SCLERODERMA (J VARGA, SECTION EDITOR)

The Immune Pathogenesis of Scleroderma: Context Is Everything

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Abstract The fundamental mechanisms that drive the pathogenesis of systemic sclerosis (SSc) remain elusive, despite over 50 years of investigation. Here, we review recent progress in the understanding of the immunopathogenesis of SSc. In particular, we consider interleukin-13 (IL13), and its upstream and downstream pathways, as an example of an immune system-derived mediator involved in fibrotic and vascular pathology. Emerging results linking pattern-recognition receptors and interferon pathways to SSc are also stressed. We discuss genetic data linking the immune system to SSc risk and efforts to apply animal models to subsets of patients recently resolved by gene expression profiling. These developments will help build a context for better understanding of previous observations and design of the next generation of studies that may eventually lead to effective treatment.

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Introduction

The fundamental mechanisms of initiation of SSc and of its progression remain elusive, despite over 50 years of investigation. One of the challenges in this regard is that SSc involves aspects of inflammation, vascular biology, and growth factor-propagated fibrosis. Thus, the pathologic mechanisms involved invoke the full complexity of each of these. Despite the co-occurrence of these seemingly disparate forms of pathology in SSc, accumulating evidence, for example the discovery of a strong association of human leukocyte antigens (HLA) with disease risk and direct observation of histologic and molecular immunopathology, increasingly suggest that immune dysregulation is a proximal cause of SSc. From this perspective, immune dysregulation is poised to emerge as a unifying explanation for the fibrotic, vasculopathic, and inflammatory manifestations of SSc.

Here, we review recent progress in the understanding the immunopathogenesis of SSc, focusing on pathways that link inflammation with pro-fibrotic signaling and, potentially, the vasculopathy observed in SSc. We consider interleukin-13 (IL13) and its upstream inducer IL33 in detail, as examples of immune system-derived mediators involved in fibrotic and vascular pathology. We also discuss recent genetic data linking the immune system to SSc risk, efforts to apply animal models to specific SSc patient subsets, and new data regarding pattern-recognition receptors and interferon pathways in SSc. In particular, patient stratification efforts are emphasized on the basis that they provide an explicit molecular demonstration of the longstanding clinical observation that SSc is a heterogeneous

disorder. This heterogeneity in turn suggests that consideration of context, in terms of the clinical, molecular, and genetic background of the patients and animal model systems under investigation, will be critical to interpreting the results of individual studies and ultimately generating the insight necessary to develop successful new treatment.

Recent Genetic Evidence Implicating the Immune System in SSc

Despite its classification as an autoimmune disease, the primary involvement of the immune system in the pathogenesis of SSc remains a topic of debate. The uniform observation of increased extracellular matrix production and early vascular phenomena, for example Raynaud's, has led to the suggestion that this disease is a primary disorder of either fibroblast dysfunction or endothelial cell injury [1, 2]. Although autoantibody production characterizes most SSc patients, some have argued this is an epiphenomenon as a consequence of chronic tissue damage [3]. Furthermore, classic immunosuppressive medication is of limited benefit [4], in contrast with other autoimmune conditions.

Recent genetic evidence that implicates immune system dysfunction as a primary driver of SSc pathogenesis have affected this debate. Genome wide association studies (GWAS) of single nucleotide polymorphisms (SNPs) enable high-resolution screening for genetic risk factors of complex diseases. To date, three GWAS studies have been performed on SSc patients. Each of these identified strong associations within the major histocompatibility locus, which most probably indicates that adaptive antigen-driven immunity contributes to SSc pathogenesis [5, 6••, 7•]. In these and followup studies, associations of SSc risk or SSc clinical subphenotypes were identified in genes encoding six proteins implicated in the immune response:

1. CD247, the zeta chain of the T-cell receptor [6••, 7•];
2. STAT4 and IRF5, transcription factors mediating immune responses [6••, 7•, 8];
3. IL12RB2, a subunit of the IL12 receptor complex [9];
4. CD226, an immunoglobulin superfamily member involved in NK cell and lymphocyte activation [10]; and
5. TNFSF4 (OXL40), a co-stimulatory molecule expressed on antigen presenting cells [11].

Interestingly, some of these risk alleles are shared with other autoimmune diseases, suggesting that a common pathway leading to loss of tolerance may underlie phenotypically different diseases. As the genetics of SSc are a rapidly developing field, the authors encourage the reader to seek dedicated reviews on this topic [12, 13]. Although neither the mechanistic effects of the genes implicated in SSc

pathogenesis nor an integrated picture of how these risk alleles interact with one another has emerged, these data indicate the immune system is involved in the development and manifestations of this disease.

