarette smoke, mechanical strength, ischemia-reperfusion, and T-cell receptor ligature] have been shown to increase *Egr-1* expression (373). TGF-β also induces *Egr-3* by canonical Smad3 signaling, and *Erg-3* overexpression stimulates CI gene expression (376).

Antifibrotic Mediators

Bone morphogenic protein-7, although a member of the TGF-β superfamily, stimulates fibroblast chemotaxis like TGF-β1, but does not induce CI, fibronectin, hyaluronan, or TIMP synthesis (377). BMP-7 also inhibits fibrogenic properties of TGF-β1 (378) and signals through a receptor complex structurally different from that of TGF-β and utilizes SMAD1/5/8 (365). *IL-10* inhibits both proliferation and CI synthesis by fibroblasts (379). Certain IL-10 genotypes have been associated with development of SSc in Caucasian and Japanese subjects (380). *TNFα* inhibits CI, stimulates MMP-1 synthesis by fibroblasts, and is a potent chemoattractant for these cells (381). *IFNγ* is a potent inhibitor of expression of CI and CIII mRNA and protein by cultured SSc fibroblasts *in vitro* (382). To what extent BMP-7, TNFα, IL-10, IFNγ, or other antifibrotic mediators or mechanisms try to counter the drivers of fibrosis such as TGF-β, IL-4/IL-13, IL-6/IL-6R-Gremlin-1 in SSc is unknown but provides candidates to be the focus of future studies.

Effect of blocking *TNFα* with etanercept was assessed in the BLM scleroderma mouse model. Compared to vehicle-treated mice, the etanercept-treated mice had less dermal fibrosis and lower serum levels of TGF-β1 than controls not treated with etanercept (383). Etanercept has not been efficacious in ameliorating dermal fibrosis in patients with SSc (384) (see **Table 3**).

Peroxisome proliferation-activated receptor gamma-γ, when engaged by ligands of different types, blocks TGF-β-mediated fibrotic responses *in vitro* in cultured fibroblasts and in various fibrotic animal models *in vivo* (81, 82). PPARG rs310746 is associated with SSc (83).

In the *cGVHD* murine model of scleroderma induced by transferring splenocytes from B10.D2 donor mice into BALB/c recipients, tolerizing the recipient BALB/c mice *by oral administration of protein extract of BALB/c spleens* for 11 days after transfer of B10.D2 splenocytes was associated by upregulation of IL-10 and downregulation of IFNγ production by T cells from the BALB/c recipients and protected the recipient BALB/c mice from dermal fibrosis and other manifestations of cGVHD (385). IL-10 was likely produced by Tregs induced by oral tolerance induction by the BALB/c spleen extract and was likely responsible for suppression development of fibrosis (379).

Genome-Wide Gene Expression of Skin

The fibrogenic role of *TGF-β*, *IL-13/IL-4*, and *Egr-1* in patients with SSc has been assessed by performing genome-wide gene expression studies on lesional and non-lesional skin biopsies from patients with dcSSc, lcSSc, morphea, and healthy controls. These studies show four intrinsic subsets of gene expression termed "diffuse proliferation" (further divided into diffuse1 and diffuse2) and containing only dcSSc patients; inflammatory group containing dcSSc, lcSSc, and morphea; limited group containing lcSSc; and a normal-like group containing normal, dcSSc, and lcSSc patients (386). Further comparisons were made subjecting TGF-β, IL-13/IL-4, and Egr-1-stimulated normal dermal fibroblasts in culture to gene expression microarray analysis and comparing these fibroblast microarrays to gene expression arrays of biopsies of skin from SSc, morphea, and normal donors. TGF-β responsive gene signature was found in 10 out of 17 patients with dcSSc (59%) and none of 7 lcSSc, none of 3 morphea, and none of 6 healthy controls (387). The dcSSc patients with the TGF-β-responsive signature had higher MRSS and likelihood of having ILD (387). The TGF-β signature-positive dcSSc patients also were in the diffuse-proliferation subset; however, one in the diffuse-proliferation subset did not have the TGF-β signature. This suggests that only a subset (and not all) SSc patients have the TGF-β signature. The fibroblast *Egr-1*-responsive gene signature was present in the skin biopsies from diffuse-proliferation subset of dcSSc patients, but was not present in biopsies of patients with lcSSc, morphea, or healthy controls (388). The *IL-4* response signature overlapped approximately 60% with the IL-13 response signature, which were both enriched in the SSc inflammatory subset (389). Expression in skin biopsies from SSc patients of the IL-13 pathway activation [as well as transcripts of IL-13 receptor components (*IL-13RA1* and *IL-4RA*)] correlated with MRSS (389). Expression of *CCL2* (MCP-1) transcripts also correlated with MRSS and *IL-13RA1* (389). This study also