Molecular Stratification of SSc Patients

SSc is a heterogeneous disorder with a wide range of clinical manifestations and variations in severity [14–16]. Major obstacles to elucidation of the involvement of the immune system in the pathogenesis of SSc include a lack of insight into molecular aspects of different manifestations of the disease and poor understanding of which subsets of patients might be best represented by a given animal model of this disease. Given the relative accessibility of the skin to tissue harvesting, recent efforts to classify patients have focused on transcriptional profiling of skin biopsies [15, 17, 18••]. Whitfield and colleagues performed unsupervised clustering of skin gene expression profiles from patients with diffuse SSc (dSSc), limited SSc (lSSc), and morphea, and identified an *inflammatory* subset of patients with highly enriched expression of chemokines, chemokine receptors, interferon response genes, and transcripts characteristic of immune cells [17, 18••]. Interestingly, according to these two reports the *inflammatory* subset cuts across traditional clinical stratifications of SSc, encompassing an estimated 17 % to 36 % of patients with dSSc, 28 % with lSSc, and 100 % with localized scleroderma. In contrast, for a second *fibroproliferative* subset, largely composed of patients with clinical diffuse disease, upregulation of genes involved in cell proliferation predominates [17, 18••]. Accordingly, higher numbers of T-cells and Ki-67 positive proliferating cells are observed for patients in the *inflammatory* and *fibroproliferative* subsets, respectively [17]. A recent longitudinal analysis demonstrates that these expression profiles are stable, suggesting that these subsets represent different disease entities as opposed to different time points along the course of a single disorder [18••]. These findings suggest that stratifying patients according to these molecularly defined subsets may reduce a major source of heterogeneity in studies of SSc and, accordingly, increase the robustness of future clinical studies. Furthermore, they provide a basis for understanding the different animal models of SSc and a means of converting pathogenic pathways identified in these models to personalized treatment for patients.

The Sclerodermatous Graft Versus Host Disease Model and its Relationship to the *Inflammatory* Subset of SSc

Investigation of the causes of SSc relies heavily on the use of mouse models, and systematic consideration of these

models has been the topic of recent reviews [19, 20]. The sclerodermatous graft versus host disease model (SclGVHD) is based on the observation that clinical manifestations for patients with chronic GVHD overlap those for patients with SSc, including skin sclerosis and myofibroblast accumulation [21, 22]. This model is generated by transferring splenocytes into irradiated or immunodeficient mouse strains allogenic at multiple minor histocompatibility loci [23]. Notably, the specific tissue manifestations of the disease vary depending on the MHC haplotype shared between the graft and host. When the model is run on donor–host pairs that share the H-2^k or H-2^b MHC haplotype instead of the "native" BALB/c H-2^d haplotype, skin manifestations are less prominent and, instead, mice develop constitutional symptoms and diarrhea [24]. This implies that shaping of the thymic repertoire by specific MHC alleles may be an important determinant in the range of tissues affected in individual SSc patients.

The version of SclGVHD using $Rag2^{-/-}$ immunodeficient hosts on the BALB/c background is particularly well characterized [23, 25••]. These mice develop skin thickening and fibrosis characterized by dermal infiltration of graft CD4$^+$ T-cells. There is also a resident dermal macrophage population that expands over the first few weeks of the disease. Fibrosis is also observed in the renal interstitium and intestinal lamina propria and Scl-70 autoantibodies, a characteristic serologic marker of human SSc, are produced. Interestingly, this clearly immune-driven model includes some of the vasculopathic manifestations of SSc, with luminal narrowing in the capillaries of the skin and kidney, associated with increased expression of the vasoconstrictor endothelin-1 [23]. Human chronic GVHD seen after BMT, however, lacks the vascular rarefaction seen in SSc skin [22]. These data suggest that murine SclGVHD may differ from human chronic GVHD in its vascular manifestations, with the former more accurately modeling SSc. Further investigation is needed to understand this potential difference between human chronic GVHD and murine SclGVHD.