assessed gene expression profiling in skin of a sclerodermatous graft-versus-host disease (scl GVHD) model in *Rag2⁻/⁻* mice, which were found to also exhibit the IL-13 pathway activation resembling that in SSc patients of inflammatory subset (389). This observation is interesting, given that it has been hypothesized that fetal–maternal or maternal–fetal microchimerism might induce a cGVHD state in some patients with SSc as described above. Since IL-6 and IL-6R induces Gremlin-1 protein (which then signals through the canonical Smad-dependent pathway), it raises the question as to whether some of the TGF-β signature in the dcSSc diffuse-proliferation subset (discussed above) is actually due to *Gremlin-1*. Further studies would need to be done comparing Gremlin-1-induced gene signature in dermal fibroblasts with that of TGF-β1 to sort this out.

A more extensive genome-wide expression profiling skin biopsies involving analysis of additional pathway-specific gene signature for *PDGF*, *S1P*, *PPAR-γ*, *TNFα*, *IFNα*, *NFκB*, *IL-13*, *IL-4*, *poly (I-C)*, and *inomycin-phorbol 12-myristate 13-acetate (inomycin-PMA)* was recently conducted by this group (390). Results showed IFNα signaling was strongly associated with early disease, compatible with the notion that innate immune response may be a feature in early disease which was contrasted with TGF-β signaling being a feature of later disease with worse MRSS (390). Surprisingly, PDGF signaling was most strongly associated with the fibroproliferative subset (more so than TGF-β), and the inflammatory subset exhibited strong activation of innate immune pathways including enrichment of IL-4, S1P, NFκB, LPS, poly(I-C), and TGF-β gene signatures (390). The findings support an earlier hypothesis by Gabrielli et al. that a stepwise process of SSc development begins with inflammatory (e.g., IFNα signaling) and continues with fibrosis (e.g., PDGF and TGF-β signaling) and ends in atrophy (391). IL-4 pathway was significantly enriched in the inflammatory subset more than IL-13, and suggests a T$_H$2 enhancement of immune response in patients within the inflammatory subset (390).

Most patients with dcSSc have some resolution with the passage of time of dermal fibrosis after the onset of their disease. This has been observed in several different studies clinically as decreases in the MRSS. In a large, single SSc center in the UK, 131 patients with dcSSc had MRSS measured repeatedly up to 36 months after onset of their disease (392). Three patterns were discernable as follows: those with high baseline MRSS that did not improve over 36 months from baseline (38%); those with high baseline MRSS that improved over 36 months from baseline (21%); and those with low baseline MRSS that improved over 36 months from baseline (35%). The reason for these three clinical trajectories of change in MRSS over time is not apparent, but could be a function of different genetic backgrounds, different triggers, or other environmental modifications that either ameliorate or contribute to perpetuation of the disease. The patients received different medications; however, clinical MRSS response or survival could not be attributed to any of the medications (392). The fact that most of these patients with dcSSc had improvement in their MRSS suggests that the myofibroblast phenotype responsible for excessive ECM deposition does not persist, that the fibrotic skin can revert toward normal, and that a normal-like homeostasis can be re-established in such patients. This study suggests that

those dcSSc patients with persistently high MRSS likely have a continuous presence of a driver of dermal fibrosis that constantly stimulates the fibroblasts to maintain the myofibroblasts phenotype with maintenance of increased ECM in their dermis. Application of the genome-wide gene expression studies of skin biopsies, in a cohort such as this one in which patients have skin biopsied repeatedly over several years, may shed light on the mechanisms responsible for the three different MRSS trajectories over time, and would answer the question whether the inflammatory subset morphs into the fibroproliferative subset.