Although versions of the SclGVHD model using irradiated versus $Rag2^{-/-}$ immunodeficient hosts have not been directly compared, one notable difference is that only the irradiated version develops significant lung fibrosis [23, 26]. One possible explanation is that inflammatory "danger" signals, for example those elicited by radiation injury, are important for the induction of a fibroinflammatory program in the SclGVHD lung. Interestingly, lung involvement in SSc patients is also variable, and studies suggest it may be linked to gastroesophageal reflux, leading to acid aspiration and epithelial injury [27]. Taken together, these findings suggest a "second hit" hypothesis to explain the variable lung involvement among SclGVHD models and SSc patients whereby immune system activation must be accompanied by tissue injury to incite this organ-specific manifestation.

Given the complexity and heterogeneity of SSc, an important part of the evaluation of any relevant animal model is determination of whether it accurately reflects the human disease, and, if so, which subset of disease it models best. Although these judgments have typically occurred at the level of gross and histologic assessment of the disease, the advent of molecular stratification of SSc subsets has enabled a more systematic molecular approach to this question. In particular, SclGVHD seems to best approximate the *inflammatory* subset of patients [25••]. Thus, the SclGVHD model is an example of immunopathology resulting in fibrotic and vasculopathic manifestations similar to those seen in SSc, and at the molecular level best represents patients with the *inflammatory* skin gene expression profile.

IL13 as a Pro-Fibrotic Mediator in SSc: Background

Several pro-fibrotic factors with a peripheral relationship to the immune system are under investigation in SSc, including TGFβ, CTGF, PDGF, and endothelin; these are reviewed elsewhere [28–31]. In addition to these, IL13 has emerged as a putative immune system-derived mediator of fibrotic disease in SSc. IL13 is a 15-kDa secreted cytokine encoded at chromosome 5q31. IL13 was discovered, with IL4, as a predominant product of Th2-skewed T cells [32]. However, in addition to T cells, other cell types produce IL13, including mast cells, macrophages, and dendritic cells [25••, 33–35]. Another potential source of IL13 is a recently described cell population called the "nuocyte" [36], so named because of their production of IL13, with "*Nu*" being the 13th letter of the Greek alphabet. Nuocytes are induced by IL33 and IL25, and are grouped within the growing category of innate lymphoid cells (ILC) [37•, 38•, 39•], a term derived from their responsiveness to IL-7, a prototypic lymphoid growth factor, but persistence in mice lacking adaptive immunity.

IL13 has effects that partially overlap those of IL4, and this is reflected in the mechanisms of IL13 signaling. IL4 signals through two receptor complexes, a type I receptor comprised of IL4RA and the common γ-chain, and a type II receptor comprised of IL4RA and IL13RA1 [40]. IL13 only utilizes the type II receptor, which activates the STAT6 transcription through TYK2 and JAK2 [41]. In addition to IL13RA1, there is a related receptor, IL13RA2, which does not partner with IL4RA. IL13RA2 has two variants: a secreted form and a transmembrane form with a short cytoplasmic tail devoid of known signaling motifs [42]. Early studies on IL13RA2 did not find that it conferred responsiveness to IL13, which led to speculation that it functions as a decoy receptor [43–45]. Accordingly, increased levels of IL13-driven disease are observed for IL13RA2 knockouts[46, 47], and soluble IL13RA2-Fc fusion protein

therapeutically blocks IL13 [48]. In contrast, IL13RA2 promotes fibrosis in some TGFβ-dependent models, suggesting it may have signaling capacity [49, 50]. Further study of the mechanisms of regulation of IL13RA2 and the pathways it engages is needed to generate a clear picture of its biological relevance to IL13 signaling.

IL13 as a Pro-Fibrotic Mediator in SSc: Translational Studies

Keeping in mind the caveats discussed in this review regarding the heterogeneity of SSc, if IL13 is involved in the pathogenesis of SSc it should be detectable in patients at levels above those in controls. In the serum of both dSSc and lSSc patients, levels of both IL4 and IL13 were increased [51]. In the skin of SSc patients, the total number of IL13-expressing cells, as judged by immunohistochemical staining, was elevated, and some of the positive cells also stained for a macrophage marker, suggesting that resident dermal macrophages are a source of IL13 in the skin of SSc patients [25••]. In another study, IL13 expression was increased in peripheral blood CD8+ T-cells in patients with SSc [52]. IL13 levels correlated with the biomarkers CRP and ESR, suggesting that IL13 may be linked to inflammatory mechanisms. In a follow-up study, CD8+ T-Cells from SSc patients were found to upregulate the Th2 transcription factor GATA3, which promotes IL13 expression and inhibits differentiation toward IFNγ-producing Th1 cells [53]. The proportion of GATA3-expressing CD8+ T-cells correlated with measures of disease severity, for example modified Rodnan skin scores (mRSS) and interstitial lung disease. In addition to IL13 itself, mRNA levels of the genes encoding the IL13 receptor complex (IL13RA1 or IL4RA) and an IL13 regulated gene, CCL2, were shown to correlate with both the presence of SSc and disease severity as measured by the mRSS [25••]. Last, an IL13 responsive gene profile derived from treating fibroblasts in vitro with IL13 was significantly enriched in the inflammatory subset [25••], suggesting that IL13 pathway activation may be most relevant to this group of patients.