Vitamin D and Fibrosis

Vitamin D has a variety of antifibrotic actions. Studies *in vitro* have demonstrated *1,25(OH)$_2$D3* inhibits growth of murine fibroblasts (393–397), inhibits fibroblast-mediated contraction of CI gels (largely a TGF-β-stimulated function) (398), inhibits fibroblast synthesis of IL-6 and IL-8 (399, 400), and inhibits production of plasminogen activator (401). It was also observed that 1,25(OH)$_2$D3 *in vitro* inhibited CI and CIII synthesis by fibroblasts grown from different human tissues including bone marrow, lung, and skin (402, 403). In mice, *in vivo* administration of 1,25(OH)$_2$D3 has been shown to ameliorate renal interstitial fibrosis, glomerulosclerosis in rats, and reduce conversion of adipose tissue to fibrous tissue in mouse skin exposed to chronic UV irradiation (404, 405).

The cutaneous formation and metabolism of VitD in patients with SSc has been reported to be normal (406–408). In one report, fibroblasts grown from biopsies of lesional skin from patients with SSc and from healthy volunteers were inhibited in proliferation and CI synthesis to a similar extent by 1,25(OH)$_2$D3 addition to the fibroblast culture (409). The VitD receptor (VDR) in SSc lesional skin fibroblasts is reported to be decreased, likely due to TGF-β's ability to downregulate the VDR (410).

Studies using a mouse mesenchymal multipotent cell line revealed that 1,25(OH)$_2$D3 promoted increased expression and nuclear translocation of the VDR; decreased expression of TGF-β1 and plasminogen activator inhibitor (SERPINE 1); decreased expression of CI I, III, and other CI isoforms; and increased expression of several other antifibrotic factors including *BMP-7*, *MMP-8*, and *follistatin* [an inhibitor of the pro-fibrotic factor, myostatin (411)]. Studies in rat interstitial myofibroblasts showed that 1,25(OH)$_2$D3 inhibited in a dose-dependent manner (10^{-9}–10^{-6} M) TGF-β1-induced *de novo* αSMA expression and suppressed CI and TSP-1 expression induced by TGF-β1, which was shown to be mediated by upregulated HGF (412).

Slominski et al. have discovered the skin and other tissues in humans synthesize other VitD derivatives [including *20(OH)D3*, *20,23(OH)$_2$D3*, and *17,20(OH)$_2$pD*] that, also like 1,20(OH)$_2$D3, *in vitro* inhibit CI and hyaluronan synthesis by fibroblasts grown from normal or SSc lesion skin (144). Unlike VitD3, 25(OH)D3, or 1,25(OH)$_2$D3, these novel endogenously produced VitD analogs are non-calcemic when given in high doses to mice. 20(OH)D3 also suppressed development of dermal fibrosis in the BLM, scleroderma mouse model (413). These results suggest multiple endogenous forms of VitD3 have antifibrotic properties that may prove useful in SSc as therapeutic agents in the future.

Lysophospholipids and Fibrosis

Lysophosphatidic acid induces fibroblast chemotaxis and proliferation (414). LPA induces αvβ6 integrin-mediated TGF-β activation by engaging LPA$_2$ receptors on epithelial cells and makes fibroblasts resistant to apoptosis, which is a characteristic of SSc lesional fibroblasts that would prolong their survival (415, 416). Evidence that LPA is involved in myofibroblast formation in SSc lesional skin was suggested by the finding that fibroblasts cultured from skin of SSc patients exhibited increased LPA-activated chloride current, which is a hallmark of LPA-induced myofibroblasts (417). *AMO95*: a selective small molecule inhibitor of LPA$_1$ signaling (AMO95) protected mice from developing BLM-induced skin fibrosis and increased regression of established BLM-induced skin fibrosis (418). Contrary to the results in the BLM skin fibrosis model in which LPA$_2$ knockout did not affect dermal fibrosis, in the BLM lung fibrosis model, LPA$_2$ knockout mice exhibited reduced lung injury, fibrosis, and fibronectin deposition in BLM-treated lungs (419). *S1P* facilitates migration of fibroblasts in response to a chemotactic gradient of fibronectin in a S1P$_2$ receptor-dependent manner (420). S1P signals through the Smad pathway utilized by TGF-β1 in fibroblasts and other cell types and mimics TGF-β1 pro-fibrotic effects in that it decreases MMP-1 and increases TIMP and CI production by fibroblasts (421–423). SSc dermal fibroblasts express more S1P$_3$ receptors than control donor fibroblasts and exhibit an exaggerated pro-fibrotic response to TGF-β1 (421). Furthermore, S1P levels are elevated in sera of patients with SSc (214). As mentioned above, S1P gene signature is prominent in the inflammatory subset (390). Fingolimod (*FTY720*) has both agonist and antagonist effects in different S1P receptors and modulates lymphocyte trafficking, monocyte/macrophage biology, dendritic cell biology, and enhances Treg function at marginal zone B lymphocytes (144). When administered to chronic scleroderma graft-versus-host disease (cScl-GVHD) mice, FTY720 in either preventative or therapeutic protocols reduced fibrosis, expanded splenic myeloid suppressor cells, increased Tregs and B regulatory cells (Bregs), protected against vascular damage, reduced serum S1P and E-selectin levels, reduced numbers of inflammatory cells in skin, and reduced dermal expression of mRNA for TGF-β1, MCP-1, MIP-1α, RANTES, TNFα, IFNγ, IL-6, IL-10, and IL-17A (424). FTY720 also returned phosphatase and tensin homolog (PTEN) and Smad3 phosphorylation to normal levels in cScl-GVHD mice (424). Although FTY720 is approved to treat MS, its use in SSc clinical trials has not been reported.

The Endocannabinoid System and SSc

The ECS is an endogenous regulatory network made up of multiple GPCRs and a series of endogenous arachidonic acid derivatives, which act in an autocrine fashion and seem to play a homeostatic role affecting diverse key biologic and physiologic processes including angiogenesis, cell proliferation, apoptosis, differentiation, metabolism, immune function, and vascular tone that may have implications for SSc pathogenesis and potential therapeutic targets. The term "endocannabinoid" generally refers to the first two characterized endocannabinoids (ECs), anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), though a number of endogenous cannabinoid receptor agonists have since been discovered.

AEA and 2-AG may be degraded into free arachidonic acid by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively, but may also be metabolized by lipoxygenases, cyclooxygenase 2 (COX-2), and P450 epoxygenases, and acyl transferases yielding a vast library of EC analogs with different actions and target receptors (425). Some of these metabolites engage EC receptors, while others have been demonstrated to modulate the activity of ECs via "entourage effects" at cannabinoid receptors 1 (CB1) and 2 (CB2), inhibition or potentiation of ECS degradation, activation of downstream targets such as *PPARγ* or metabolic interconversion (426).