Pulmonary arterial hypertension (PAH) is a complication of SSc resulting in particularly high morbidity and mortality [54]. PAH is most commonly associated with lSSc, although it can be observed in dSSc also. Among lSSc patients, IL13 levels were increased in patients with PAH, and expression of the IL13 target gene MRC1 in circulating monocytes correlated with pulmonary arterial pressures [55]. Serum IL13 levels also correlated with abnormalities in the capillary loops visible through the nails of SSc patients, an early feature of SSc that may precede other manifestations [56, 57]. Thus, an association between IL13 expression and the presence of SSc has been observed in several studies, and

several studies suggest that IL13 levels correlate with vasculopathic manifestations of scleroderma, for example PAH and nailfold capillary abnormalities.

Studies of the genetics of scleroderma have yielded conflicting results regarding the effect of polymorphisms in the IL13 pathway. Granel et al. showed that polymorphisms in IL13 or IL13RA2 contribute to genetic risk of SSc [58, 59]. However, none of the SSc GWAS studies identified signals within loci relevant to the IL13 pathway and a recent study targeting polymorphisms in IL4, IL13, and the genes encoding the type II IL4 receptor (IL13RA1, IL4RA) failed to find an association with SSc or clinical subtype [60]. These conflicting studies await further evaluation using independent cohorts.

IL13 as a Pro-Fibrotic Mediator in SSc: Mouse Models

Perhaps one of the strongest demonstrations of the potent pro-fibrotic activity of IL13 is the observation that transgenic overexpression of IL13 in the skin and lungs of mice is sufficient to drive chronic inflammation and fibrosis [61, 62]. With regard to more specific SSc models, both IL13 and a receptor IL13RA2, are elevated in skin treated with the pro-fibrotic agent, bleomycin [63]. Furthermore, bleomycin-induced skin sclerosis and lung fibrosis are attenuated by genetic deletion of Il13 or therapeutic neutralization, respectively [50, 64, 65].

In the SclGVHD model, dermal infiltrating CD4+ T-cells and a dermal macrophage population were identified as sources of IL13 (Fig. 1) [25••]. Accordingly, hosts lacking the common IL4 and IL13 receptor subunit, IL4RA, were completely protected from disease. Hosts lacking IL13 were partially protected, which likely reflects continued production of IL13 by graft CD4+ T-cells. Moreover, the IL13 response profile enriched in inflammatory patients is also enriched in SclGVHD mice but not the IL13 unresponsive $IL4ra^{-/-}$ hosts. IL13 seems to act indirectly via host cells to shape the responses of the graft-derived lymphocytes, because regulatory T cells and total CD4+ T-cell IL-10 expression were increased in IL4RA-deficient hosts. Furthermore, a mixed phenotype of classical and alternative activation markers [66], which may be driven by IL13, were observed in macrophages in the skin of SclGVHD [25••]. Interestingly, similar phenotypes have been observed with tumor-associated macrophages, suggesting a parallel between the fibrotic stromal reaction seen with some tumors and the fibrotic disease seen in SSc [67]. To further identify the mediators downstream of IL13, the overlap between genes upregulated in the skin of SclGVHD mice, the human inflammatory subset of SSc, and IL-13-treated fibroblasts was considered [25••]. The chemokine CCL2 emerged as the only gene present in all three data sets, consistent with the

action of CCL2 as a secondary mediator of disease in the IL-13 transgenic lung model [68]. Accordingly, CCL2 is upregulated in both macrophages and stromal cells by an IL4RA-dependent pathway and blockade of CCL2 prevents SclGVHD [25••]. Taken together, IL13 derived from dermal macrophages and infiltrating lymphocytes promotes SclGVHD by inducing CCL2 expression, affecting macrophage polarization and indirectly inhibiting immunoregulatory T-cell expansion (Fig. 1).