Efforts to identify new cannabinoid receptors are ongoing; but CB1, CB2, and GPR55 are among the most extensively studied, and recent studies clearly demonstrate that all three of these receptors are activated by AEA and play a key role in transduction of EC signaling. Interestingly, CB1, CB2, and GPR55 have also been shown to modulate one another's activity via heteromerization, cross-antagonism, and other strategies (427, 428). GPR18 (also known as "abnormal cannabinoid receptor"), another candidate cannabinoid receptor, may play a role in EC-mediated central blood pressure control and peripheral vascular tone (429, 430). Unlike CB1, CB2, and GPR55; however, activation of this receptor requires FAAH-mediated metabolism of AEA or 2-AG to *N*-arachidonoyl-glycine (431). It is interesting to speculate that if there is reduced FAAH in tissue-expressing GPR18 in patients with SSc as has been found in SSc dermis, then this might contribute to vasoconstriction and hypertension (432). Substantial cross talk has also been established between the ECS and a network of non-selective action channels known as the transient receptor potential vanilloid (TRPV) family, which serve to integrate mechanical and environmental stimuli with local autocrine signals to effect a variety of cell processes (433, 434).

Endocannabinoid System as a Therapeutic Target in SSc

The ECS is an appealing potential target for treatment of SSc, as it modulates endothelial cell function, vascular tone (including pulmonary artery vasodilation), the innate immune response to injury, autoimmunity, and fibrogenesis (435–440).

Endocannabinoid Modulation of the Immune System

The general effect of cannabinoids on cells of the immune system is to act as immunosuppressive and anti-inflammatory agents. Although immune cells express more CB2 than CB1, both receptors and other non-CB receptors (such as PPARγ and GPR55) have been implicated in effecting immunomodulatory actions of cannabinoids (441, 442). CB1 mRNA and protein expression in/on immune cells is responsive to cellular activation signals, i.e., cell type, cannabinoid ligand type, and immune stimulus-dependent (443). Of relevance to SSc, IL-4 is specifically able via STAT5 pathway to induce CB1 mRNA in human T cells (444). Dendritic cells exposed to cannabinoids undergo NFκB-dependent apoptosis and reduce production of IL-12, which is important in priming Th0 cells to a Th1 orientation (445, 446). Cannabinoids induce apoptosis of T cells via CB1 and CB2 engagement and effect a Th2 polarization (e.g., increased IL-4 by T cells) while decreasing Th1 polarization (e.g., decreased IL-12 by DC) but

also suppress activation, differentiation, and expansion of T cells (443, 447, 448). B cells are affected by cannabinoids in several ways, including direct effects on activation, differentiation, and proliferation but also via effects on T cells that provide help to B cells (449). The effect of cannabinoids on B cells to suppress IgM and enhance IgE production apparently is mostly via engagement of CB2 (450). Production of pro-inflammatory cytokines (including TNFα, IFNγ, IL-2, and IL-1β) is suppressed *in vitro* and *in vivo*, by engaging CB2 by cannabinoids or other CB2 agonists (451–453). Of particular relevance to SSc is that mast cell activation is inhibited by 2-AG (454). The CB2 agonist, Gp1a, was found to suppress clinical disease in the EAE mouse model with a reduction in Th1 and Th17 cells in peripheral lymphoid organs. Analysis of the CD4$^+$ cells *in vivo* in the periphery revealed Gp1a-treated mice had lower levels of expression of T but also RORγt (Th17 marker) and exhibited increased Foxp3 and GATA-3 expression (455). Under polarizing conditions *in vitro*, Gp1a suppressed Th1 and Th17 development of CD4$^+$ T cells (455). The role of the ECS in innate and adaptive immune dysregulation in SSc is an area for further investigation, and these results with this CD2 agonist suggest similar agents might decrease autoimmunity and autoantibody production in SSc.

Endocannabinoid Modulation of the Vasculature

The upregulation of *ICAM-1* and *VCAM-1* on the endothelium of human coronary arteries by treatment with TNFα or LPS is inhibited by the CB2 agonist drug, *JWH-133* (437). Engaging CB2 in rat coronary arteries by AEA induced the coronary arteries to dilate (456). Blocking CB1 on isolated human coronary smooth muscle cells by the CB1 antagonist, *rimonabant*, reduced ability of the smooth muscle cells to migrate and proliferate in response to PDGF (438). The ECs (*AEA* and *virodhamine*) were found to have a potent vasodilatory effect on preconstricted isolated human pulmonary artery rings that was endothelium-dependent and likely involved PGE$_2$ (436). The effect of AEA and virodhamine was CB1- and CB2-independent but involved a third receptor termed "endothelial cannabinoid receptor" (436). These studies suggest that a target for development of treatment for PAH in SSc might be based on virodhamine-like drugs that are agonist for the endothelial cannabinoid receptor.

Endocannabinoid Modulation of Fibrosis

C57BL/6 mice with either *TRPV1* receptor or *calcitonin G-related peptide (CGRP)* knocked out compared to WT mice developed enhanced dermal fibrosis after repeated subcutaneous injection of BLM (434). This suggests that *TRPV1* receptor and *CGRP* have antifibrotic effects and may have relevance to patients with RP and SSc since skin biopsies from patients with SSc have reduced numbers of *CGRP*-immunoreactive C fibers and would likely have reduced vasodilatation from *CGRP* in response to stressors that trigger RP (434). It is unclear whether other *TRPV1* receptor engagement or *CGRP* effects are operative to protect against fibrosis. *TRPV4* has been implicated as a mechanosensor in endothelial cells and fibroblasts, and has been shown to stimulate myofibroblast differentiation in rat cardiac fibroblasts via integration of mechanical and soluble (autocrine) signals, and pretreatment with the TRPV4 antagonist, *AB159908*, resulted in significant inhibition

of TGF-β1-induced myofibroblasts differentiation of cardiac fibroblasts (457). *5,6-EET* (generated by activation of *PAR-2* by mast cell tryptase or Factor Xa) has been implicated as the most likely autocrine mediator contributing to activation of *TRPV4*, though other autocrine signals may be involved as well, with the known *TRPV4* agonist *N*-acyl taurine being another possible candidate. It is worth noting that this system of channels seems to be dysregulated in dcSSc fibroblasts with profound downregulation of *TRPV2* (and possibly *TRPV1*) and overexpression of *TRPV4*. While the role of *TRPV1* and *TRPV2* is less clear, *TRPV4* is known to stimulate myofibroblast differentiation in response to activation by mechanical stress and arachidonic acid derivatives. In normal wound repair, release of the myofibroblast from this mechanical stress signal plays a role in inducing apoptosis or, alternatively, may help drive the myofibroblast back into a quiescent fibroblast. Given that TRPV4 remains overexpressed in the dcSSc fibroblast, this may suggest that certain autocrine signals are present, which alter the cellular milieu in favor of constitutive activation of *TRPV4*, thus rendering the myofibroblast incapable of responding appropriately to mechanostress signaling. AEA and 2-AG activation of *TRPV4* is indirect and requires hydrolysis of these compounds to free arachidonic acid, which is then converted into the potent *TRPV4* agonist 5,6-EET. The fatty acid amide *N*-acyl-taurine was recently discovered to be a potent agonist of TRPV4, as well, and is likely overexpressed in SSc owing to underexpression of FAAH (432). This compound was shown to be elevated 10-fold following experimental inactivation of FAAH, with highest levels noted in the lungs and kidneys (458). Furthermore, inaction of FAAH in mice increases dermal fibrosis in response to subcutaneous administration of BLM (432).

Serine proteases activate PARs, which have been associated with fibrosis of internal organs (459–461). *PAR-1* is expressed by keratinocytes, endothelial cells, and fibroblasts, while *PAR-2* is expressed in suprabasal keratinocytes in SSc lesional skin and in healthy donor skin (462). There is more expression of PAR-1 by fibroblasts in biopsies of SSc lesional skin than by fibroblasts in biopsies of normal donor skin, and PAR-2 was expressed only by SSc lesional skin fibroblasts and not by normal donor fibroblasts (462). A large portion of fibroblasts in samples from SSc lesional skin were myofibroblasts, staining positive for αSMA suggesting that PAR-1 and PAR-2 may be involved in fibrosis development in SSc (462). PAR-1 is increased on SSc-associated ILD myofibroblasts, and when it is inhibited by the direct thrombin inhibitor, dabigatran, there is abrogation of formation of myofibroblasts, αSMA, and production of CI (463). Agonists of PAR-2 include mast cell tryptase and Factor Xa, and mast cells have been demonstrated to stimulate human lung fibroblast proliferation via activation of PAR-2.