IL33: An Emerging Profibrotic Cytokine Upstream of IL13

As evidence gathers that IL13 has a variety of pro-fibrotic functions that may be relevant to the pathogenesis of SSc, it becomes of great interest to understand how IL13 expression is induced. Recent evidence implicates IL33 as a broad upstream inducer of IL13 in the immune system. IL33 is an IL1 family member constitutively expressed in cell nuclei [69, 70]. Necrotic cell death initiates IL33 release, leading to the suggestion that IL33 acts as an innate "danger signal" driving the pro-inflammatory effects of necrosis. IL33 signals through a dimeric receptor composed of an IL33-specific receptor chain (ST2) and a subunit shared by several IL-1 family members (IL-1RAcP) [71]. Whereas the precursor forms of other IL1 family members are activated by caspase-1/inflammasome-dependent cleavage, cleavage of pro-IL33 by caspase-1 is inactivating [72]. Instead, other proteases, for example neutrophil elastase or cathepsin G, may mediate maturation of pro-IL33 to the bioactive form [73].

IL33 expression has been demonstrated in a range of cells implicated in the pathogenesis of SSc—endothelial and epithelial cells, and fibroblasts and smooth muscle cells [74, 75]. This suggests that vasculopathic insults, which could be an initial event in SSc, may lead to IL33 release via ischemia. Alternatively, IL33 could be released via inflammation or toxin-induced tissue damage. Ultimately, IL33 would induce a fibroinflammatory response via IL13 and other downstream factors. Thus, IL33 release activated by different upstream events could explain the observation that seemingly disparate stimuli (e.g. bleomycin, SclGVHD, ischemia) lead to fibrotic pathology. With regard to human translational studies, depletion of nuclear IL-33 has been observed in skin endothelial cells and keratinocytes of early SSc patients despite similar or increased levels of IL33 transcripts, leading to speculation that microenvironmental stressors in SSc skin result in release of IL33 [76]. Moreover, expression of both the IL33-specific receptor ST2 and levels of circulating IL33 are increased in the serum of SSc patients [76–78]. In an Italian cohort, IL33 levels were higher in patients with early disease, and in those with active nailfold vascular changes [78], an observation that emphasizes the possible link between IL13 and SSc vasculopathy [55–57]. In a Japanese cohort, IL33 levels correlated with skin scores and inversely with forced vital capacity, a measure of lung fibrosis [77].

Since the discovery of IL33 as the ligand utilizing the ST2 receptor, IL33 stimulation has been linked to the induction of Th2 cytokines, including IL13 [70]. Although best described as activating IL13 expression in Th2 cells [70], B-cells [79], and a subset of ILCs, termed nuocytes [37•, 39•], IL-33 also stimulates IL-13 production by innate immune cells, for example mast cells [80] and basophils [81]. Consistent with the known pro-fibrotic effects of IL13, subcutaneous injection of IL-33 is sufficient to drive skin fibrosis, and this response is attenuated in IL13-deficient

Fig. 1 IL13 in the skin of *inflammatory* SSc patients. This figure depicts the possible cellular sources, targets, and effects of IL13 in the skin of *inflammatory* SSc patients, as determined in animal models and SSc patient samples
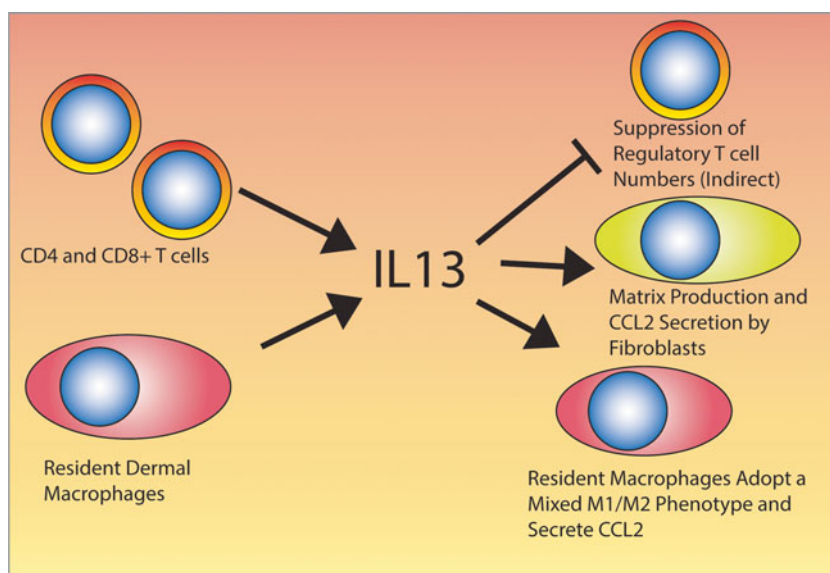
mice [82]. Thus, these studies suggest a hierarchy among pro-fibrotic cytokines whereby tissue injury activates IL-33 release, which in turn induces the effector cytokine IL-13 in a variety of target cells. Although the above studies suggest that IL33 is involved in the development of SSc, the involvement of this cytokine in pathogenesis must be investigated further.

Insights From Chemical-Induced Fibrosis Models

Several animal models of SSc rely on the administration of compounds with known pro-fibrotic toxicity. With comparison with other complimentary models and correlation with human SSc to provide context, chemical models provide unique insights. In addition to the widely studied agent bleomycin [83], other chemical agents have been used as a model of fibrosis. In particular, building on observations that a type I interferon gene expression signature is elevated in patients with SSc [84, 85], and that the TLR3 and RIG1 ligand poly I:C is a potent inducer of type I interferons [86], subcutaneous infusion of poly I:C was found to induce fibrotic and inflammatory dermal changes [87••]. These changes included induction of the vasculopathic mediator endothelin-1, and parallel studies revealed a concordant increase in endothelin-1 mRNA expression in the skin of SSc patients [88]. Additionally, poly I:C was also found to induce TGFβ target genes, suggesting that TLR3 or other dsRNA sensors are a point of interaction between inflammatory signaling and pro-fibrotic growth factors. Although it is currently unclear how TLR3 is involved in the pathogenesis of SSc, these results reveal a potential pathway whereby tissue damage or viruses may contribute to SSc skin disease [89].

Conclusions

Although it has long been suspected that SSc is an immune-mediated disease, only in recent years is evidence of this starting to emerge. Perhaps the reason for this slow progress is not that the volume or quality of studies has been lacking, but rather that appropriate contexts for combining the disparate observations made in this heterogeneous systemic disorder has previously been lacking. These various contexts encompass the genetic, molecular, and clinical framework that places each individual study in perspective. In that respect, GWAS studies have implicated polymorphisms in the HLA locus and other immune-associated genes as risk factors for the development of SSc, providing strong evidence that the immune system is a central mediator of SSc [5, 6••, 7•]. Similarly, use of molecular data to allocate SSc patients to subsets revealed significant heterogeneity of

these patients and enabled identification a particular *inflammatory* subset with evidence of immune activation [15]. The results from these subset studies correlate with those from animal models revealing that specific cytokines, for example IL13, are critical mediators of fibroinflammatory pathology in models of SSc; these findings may be most relevant for this *inflammatory* subset of patients [25••]. All of these studies providing evidence for the immune-mediated pathogenesis of SSc build upon many years of observation of mixed lymphoid infiltrates present in the skin and lungs of SSc patients [90, 91].

An important achievement leading to understanding of SSc is the placing of the observations of the past 30 years in the context of these newly defined patient subsets. For instance, the number of cytokines and other mediators observed to be elevated in SSc is so large that constructing a single coherent model that incorporates each of these is challenging. From this perspective, subdividing SSc patients may deconvolute this complexity to the point where the contributions and mechanistic interrelations of the pathways involved for individual patients can be resolved. Similarly, molecular stratification of patients may help build a more unified picture of the genetic risk for SSc, as the power of previously published studies was probably diluted by inclusion of patients that varied in their underlying mechanism of disease.

Definition of context is also critical in ongoing investigations into the mechanistic contribution of fibroinflammatory mediators to animal models and use of this understanding to improve patient care. Given the complexity of the disease and the large number of potential mediators, we suggest specific priorities to enrich our collective ability to relate and compare parallel studies. First, wherever possible, consistency among investigators in terms of methods and models examined should be encouraged. Second, although it is desirable to develop new SSc models, replication of findings across existing models should be highly valued. Third, further attempts should be made to develop the relationships and hierarchies among individual mediators and cellular participants to determine which are upstream, downstream, and orthogonal to one another. Fourth, establishing the relationship of the different animal models within the heterogeneous nature of SSc is critical. Thus, future studies of the immunopathogenesis of SSc should, ideally, use models with rigorously defined relationships to SSc patient subsets. Last, priority should be given to corroborating findings from animal models by use of patient-derived samples.

In summary the most notable advances in our understanding the immunopathogenesis of SSc in recent years are an improved contextual structure comprising genetic data, molecular patient stratification, and an emerging understanding of how SSc animal models relate to the human

disease. These resources will orient the future study of SSc, enabling investigators to build momentum toward understanding this complex disorder.

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