It is worth noting the importance of COX-2 in the metabolism of ECs – specifically regarding 2-AG, which is more readily metabolized by COX-2 than AEA and has also been shown to activate PPARγ via a mechanism that is COX-2 dependent. Recent studies suggest the COX-2 metabolite of 2-AG, 15-deoxy-PGJ2-G, activates PPARγ (464) and that inhibition of IL-2 secretion by AEA in murine splenocytes is attenuated by COX-2 inhibition and also partially antagonized by PPARγ inhibition (465). Further work should be directed at evaluating PPARγ as a downstream

target of the oxygenated metabolites of AEA and 2-AG, as this nuclear receptor is known to modulate fibrogenesis (likely by being a transcriptional repressor of TGF-β), autoimmunity, and a wide range of other physiologic processes (82, 466). 2-AG is oxygenated by COX-2 and other PG synthases to produce several different glycerol-esters of the prostaglandins (PG-Gs). During the early stages of inflammation, in which microsomal prostaglandin E2 synthase (mPGES)-1 is high, one would expect to see higher levels of PGE_2-G. Similarly, during resolution, in which PGD_2 and its spontaneous degradation products predominate, one would expect to see higher levels of PGD_2-G and its metabolites. Interestingly, the EC analog of 2-AG, which is known as 15-deoxy-PGJ_2-G, also has been shown to activate PPARγ and this cyclopentanone-EC derivative may be the mediator of 2-AG's activation of PPARγ. Further characterization of the P450-derived epoxides of AEA and 2-AG is needed, especially given the importance of P450, epoxygenase in PAR-2-mediated sustained activation of TRPV4, which may play a role in perpetuating fibroblast activation.

The efficacy of several cannabinoid agonists in attenuation of fibrosis has been demonstrated in both the BLM and hypochlorite murine models of SSc. Additionally, these compounds have been shown to counter several behavioral abnormalities of SSc fibroblasts, including reversal of myofibroblast differentiation and decreased resistance of SSc myofibroblasts to apoptosis, with the ultimate effect of decreased ECM deposition and attenuation of fibrogenesis. The exact mechanism by which these cannabinoid agonists exert their antifibrotic action is still a matter of debate, but it seems to be mediated in part by activation of CB2 and PPARγ. CB1 and CB2 are both overexpressed in dcSSc fibroblasts. Twenty-four hour incubation with the CB1/CB2 agonist, *WIN55,212-2*, resulted in agonist-induced inhibition of both CB receptors, which was reversible after agonist withdrawal (439). After 10 µM WIN55,212-2 incubation, a reduction in CI mRNA and protein was observed in both dcSSc and healthy fibroblasts (439). TGF-β and CTGF mRNA expression, as well as IL-6 levels were also substantially decreased after exposure to WIN55,212-2 (439). Analysis for αSMA by Western blotting, RT-PCR, and immunocytochemistry showed that WIN55,212-2 induced reduction in αSMA expression by 43% and increased by twofold the number of apoptotic fibroblasts from patients with dcSSc but not in fibroblasts from healthy donors (439). Pre-incubation of dcSSc and healthy fibroblasts with synthetic cannabinoid receptor antagonists *AM281* (CB1 antagonist) and *AM630* (CB2 antagonist) did not significantly reverse the effects of WIN55,212-2 on CI neosynthesis, inhibition of IL-6, or fibroblast apoptosis, indicating that the antifibrotic actions of WIN55,212-2 are mediated, in part, by pathways not involving CB1 and CB2, perhaps as the authors suggest by transducing pathways involving p-ERK (439). Incubation of dcSSc fibroblasts and healthy controls with WIN55,212-2 was noted to result in decreased phospho-ERK-1/2 protein expression in both groups (439). WIN55,212-2 was found to prevent BLM-induced dermal fibrosis in DBA/2J mice *in vivo*. Levels of phospho-Smad2/3 were analyzed and found to be significantly lower after WIN55,212-2 exposure. Subcutaneous inflammatory cell infiltration, dermal thickness, and CI content were comparable to the control group (440). BALB/c mice injected daily for 6 weeks with PBS or hypochlorite were injected intraperitoneally

with PBS or with WIN55,212-2, an agonist of CB1 and CB2, or with JWH-133, a selective agonist of CB2. Both WIN55,212-2 and JWH-133 prevented development of skin and lung fibrosis, as well as fibroblast proliferation and formation of autoantibodies (467).

As mentioned above, FAAH levels are markedly reduced in biopsies of SSc lesional skin compared to skin from healthy donors, and mRNA for FAAH expression in cultured lesional skin fibroblasts from patients with SSc was also reduced from that expressed by cultured normal donor dermal fibroblasts (432). The induction of BLM skin fibrosis in *FAAH* null mice or in normal mice treated with the FAAH inhibitor, *JNJ1661010*, resulted in a marked increase in skin fibrosis at the BLM injection site (432). Furthermore, blocking CB1 receptor in BLM-treated mice with FAAH blocked by JNJ1661010 resulted in a marked increase in skin fibrosis at the BLM injection site (432). Additionally, block-ing CB1 receptor in BLM-treated mice with FAAH blocked by JNJ1661010 prevented the enhanced fibrosis induced by BLM treatment, whereas blocking CB2 further enhanced skin fibrosis in BLM-treated mice with FAAH blocked by JNJ1661010, suggesting CB1 mediated fibrosis whereas CB2 dampened fibrosis as a result of increased EC present because of blocking FAAH (432).

The role of the CB1 receptors in the BLM skin fibrosis model and Tsk-1/+ mice model was assessed using *CB1*KO mice (468). WT and *CB1*KO mice were treated with BLM and with the CB1 selective agonist *N*(a-chloroethyl)-5Z, 8Z, 11Z, 14Z eicosatetraena-mide (ACEA) (468). *CB1*KO mice were protected from developing BLM dermal fibrosis; however, crossing *CB1*KO mice with Tsk-1/+ mice did not prevent fibrosis (468). This suggested that fibrosis associated with inflammation was dependent on CB1 expression or leukocytes. Indeed, chimeric bone marrow studies revealed that CB1 on leukocytes was essential for leukocyte infiltration and fibrosis in the BLM skin fibrosis model induced by the CB1 agonist (468).

These preclinical studies of CB receptor agonists/antagonist provide useful information for translation of these or similar CB receptor active agents for treatment of SSc.

Conclusion

Systemic sclerosis is one of the most complex systemic auto-immune diseases that target the vasculature and connective tissue-producing cells (namely fibroblasts/myofibroblasts) and components of the innate and adaptive immune systems – all three of which themselves interact and affect each other. The disease is heterogeneous in its clinical presentation that likely reflects different genetic background or triggering factor influences on the vasculature, connective tissue cells, and immune system. The roles played by other ubiquitous molecular entities (such as lysophospholipids, ECs, and their diverse receptors) in influencing the vasculature, immune system, and connective tissue cells are just beginning to be realized and studied and may offer new therapeutic approaches to treat SSc.

Author Contributions

Statement pertaining to each author's contribution. Drs. DP and AP wrote the "Introduction," Dr. MB wrote the section on "Genetics and GWAS," Dr. AP wrote the sections on "Immune System in SSc Pathogenesis," Dr. DP wrote the section on "Vascular Abnormalities in SSc," Dr. AP wrote the section on "Fibrosis in SSc" and "Animal Models of SSc/Scleroderma," Dr. BP wrote the section on "The ECS and SSc." All authors reviewed the final manuscript.

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